The Early Local and Regional Immune Response to Recall Inoculations of Mycobacterium Leprae in the Skin of Rhesus Macaques (Macaca Mulatta).

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DEDICATION

I dedicate this work to my brother Jérôme who passed away from cancer while I was acquiring data to test the hypothesis of the study reported herein. The goals and philosophy of biomedical research to alleviate disease and suffering of humans and our fellow animals take their full and very concrete meanings when a beloved one, in the dawn of happiness and a new period of his life, is full blown by disease.
ACKNOWLEDGEMENTS

I wish to acknowledge my major professor, Dr David Scollard, for his communicating enthusiasm concerning leprosy research. He has the most important qualities that I was anticipating before starting this research project. His immuno-pathological expertise and his experience in this area of research were invaluable. His understanding, kindness and support were also invaluable during the many hard times of this work.

My thanks are also directed to my committee members, Dr Gary Baskin, Dr Doo-Youn Cho, Dr Tom Gillis, Dr Kathy O’Reilly and Dr Roger Laine, who always had valuable advises and constructive comments. I feel gifted and lucky to have benefited from such a quality of scientific advisers.

This project was also a prime example of collaboration and I would like to warmly thank the persons and teams that were involved. First the kind and warm people from the Laboratory Research Branch of the Gillis W. Long Hansen’s Disease Center where I did this research project. Among them, Joe Allen, Angelina Deming, Gregory McCormick, Melvyn Morales and Felipe Sandoval were always ready to give their help, time and expertise for this project to be more doable. Also the people from Dr Tom Gillis’ and Dr Diana Williams’ laboratory, in particular Laynette Spring, Sheryl Lewis and Naoko Robbins, who welcomed me in their “kingdom” and were always available to answer my questions.

Second, the team of the Tulane’s Delta Primate Research Center at Covington, Louisiana, in particular Dr Marion Ratterree who performed the sampling on the rhesus monkeys, and the animal caretakers of the center. I also would like to thank Dr Bobby Gormus, without the monkeys of whom this project would never have been possible, and for the scientific interaction we had when trying to interpret our results and anticipate future directions for investigations. Dr Gary Baskin took care of the main study end-point and his contribution was critical to a second level of interpretation of my database, adding an unexpected, yet very interesting scientific meaning to this study.

Third, several persons from the Department of Veterinary Microbiology and Parasitology also were very helpful for an easier completion of this work. Marilyn Dietrich acquired the data for the flow cytometry study and was very helpful and knowledgeable interlocutor when discussing flow cytometry
issues. Chad, who was working in Dr David Horohov’s laboratory, very nicely helped me troubleshooting technical problems encountered during molecular biology procedures. Dr David Horohov was also present for constructive discussions in the field of the reverse transcription and polymerase chain reaction technology. Lastly, Dr Elmer Godeny was always ready for logistic collaboration and we helped each other several times for sampling of rhesus monkey peripheral blood.

Lastly, Michael Kearney efficiently and fruitfully assisted me in the statistical analysis of the generated data. Harry Cowgill and Gregory McCormick helped me, the latter very substantially, in the processing of the illustrations. I am truly indebted to all those people.

I would also like to thank Dr Henry Wayne Taylor, who gave me the opportunity to follow this combined doctoral and residency program and all the folks from the Department of Veterinary Pathology for their kindness and valuable help and scientific interactions. The knowledge and outstanding will to teach of the faculty members also helped me to achieve an even better level of expertise in the field of diagnostic veterinary pathology.

Finally, and very importantly, I would like to thank my spouse, Christine, for constant support and understanding, the latter being greatly helped by her graduate student condition: she perfectly knew what I was talking about, except maybe when I was going into specialized technical details. Her interest for scholarship, adventures and discoveries had helped us to enjoy a life filled with hardship and rewards as well as discovery of oversea lands. My daughter Audrey, who was born during this program, had also been quite “wise” for a toddler and 3 year-old to understand that sometimes, her “Dada” had to go working instead of playing with her.
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LIST OF ABBREVIATIONS

AIDS: acquired immuno-deficiency syndrome
APC: antigen-presenting cell(s)
BB: borderline leprosy
BL: borderline lepromatous leprosy
BT: biotinyl tyramide
BT: borderline tuberculoid leprosy
CD: cluster of differentiation¹
CDC: Center for disease control, Atlanta, GA
cDNA: complementary DNA
CMI: cell mediated immunity
CS: cell suspension(s)
DC: dendritic cell(s)
DN: double negative
DNA: deoxyribonucleic acid
Dnase: deoxyribonuclease
DP: double positive
DPI: days post-inoculation
DTH: delayed type hypersensitivity
DZ+ = ML+ = animals that developed persistent *Mycobacterium leprae* infection
DZ- = ML- = animals that did not develop persistent *Mycobacterium leprae* infection
ENL: erythema nodosum leprosum
FACS: fluorescent activated cell sorting
FC: flow cytometry
FITC: fluorescein isothiocyanate

¹ standard international nomenclature of cell surface molecules/receptors/cell subset markers
HE: Hematoxylin and eosin
HIV: human immunodeficiency virus
IFN: interferon
IHC: immunohistochemistry
Ig: immunoglobulin
IL: interleukin
KO: knock out = gene-targeted deletion
LL: polar lepromatous leprosy
LN: lymph node(s)
LNCS: lymph node cell suspension
LST: lymphocyte stimulation test
LTT: lymphocyte transformation test
MACS: magnetic cell sorting
MGCs: multinucleate giant cells
ML = M. lepra = Mycobacterium leprae
ML+ = DZ+ = animals that developed persistent Mycobacterium leprae infection
ML- = DZ- = animals that did not developed persistent Mycobacterium leprae infection
MoAb: monoclonal antibodies
MΦ: macrophage(s)
μm: micrometers
PBMC(s): peripheral blood mononuclear cell(s) ("buffy coat")
PCR: polymerase chain reaction
PE: phycoerythrin
PerCP: peritidin chlorophyll protein
RNA: ribonucleic acid
RNase: ribonuclease
RR: reversal reaction
RT: reverse transcription
RT-PCR: reverse transcriptase-polymerase chain reaction

SAIDS: simian acquired immuno-deficiency syndrome.

SISCS: skin inoculation site cell suspension

SIV: simian immunodeficiency virus

SIV+: animals inoculated with simian immunodeficiency virus

SIV-: animals not inoculated with simian immunodeficiency virus

TB: tuberculosis

TCR: T cell receptor

TNF: tumor necrosis factor

TT: polar tuberculoid leprosy
ABSTRACT

Epidemiological studies have failed to show any important impact of human immunodeficiency virus (HIV) infection and disease on leprosy, in contrast to the well documented increase in incidence and morbidity with other mycobacteria in these patients, notably *Mycobacterium tuberculosis*. The early events following exposure and repeated exposure to *Mycobacterium leprae* (ML) have not still been studied. Previous studies of first inoculations of ML in the skin of rhesus monkeys have indicated delayed recruitment of CD4+ cells and delayed interleukin (IL) 2 secretion in skin inoculation sites in simian immunodeficiency (SIV) positive animals.

The development of the secondary immune response against a challenge of ML was studied over a period of 2 months in the skin of 9 rhesus macaques, all inoculated previously with ML, 3 of which were SIV positive slow progressors. Cell recruitment and types of cytokine response in the skin and draining lymph nodes (LN) were investigated using flow cytometry (FC), immunohistochemistry, and reverse transcription polymerase chain reaction to detect relative levels of messenger ribonucleic acid (mRNA) expression for IL2, IL4, interferon gamma (IFNγ) and IL10. Results were analyzed according to SIV status and persistence of ML infection.

An early vigorous expression of IL2 and IFNγ mRNA, indicative of a strong T helper (Th) 1 cytokine profile, was seen in the skin of macaques that cleared ML infection. Animals that developed persistent ML infection (ML+) had delayed, weak Th1 response, and no evidence of early Th2 response in the skin. In contrast, early up regulation of IL4 was only observed in the LN of ML+ macaques. Recruitment of CD4+ lymphocytes into the skin was significantly lower and delayed in ML+ animals. CD4:CD8 ratio were significantly lower in the blood in SIV+ monkeys. However, no significant differences in CD4+ lymphocyte recruitment or expression of cytokine mRNA were observed between SIV- and SIV+ monkeys.

Despite evidence of systemic immunodepression, the slow progressing SIV+ rhesus macaques are still capable of mounting an adequate response to ML re-inoculation in the skin. A Th2 response is not generated in the skin early after re-infection in animals which developed progressive infection.
1 INTRODUCTION

"Different forms of clinically established (‘determinate’) disease are associated with different type of immune reactivity, but it is not completely known to what extent these differences have been of primary importance for the course after exposure to *M. leprae* or a consequence of the development of the infection." Morten Harboe, ‘Leprosy’, 1994.

In mycobacterial diseases in general and in leprosy in particular, both the ‘natural’ and ‘acquired’ arm of cellular immunity are believed to be important. Their respective importance, however, remains, to most leprosy investigators’ knowledge, largely speculative and difficult to define since proper cooperation between different cell types and coordination of macrophages and lymphocytes are critical to the development of strong cell-mediated immunity (CMI).

Rodent animal models, such as nude (*nu/nu*) or ALY (alymphoplasia) mice, teach us that T-cell mediated adaptive immunity is important when coping with mycobacteria (Orme, Andersen et al. 1993) and *Mycobacterium leprae* (Yogi, Endoh et al. 1998). In the clinical fields, there are rare reports of renal transplantation in leprosy patients who developed relapse of the disease after immunosuppressive regimens (Adu, Evans et al. 1973; Teruel, Liano et al. 1985). However, with a few exceptions (Borgdorff, van den Broek et al. 1993), increased incidence of leprosy and down-grading of leprosy has not been observed in immuno-compromised, human immunodeficiency virus (HIV)-infected individuals (Pönninghaus, Mwanjasi et al. 1991; Frommel, Tekle-Haimanot et al. 1994; Munyao, Bwayo et al. 1994; Lienhardt, Kamate et al. 1996).

Previous studies have shown that the T cell subsets in tuberculoid (paucibacillary) versus lepromatous (multibacillary) lesions (Modlin and Rea 1994), and the type of cytokine secretion associated with these lesions are different (Yamamura, Uyemura et al. 1991). Those valuable studies in human patients with fully developed lesions however, probably represent only the emerged portion of the iceberg, at a stage in pathogenesis when limited immunomodulatory interventions can be taken in addition to poly-chemotherapy. For obvious ethical and practical reasons, it is impossible to study longitudinally the early, silent phase of leprosy, before lesion development, after documented exposure.
The exact documented time of exposure is seldom known. Likewise, the incubation period of leprosy, believed to be quite long, is not precisely known. Some studies are currently under way to characterize the cytokine profile and inflammatory infiltrate composition from single lesions of leprosy, believed to be early lesions, in leprosy patients in Brazil (T. Gillis, personal communication) and in India. However, only animal models can help to provide insights concerning the initial host response soon after exposure or re-exposure.

It was recently discovered that helper T lymphocytes do not up-regulate cytokines important for an immune response in a random and / or an independent manner but mature into two main types of secreting lymphocytes, termed type 1 and type 2, which are defined by the secretion of consistent sets of cytokines (Mosmann, Cherwinski et al. 1986). The type 1 T helper lymphocyte (Th1) cytokine response is characterized by the recruitment of lymphocytes secreting a defined array of cytokines, i.e. interleukin (IL) 2, interferon gamma (IFNγ) lymphotoxin (LT / TNFβ) and granulocyte macrophage colony stimulating factor (GM-CSF). In contrast, the type 2 helper T lymphocyte cytokine response (Th2) is characterized by the secretion of IL-4, IL-5, IL-3, IL-6 and IL-10. These T cells evolve from immature precursor T cells able to secrete a non-committed array of cytokines and is termed 'Th0'(Mosmann, Cherwinski et al. 1986). However, when T cells are engaged in one of the 2 types of cytokine secretion, they are in an irreversible manner, at least at the clonal level. A debate still exist as to the existence of a mature Th0 subset (Lucey, Clerici et al. 1996). The 2 types of T cells provide help for different cell subsets and favor maturation of immune cells in a different manner, resulting in different types of immune response and are thought to regulate each other (Mosmann and Coffman 1987), although this is still matter of debates (Muraille and Leo 1998).

The Th1 cytokine response, otherwise called 'type 1' response when the cytokine response is studied from inflammatory cells, has been demonstrated to play an important role in controlling most mycobacterial infections (reviewed in (Daugelat and Kaufmann 1996; Lucey, Clerici et al. 1996)). Type 1 cytokines contribute to the control and elimination of *Mycobacterium tuberculosis* and *M. avium* through activation of the macrophages. In tuberculosis, the initial response to a respiratory challenge with *M. tuberculosis* is a type 1 response that subsequently evolves into a type 0 response characterized by the apparition of IL-4 mRNA expression in the lung with progression of disease towards pneumonia.
(Hernández-Pando, Orozco et al. 1996). Interestingly, there is correlation between lesion type and severity and intensity of down-regulation of type 1 cytokine and up-regulation of type 2 cytokines.

In leprosy where the disease evolution is very slow, it would be desirable to know the cytokine profile of microscopic lesions before they are grossly visible, at the primary site of infection, to know whether this is determinant for the subsequent expression of the disease or is independent of it. That is where the value of performing longitudinal studies on primate animal models becomes most obvious. With such models, it is theoretically possible to study the kinetic of the local cytokine response from the time of inoculation and to generate inferences on the importance of the type of cytokine response on the subsequent outcome after exposure. Such studies might indicate whether early lesions all have a type 1 cytokine milieu or an uncommitted type 0 mixture of cytokines with similar levels of expression. Do type 2 cytokine responses appear at the beginning of a primary or a secondary exposure to M. leprae? Are there shifts from type 1 to type 2 or vice-versa in the same animals inoculated over time? Is the type 2 cytokine milieu causative for developing a lepromatous multibacillary disease or a consequence of it, being part of a specific immune down regulation system (i.e., T helper 2 lymphocytes might be the long sought “suppressor” T lymphocytes)? Does the type of cytokine milieu correlate with any preferential recruitment of T cells or T cell subsets?

Kinetic studies of cytokine expression are critical to the understanding of leprosy because two cases can theoretically exist. First, it may be that the initial cytokine milieu at the site of inoculation might be appropriate and the same in intensity and timing, regardless of the outcome after Mycobacterium leprae infection or the existence of an immunocompromising chronic viral infection. In that case, if only the animals thereafter developing a persistent infection do shift to a type 2 cytokine milieu, then further studies of initial cytokine milieus are no longer critical to understand the immunopathogenesis of Hansen’s disease. Rather, focusing on defects in macrophage recruitment, macrophage activation and macrophage-lymphocyte interaction (such as antigen presentation, and immune response (MHC) genes) and lymphocyte activation and recruitment, would be more desirable. The second possibility is that the animals that encounter the leprosy bacillus either have an initial non-committed or a variably committed cytokine milieu. In that case, understanding what determines the type of cytokines that will be initially secreted will be of great importance to fight Hansen’s disease. In inoculated

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animals, the effect of variable immunosuppressive agents, including immunosuppressive chemotherapeutic agents and immuno-compromising infectious agents such as the lentiviruses that induce immuno-deficiency should also be studied in terms of type and timing of lymphocyte recruitment and the nature of the cytokine response.

In mycobacterial diseases, in particular tuberculosis and diseases secondary to opportunistic mycobacteria of humans, such as *Mycobacterium avium*, there is a striking acceleration of the evolution and a worsening of the clinical signs of tuberculosis in human immunodeficiency virus (HIV) infected individuals (Havlir and Barnes 1999) and an increased incidence of the opportunistic mycobacteriosis in HIV patients (Benson and Ellner 1993). A debate is still present among leprosy researchers as to whether HIV has any effect on the incidence, evolution and spectrum of dually infected HIV-*M. leprae* patients (Pönninghaus, Mwanjasi et al. 1991). Epidemiological studies and immunological studies thus far have failed to show any impact of HIV infection and disease on leprosy (Pönninghaus, Mwanjasi et al. 1991; Frommel, Tekle-Haimanot et al. 1994; Sampaio, Caneshi et al. 1995). However the question is still matter of some debates, especially concerning the incidence of immuno-pathological reeactions to leprosy (Borgdorff, van den Broek et al. 1993; Bwire and Kawuma 1994). The controlled experimental model of rhesus macaques (*Macaca mulatta*) dually infected with *M. leprae* and SIV might help to clarify the situation of human leprosy in the context of HIV infection.

These thoughts, arising from the review of the CMI against *M.leprae* and the interactions of mycobacteria with immunosuppressive lentiviruses, are the basis for the hypothesis of this study that can be formulated as follow:

"The immunodeficiency resulting from the chronic progressive infection with the simian immunodeficiency virus (SIV) is responsible for an inappropriate T-lymphocyte response against mycobacteria, including *Mycobacterium leprae* in rhesus macaques (*Macaca mulatta*)"

There are additional reasons to study the early immune response in rhesus macaques after exposure to *M. leprae* in SIV- and SIV + animals. There is a need to better characterize the immune response to *M. leprae* in rhesus macaques (*Macaca mulatta*), where thus far only pathologic (Baskin 1990) and disease outcome (persistent *M. leprae* infection with nerve lesions versus rejection of the infection), lymphocyte proliferative assays and antibody levels in the peripheral blood have been

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performed (Gormus, Murphey-Corb et al. 1998; Gormus, Xu et al. 1998). There are also renewed interests in studying the CMI to mycobacterial disease since the HIV/AIDS pandemic. In addition, leprosy research suffers from the scarcity of experimental animal models that exhibit the spectrum of leprosy (i.e. tuberculoid and lepromatous) and is fully characterized immuno-pathologically, regardless of their similarity and discrepancy with the human disease.

In this context, my research goals are:

1 – **Objective 1**: to ascertain that the rhesus monkeys (*Macaca mulatta*) can effectively mount a cellular immune response locally and regionally against *Mycobacterium leprae*.

2 – **Objective 2**: to compare the kinetics of T lymphocyte recruitment at cutaneous inoculation sites and the fluctuation of T lymphocyte subsets in the regional lymph nodes draining the inoculation sites and in the peripheral blood.

3 – **Objective 3**: To assess the mRNA expression of 4 important cytokines in the developing lesions in the skin and mRNA up-regulation or down-regulation of these same 4 cytokines in the regional draining lymph nodes.

### 1.1 LITERATURE CITED


2 REVIEW OF THE LITERATURE ON THE IMMUNOPATHOLOGY OF LEPROSY

2.1 CELL MEDIATED IMMUNITY (CMI) TO MYCOBACTERIA, WITH PARTICULAR REFERENCE TO MYCOBACTERIUM LEPRAE

2.1.1 CURRENT DEFINITION OF AND CONCEPTS ABOUT CELL MEDIATED IMMUNITY (CMI)

Cell mediated immunity (CMI) has been classically defined as the protective functions and structural changes of a host directed against an invading harmful micro-organism, primarily mediated by macrophages and lymphocytes that matured in the thymus, the T lymphocytes, all recruited at site or at the vicinity of the invading micro-organism. The structural microenvironment where CMI takes place is called a granuloma. It is composed predominantly of macrophages and T lymphocytes that interact either through surface receptors and cytokines in order to kill or at least contain the invading microorganism (Kaufmann 1994).

Cells acting in a granuloma can be arbitrarily divided into 2 main categories. First, the effector cells, the function of which is to kill the pathogenic parasitic organism (the pathogen) or the infected cell and second, the orchestrating cells, whose function is to increase (help) or diminish (suppress) the inflammatory and immune response through various surface signals and soluble mediators directed at the effector cells. The relative importance of the effector cell versus the orchestrating cell is difficult to assess. In a perfect world, the function of the effector cell would be 100% effective, without any help provided by other cells. However, due to evading strategies employed by most pathogen of medical importance, adaptive immunity and helper T cells are important to successfully contain intracellular parasites. The orchestrating 'helper' immune cell is in charge to tune the efficacy of the cellular immune primary and secondary response, and to a certain extent modulate the launch of the humoral response, without excessive toxicity to the host, against a given pathogenic organism. These various signals themselves are believed to be elicited by the invading microorganism and the effector cells from the first line of defense coping with the invader. They may also be dependant upon the degree of tissue injury elicited by the invading microorganism ("non-self element") and the first line of defense represented by granulocytes, mast cells and macrophages (Matzinger 1994). Also the degree and type of antigen presentation and other factors of innate immunity, such as the chemokines and cytokines...
secreted by the inflammatory cells are important. Another consideration is the difference in the repertoire of ligand receptor between acquired and innate immunity: whereas the ligand-receptor diversity is very high in adaptive immunity, innate immunity is characterized by a more limited array of recognition patterns of invading micro-organism. The first consequence is that the primary handling of invading micro-organisms by effector cells of innate immunity is rarely optimal when this micro-organism is a pathogen for a specified host. The second consequence is that any genetic weakness in handling and managing a pathogenic organism by a host is more likely to occur during the primary infection, before the lymphocytes with specifically selected T cell receptors come into play.

2.1.2 Helper, Suppressor and Effector Immune Cells – Current Concepts

Classically, in a granuloma, macrophages and perforin-containing cytotoxic CD8+ T lymphocytes were considered the main effector cells, and the CD4+ T helper cells were believed to be the main orchestrating cells. This was mainly because CD4+ lymphocytes together with the professional antigen presenting cells (APC) (i.e. having MHC class 2 on their surface) are the main actors of the afferent phase of the CMI against particulate antigens. Antigen presentation and selection of specific clones of T lymphocytes were viewed as the cornerstones of adaptive cellular immunity, enabling the host to specifically recognize the invading microorganism, thereby tremendously augmenting the efficacy of the CMI response.

However, this correct but relatively simple classic view is no longer tenable because of the more recent data showing that both the CD8 T lymphocytes and the macrophages are fully capable of secreting complex arrays of mediators that increase or diminish the CMI: they are part of the immunoregulatory network (Kemeny, Noble et al. 1994). For example, not only can CD8+ cytotoxic T lymphocytes produce the same array of cytokines as CD4+ T cells, they can be grouped into the same sub-groups of cytokine secretion pattern as CD4+ T cells namely type 1 and type 2 helper CD8+ T lymphocytes, which very importantly are also cytotoxic through perforin secretion (Sad, Marcotte et al. 1995). These two subsets termed TC1 and TC2 can both induce delayed type hypersensitivity reactions, the latter with more eosinophils (Li, Sad et al. 1997). Similarly, the macrophage is also a critical cell in up-regulation of CMI locally and generally, by secreting important cytokines such as interleukin (IL)-12, IL-18, tumor necrosis factor (TNF)-α, IL-1 and the different types of interferons (IFN). On the other hand, it is now clear that CD4+ T lymphocytes are also capable of cytolytic activities in vitro and
in granulomas (Hancock 1989; Pithie, Rahelu et al. 1992) therefore showing that at least some CD4+ T lymphocytes have effector cell capabilities.

Another important debate deals with suppressor T lymphocytes, a topic of important confusion and controversy until recently (Kaufmann 1994). Suppression is defined as the decrease of proliferation in standard lectin-induced lymphocyte stimulation tests (LST) after the introduction of lymphocyte subsets responding to particular antigens (Mehra, Mason et al. 1979). Using this method, however, there is no indication of the mechanism of suppression. Several possible mechanisms may be mediated by different lymphocyte subsets. In the granuloma, two levels of suppression are theoretically possible: the first one is at the level of innate immunity and the inflammatory effector cells, most commonly through apoptosis, but also possibly through unresponsiveness, as in the foamy macrophages of lepromatous leprosy. The second level involves defective T cell presentation leading to tolerance through apoptosis or anergy of specific T cell clones. This is now believed to be through the lack of an appropriate secondary co-signal, either through a cell surface co-stimulatory signal, such as CD 86 (B7-1) - CD28 engagement, or through the appropriate array of cytokine.

These suppressor cells do exist (see the lepromatous leprosy section, page 24) but have never been conclusively and clearly identified. They were first believed to be of CD8+ phenotype (Modlin, Mehra et al. 1986), but it now appears that these are in fact only one component of a broader group of cytolytic and secretory cells of various phenotypes. These cells would be able to induce apoptosis or inhibit the activation of other immune cells through a range of ligand-receptor mechanisms and/or secretion of critical mediators, respectively.

Macrophages can also secrete suppressor factors under specific states of maturation and/or types of infection. For example, secretion of prostaglandin E2 (PGE2) is important in the suppression of macrophage activation: it induces IFN-γ unresponsiveness, down modulate the MHC class 2 expression and the oxydative burst (Krahenbuhl 1994). IL-6 has been recently identified as a potent suppressor of T lymphocyte activation secreted by bone marrow derived macrophages infected with mycobacteria such as Mycobacterium bovis strain 'bacille de Calmette et Guérin' (BCG) and M. avium (Van Heyningen, Collins et al. 1997). Interestingly, this high level of IL-6 secretion was characteristic of infection with mycobacteria. Listeria monocytogenes and Leishmania mexicana infected macrophages
did not secrete this suppressor factor. This interesting finding need to be confirmed by further studies exploring the mechanisms of up-regulation of expression of IL-6 in macrophage-infected macrophages (Russell, Sturgill-Koszycki et al. 1997). In addition, IL-4, secreted by mast cells and both CD4 and CD8 T cells, can provoke apoptosis of stimulated, but not resident, monocytes, being qualified of “major monocyte deactivating factor” by these authors (Mangan, Robertson et al. 1992). This apoptosis-promoting action is mediated through the down regulation of CD14, which is the receptor for complexes of lipopolysaccharides (LPS) and LPS binding proteins that are required for monocyte activation (Heidenreich, Schmidt et al. 1997). Interestingly, LPS, macrophage colony stimulating factor (M-CSF), TNF-α and IL-1β are all factors capable of activating monocytes/macrophages, with prolonged survival and inhibition of apoptosis (Mangan, Welch et al. 1991).

Depending upon the system studied, it appears that the apoptosis inducing mechanisms are independent of specific cell surface markers previously thought to be important, such as CD4 or CD8, but rather to the up-regulation of key factors such as, for example, the Fas/Fas ligand under the appropriate cytokine milieu. Markers of suppressive activity may exist, but they remain to be identified, as do markers of memory state of lymphocytes (London, Perez et al. 1999), (see also section S page 60). In the granuloma, IL-10, (which is currently identified as a prime cytokine in down-modulating the immune response) (Muraille and Leo 1998) and TGF-β, the main anti-inflammatory cytokine, are now believed to be very important mediator of the down-regulation of the immune response including CMI. More specifically, it is now proven that this cytolytic activity (i.e. induction of apoptosis) is not specific of any lymphocyte sub-population and is in fact part of a more general mechanism of tissue homeostasis in multi-cellular organisms (Cohen 1999).

In conclusion, it appears that in efficient CMI, several cell types can have the orchestrating and effector function, depending on the nature of the infectious process. It should also be stressed that the helper, suppressor and effector activities are loosely dependant, not to say independent of the CD4 and CD8 molecules, whose main function remains the stabilization of the TCR/CD3 – MHC/foreign molecule fragment during antigen presentation. Responses characterized by dominant CD4 and CD8 lymphocytes will depend on the dominance of phagocytized particulate antigens (Ag) versus intracytoplasmic Ag, respectively. In mycobacterial infections in humans, it is to be expected to observe
predominant CD4 T lymphocytes due to presentation of processed phagocytozed bacteria. In this system, CD4 T cells have been shown to have both helper, cytotoxic functions and, more rarely, suppressor function (Modlin, Mehra et al. 1986). CD8 T cells are also present in lesser numbers in mycobacterial infection, either because mycobacteria escape in the cytoplasm or secrete proteins, the peptide fragments of which can be presented with MHC class I molecules. CD8 have been shown in this system to have cytotoxic, suppressor and, less frequently, helper activities (Modlin, Mehra et al. 1986; Li, Sad et al. 1997).

To illustrate the role of CD4 and CD8 molecules in CMI and open further thoughts and discussion, CD4/CD8 double knockout mice have been generated and tested in intracellular infections. These mice have a larger granulomatous response against heat killed BCG and heat killed Corynebacterium parvum than did control mice. However, CD4/CD8 KO mice challenged with live BCG showed initially fewer and smaller granulomas but later more and larger granulomas than control mice. Interestingly, the level of IFN-γ mRNA was similar in CD4/CD8 knockout and control mice. Inoculation of live BCG resulted in progressively larger granuloma with higher bacterial loads within these lesions (Senaldi, Saklee et al. 1999). Therefore, and to generalize these findings, CD4 and CD8 are not essential to elicit a granulomatous response against intracellular bacteria and adequate cytokine response such as the secretion of IFNγ, but contribute to improve the host defense against live mycobacteria. This latter finding is not surprising in light of the critical role played by CD4 and CD8 for a successful antigen presentation and subsequent adaptive CMI with efficient acquired and memory cell immunity.

2.1.3 How Can CMI Be Studied?

One important drawback in studying CMI is that relatively few in vivo tests and in vitro means and techniques are presently available, and even fewer methods were successful before recombinant DNA technology and molecular definition of cytokines became available. Similarly, at the local microenvironment (i.e. the granuloma), functional tests are still impossible to perform because in vitro reconstitution of granulomas has not yet been achieved. The standard in vitro test for acquired CMI is the lymphocyte stimulation test (LST), which is a development of the former, which measures cell proliferative response through incorporation of tritiated thymidine in mononuclear cells stimulated with
specific antigens. In vivo, CMI may be measured through injection of cocktails of antigens able to elicit delayed-type hypersensitivity (DTH) reaction in the patient. However, DTH is not synonymous of CMI but rather represent an extreme, or better said, a polarized aspect of CMI (see next section). DTH may also be interpreted as the memory side of adaptive CMI and the phenomenon is probably highly dependant on the rapid secretion of appropriate chemokines.

New means to explore factors regulating CMI is to assess KO mice, to perform longitudinal studies using cytokine probes and markers either at the mRNA level or the protein level, respectively, in ex vivo or in situ studies. Those means have already permitted the achievement of tremendous advances in leprosy and mycobacterial research. The results of some of these new means will be presented in the next sections.

2.1.4 PRESENTATION OF NON-PROTEIN FRAGMENTS TO T CELLS
This breakthrough in the understanding of antigen presentation of non-peptide antigens has been recently characterized. These non-peptide antigens are mainly complex lipids (lipids and glycolipids) with phosphate residues, which are mostly presented with CD1 molecules, a recently characterized third lineage of antigen-presenting molecules (Porcelli, Morita et al. 1992; Tanaka, Sano et al. 1994; Grant, Degano et al. 1999) (reviewed by (Sugita, Moody et al. 1998)). In humans, several CD1 molecules have been identified, which are capable to mediate the generation of strong CMI to various microbial antigens (Fairhust, Wang et al. 1998), including the mycobacteria (Beckman, Melián et al. 1996). Importantly for leprosy, CD1 expression, similar to other MHC molecules, is up-regulated in patients with tuberculoid leprosy and reversal reaction whereas there was an absence of CD1 expression in lepromatous leprosy (Modlin and Rea 1994) (Sieling, Jullien et al. 1999). During culture of blood monocytes from both categories of leprosy patients, however, CD1 molecules could be induced at similar levels. This suggested that the lack of CD1 expression locally in the skin of lepromatous patients is related to local factors rather than defective CD1 expression in leprosy patients (Sieling, Jullien et al. 1999). The situation is quite similar in tuberculosis, whereby CD1+ antigen presenting cells (APC) down-regulated CD1 surface expression after infection with live Mycobacterium tuberculosis. This effect is not due to secretion of cytokine, requires live bacteria and is unique to CD1 since MHC molecules were not down-regulated (Stenger, Niazi et al. 1998). This new escape mechanism described in vitro with M. tuberculosis fairly matches the in vivo situation in leprosy since
MHC class II Ag are present and not down-regulated in patients with lepromatous leprosy (Collings, Tidman et al. 1985). However, in vivo, it is not known whether this is a direct effect of M. leprae or a consequence of the cytokine milieu, since GM-CSF, a cytokine highly expressed in tuberculoid leprosy can up-regulate CD1 expression on APC, whereas IL-10 down-regulates CD1 expression (discussed and reviewed in (Yamamura, Uyemura et al. 1991).

2.1.5 cytokine network and cytokine functions in the granuloma
2.1.5.1. Important cytokines secreted by macrophages composing the granuloma

All cells in a granuloma can secrete cytokines. Notably, eosinophils and other inflammatory cells, which are characteristically present in certain types of granuloma elicited by parasites such as Schistosoma mansoni, do secrete cytokines and other mediators. The classical view is usually that granulocytes are present earlier than the mononuclear cells at site of inflammation due to invading micro-organisms. This classic view has been challenged by recent kinetic studies, in which accumulation of granulocytes does not always precede, but sometimes follows the recruitment of mononuclear cells, depending on the type of injury elicited by the injurious agent (Buchanan and Murphy 1997). Therefore, the hypothesis implying that other cell types would be important in ‘pre-defining’ a cytokine milieu in which lymphocytes and macrophages would mature is not universally applicable or accepted in every bacterial or fungal disease.

The paragraphs below will focus on the cytokines secreted by macrophages (MΦ) within the granuloma. Macrophages (MΦ) can secrete two types of cytokines: on the one hand they may secrete IL-1, TNF-α, IL-12 and IL-18 and on the other hand they can secrete IL-10, IL-6 and TGF-β. These 2 groups of cytokines have pro-inflammatory and anti-inflammatory properties, respectively. Using the various models of inbred mice, Mills recently proposed and defined M-1 and M-2 types of macrophages, following the model of T helper lymphocytes and stressed their importance since, in many models of intracellular infections, MΦ are in the first line of defense against an infectious agent (Mills 1999), although this cannot be generalized for all infections, such as cryptococciosis where lymphocytes are recruited slightly before monocytes/MΦ (Buchanan and Murphy 1997). Mills showed that resident peritoneal MΦ from strains of inbred mice which secrete higher amounts of IL-12 and γ-IFN, and those which secrete TGF-β, have a corresponding difference in activation potential in response...
to γ-IFN and LPS (Mills 1999). Similarly, cultured dendritic cells (DC) can be induced to mature into at least 3 sub-types termed DC0, DC1 and DC2 depending of the relative amounts of IFNγ + IL12 and IL-4 cytokines in the culture media in addition to GM-CSF and IL-3. Those that matured with IL-12 and IFNγ in the medium (DC1) also secreted the highest levels of IL-12 (Sato, Iwakabe et al. 1999).

Whether this dichotomy will prove to be valid in a heterozygous outbred population of humans and other animals is worth of testing, especially with correlation with important markers of natural resistance to intracellular infection, such as the bcg / Nrampl gene. This is also a strong invitation to study the pattern of cytokine secretion of antigen presenting cells (APC), which might enable identification of the genetic factors responsible for allergic responses to common antigens.

The disruption of murine genes by specifically targeted vectors to obtain gene deficient homozygous mice, otherwise called knockout (KO) mice, is a very powerful tool to investigate whether a certain mediator is critical or not (i.e. can be replaced by redundant mediators) during granuloma formation and development of a protective CMI. Results of these in vivo experiments in mice are compelling, especially concerning cytokines in the M. tuberculosis model: IFN-γ is absolutely critical in the defense against mycobacteria since IFN-γ gene KO mice are incapable to handle Mycobacterium tuberculosis and rapidly die from the infection (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993). IL-18 and IL-12 are also essential for the establishment of a strong CMI with efficient clearance of pathogenic bacteria, and both act through the action of IFN-γ (Cooper, Magram et al. 1997; Mastroeni, Clare et al. 1999; Sugawara, Yamada et al. 1999). TNF-α is also important for granuloma formation and clearance of pathogenic M. tuberculosis (Kaneko, Yamada et al. 1999). In addition, T cell activation, as measured by DTH, was markedly reduced in IL-12 -/- mice (Cooper, Magram et al. 1997), highlighting the crucial role of this cytokine in this model system. IL-1 knockout mice have recently been produced (Horai, Asano et al. 1998) but, to the author’s knowledge, have not been tested in mycobacterial infection systems.

The case of IFNγ deserves special consideration because of its critical function in granuloma formation, clearance of intracellular bacteria through activation of MΦ (reviewed by Krahenbuhl 1994) and the fact that it is believed to be primarily secreted by T lymphocytes and natural killer (NK) cells. Of important note, MΦ can also secrete γ-IFN (reviewed in Gessani and Belardelli 1998). The in vivo
biological relevance of γ-IFN secreted by macrophage is still unclear, but the possibility that γ-IFN may have a more important role than previously thought in early phase of host response to infectious agent through NK and MΦ secretion is interesting. It would also explain the profound deficiency in handling intracellular pathogen such as *Mycobacterium tuberculosis* by γ-IFN knockout mice (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993), which somewhat differs in severity with CD4 and/or CD8 KO (Myers Caruso, Serbina et al. 1999; Senaldi, Saklee et al. 1999). However, the situation may be different in murine models and humans.

Fine dissection of the promotion of γ-IFN gene expression in T cells, the major source of this cytokine, indicates that the strongest up-regulation of γ-IFN secretion in CD4+ T cells occur when there is TCR activation through antigen presentation. However, recently, it has been shown in vitro that another way of inducing prolonged γ-IFN production independently of antigen presentation was through IL-18 and IL-12 added together in the culture medium (Yang, Murphy et al. 1999). This finding is interesting in view of the recent in vivo results obtained in CD4/CD8 double KO mice, in which levels of γ-IFN mRNA were similar to levels in control mice after infection with intracellular bacteria (Senaldi, Saklee et al. 1999).

As stated above, studies in KO mice may reveal compensatory mechanisms. Are there any IFN and/or IL12 deficient humans with disseminated intracellular infections? If none has been found and characterized so far, it is not the case concerning the receptors to these two critical cytokines. In contrast to cytokines, cytokine receptors may have less redundancy than the former. In man, children have recently been identified with IFN-γ and IL-12 receptor deficiencies, and diagnosed with disseminated mycobacterial and severe *Salmonella sp.* infection (Newport, Huxley et al. 1996; Altare, Durandy et al. 1998). Because there is now an intensive search for cytokine gene deficiencies in humans, it is likely that if a severe disease had been linked to a cytokine deficiency, it would have already been reported. It is therefore likely that cytokine mutations inducing mild or moderate cytokine malfunction with more insidious clinical consequences exist in nature and will be characterized in the future. This has already been shown for cytokine receptor deficiencies, where mild or severe clinical consequences are a function of the mutation sites (Altare, Durandy et al. 1998).
2.1.5.2 The Potential Complexity of Cytokines and Other Mediators Regulating the Granuloma

In an attempt to draw parallel models between T lymphocytes and macrophages, there is an important common concept: immune cell activation and maturation. There is a striking parallel between T cell clones which can mature from a uncommitted Th0 phenotype to mature, irreversibly polarized T cell clones (see part 2 of sme chapter, page 26-28), and monocytes, which can irreversibly mature either towards specialized phagocytic or specialized dendritic antigen presenting cells. They can exhibit a different morphology, such as epithelioid MΦ or dendritic cells (DC), and different surface receptors and cytokine secretion patterns. Increasingly, data show that monocytes, mature macrophages, activated/ epithelioid macrophages and other antigen presenting cells differ not only in their function and their relative expression of surface markers, but also in their responsiveness to cytokines. For example, human synovial fluid macrophages and human blood monocytes differ in their response to IL-4 (Hart, Ahern et al. 1993). Whereas IL-4 can suppress the release of both IL-12 and IL-10 by freshly cultured monocytes stimulated by lipopolysaccharides (LPS), synovium-derived mononuclear cells from the joints of patients with rheumatoid arthritis and LPS-stimulated monocyte-derived macrophages that had matured in vitro, down-regulate only IL-12, but not IL-10, when treated with IL-4. This difference may be attributable to a reduction in mRNA expression of the γc subunit (IL2Rγ) of the IL-4 receptor in the MΦ, which diminishes over time and correlates with a loss of IL-10 down regulation. This finding is compatible with an increase of the suppressor activity of IL-4 in the granuloma composed of mature macrophages (Bonder, Finlay-Jones et al. 1999) and offers a glimpse at the potential differences between monocyte or non stimulated, resident histiocytic cells and stimulated, mature phagocytic cells such as epithelioid MΦ and lepromatous MΦ.

In culture, bone marrow-derived dendritic cells can be induced to mature to different types of DC, depending on the composition of cytokines added to the culture medium. Specifically, adding IL-12 and IFNγ to the medium elicits DC, termed DC1, with high presentation capability as assessed by high expression of MHC molecules and co-stimulatory markers. In contrast, adding IL-4 to the medium favors the maturation of another type of DC, termed DC2. They can in turn favor the maturation / differentiation of T lymphocytes into Th1 and Th2 (see later section page 28), respectively, in a specific
manner (Sato, Iwakabe et al. 1999). The responsiveness of these different sub-types of DC to various cytokine milieus is currently being studied.

2.1.5.3 Mycobacterial Granulomas

Granulomas elicited by various mycobacteria do not present with the same morphology. The prototype of mycobacterial granuloma is the one elicited by *M. tuberculosis* in humans. It is characterized by focal lesion containing a large central area of caseous, sometimes mineralized necrosis surrounded by a wide mantle of epithelioid macrophages and a few multinucleate giant cells (MGCs), with nuclei at their periphery called Langhans' MGCs. Within and at the periphery of this mantle of MΦ are lymphocytes, mostly T cells. The granulomas seen in patients with tuberculoid leprosy are very similar to granulomas elicited by agents causing tuberculosis in mammals and birds, except that central necrosis is rarely if ever seen (Job 1994). If granulomas elicited by *Mycobacterium* infections are often discrete entities with low numbers of organisms in the lesions, other forms of mycobacterial disease exist in which the granulomas coalesce, forming sheets of macrophages loaded with an large numbers of mycobacteria. Examples are paratuberculosis of ruminants (Barker, van Dreumel et al. 1993; Jones, Hunt et al. 1997), and, in man, the lepromatous forms of leprosy (Job 1994) and mycobacterial infections in persons with the acquired immunodeficiency syndrome (AIDS).

A fascinating recent approach concerns the partial reproduction of different types of mycobacterial granulomas through the use of inbred strains of mice (Veazey 1995) and more recently through the study of gene-knockout mice. Targeted inactivation of critical genes coding for cytokines and other products believed to be important in CMI and granuloma formation have resulted in unorganized granulomas with fewer lymphocytes and uncontrolled replication of mycobacteria. For instance, no necrosis was seen in the granuloma of IL-18 deficient mice infected with mycobacteria (Sugawara, Yamada et al. 1999).

In a murine model of tuberculosis, aggregation of bone marrow macrophages was shown to be mediated by TNF (Barnes, Abrams et al. 1993). The study of TNF-α-deficient mice showed that TNF-α has an important role in protection against mycobacteria but plays an indirect role in granuloma formation (Kaneko, Yamada et al. 1999).
2.1.5.4 Conclusion

Studies of the cytokines secreted during intracellular infections indicate that an array of
 cytokines, e.g. IFN-γ, TNF-α, IL-1, IL-12 and IL-18, secreted by both lymphocytes and macrophages,
are critical for an effective granulomatous response capable of eradicating or at least containing the
invading micro-organisms. Up-regulation or suppression of the activation state of macrophages
composing a granuloma is of uppermost importance to the successful management of intracellular
infections, and is mediated by the helper and suppressor activity of cytokines secreted not only by T
lymphocytes, but also by other cell types, including the macrophagic cells.

However, many critical points in granuloma formation and successful eviction of intracellular
infection are still poorly understood, primarily due to the fact that the study of activated and mature
macrophages in vitro remains quite difficult. Macrophages apparently die rapidly after isolation from a
granuloma (J. Kraenbuhl, personal communication) whereas lymphocytes survive more easily in
standard culture conditions. Pre-culturing blood-derived monocytes in culture medium containing
defined sets of recombinant mediators for an appropriate interval before undertaking functional studies
at the protein and mRNA level of the macrophages may help to unravel the complexity of immuno-
regulatory mediators acting on the evolving granuloma (Hart, Jones et al. 1995).

2.1.6 Data on CMI of M. leprae Exposed Persons and ‘Indeterminate’
Leprosy Patients

Considerable debate still exists concerning the pathogenesis of Hansen’s disease. Evidence
from the epidemiology of the disease suggests that the vast majority of exposed individuals will never
develop the disease (reviewed by (Harboe 1994)). The incubation time is also believed, without proof
due to the present lack of reliable test of exposure, to be quite long, possibly decades (Fine 1982).
 Alternatively, healthy contacts may be exposed several times and during a possible weakening of innate
or acquired immunity, those individuals might eventually develop leprosy. There is virtually no data
and hence no evidence of difference of CMI in individuals recently exposed to M. leprae, which would
predict whether an individual will or will not develop leprosy.

The situation is further complicated in leprosy where very few pure, specific M. leprae antigens
are presently available to test CMI. CMI in leprosy has been classically studied through the injection in
the skin of killed whole M. leprae of armadillo or nude mouse footpad origin (lepromin), called the
Mitsuda test. This test measures the ability of an individual to generate a granulomatous response against extract of *M. leprae* after 2-3 weeks. In a proportion of patients, however, there is a DTH-like reaction within 2-3 days after injection of lepromin, called the "early Fernandez reaction", similar to what is seen after injection of *M. tuberculosis* protein purified derivative (tuberculin) in *M. tuberculosis*-exposed or *M. bovis* BCG-vaccinated individuals. Disrupted, purified mixtures of proteins have been developed from the leprosy bacillus, called leprosin, in an attempt to produce early Fernandez reaction without late development of granulomas at site of lepromin injection, with variable results. However, several drawbacks exist using those reagents. First, it is well established that mycobacteria share numerous antigens and epitopes, complicating the interpretation of any positive result. Second, due to the lack of *in vitro* culture of *M. leprae*, one cannot be completely sure that the CMI is not directed against antigens of mouse or armadillo origin, even though this seems quite unlikely (reviewed in Harboe 1994).

2.1.6.1 Indeterminate Leprosy

Indeterminate leprosy is characterized grossly by a usually unique discrete slightly depigmented lesion that is often overlooked or considered unimportant by the patient. Microscopically, the skin has sparse, peri-adnexial and peri and intra-neural mononuclear infiltrates of predominantly lymphocytes but no granuloma present. Neural infiltrates with nerve damage are sometimes seen. Rare acid fast bacteria are found in nerves, arrector pili muscles or in the region immediately beneath the basal layer of the epidermis (Job 1994). These cases are unfortunately too rare and unpredictable to allow systematic sampling and collection of enough cases to study surface markers *in situ* in those lesions. With such a low level of inflammation, the only technology enabling the study of cytokines with sufficient sensitivity is the powerful reverse transcriptase- polymerase chain reaction (RT-PCR) technology. To the author's knowledge, none of these studies have been performed so far.

2.1.6.2 Healthy Contacts

Healthy contacts with demonstration of exposure to *M. leprae* have been demonstrated in 1972 by positive lymphoblast transformation tests (Godal, Löfgren et al. 1972). Lymphocyte stimulation tests (LST) confirmed these results and showed increased specificity using purified protein antigens from *M. leprae* (Closs, Reitan et al. 1982). Considerable variation is seen in these healthy contacts, as a group, and it is not known whether the lowest responders might develop the disease and the highest
responders (in the range of tuberculoid patients) will remain disease free. In addition, there is so far no report of a recombinant *M. leprae* protein that does not cross-react serologically or with LST after exposure to other mycobacteria. Such peptide or lipid antigens would be of the greatest importance to compare the epidemiology of the leprosy infection versus leprosy disease.

One test may however provide an indication of developing multibacillary lepromatous leprosy. This is when a healthy contact does not present with a positive Mitsuda reaction (testing the ability to mount a CMI), even though he/she has been in contact with (a) leprosy patient(s) for years. It has been shown that these individuals are at higher risk to develop lepromatous leprosy (reviewed in Harboe 1994, page 95). If a sensitive and specific test for exposure existed (requiring antibodies raised specifically against an antigen unique of *M. leprae*), an important question concerning *M. leprae* pathogenesis could be answered. Specifically, it would indicate whether these individuals are already showing a strong down modulation of CMI (type 2 milieu – see section 2 page 40) with detectable antibodies or are unable constitutively (without priming by infection) to mount any innate and acquired immunity against the pathogen with a complete absence of acquired immunity.

2.1.7 FULLY ESTABLISHED 'DETERMINATE' LEPROSY

Fully developed, ‘determinate’ leprosy is characterized by several clinical and pathologic manifestations that correspond to markedly different immunological features, which have been linked and integrated by the classification of Ridley and Jopling (Ridley and Jopling 1966) (Figure 2.1), fully establishing the concept of a clinical, pathologic and immunological spectrum. This spectrum has two polarized, rather stable manifestations: Polar lepromatous (LL) and polar tuberculoid (TT) of Hansen’s Disease. In between those two extremes are intermediate manifestations named borderline lepromatous (BL), borderline leprosy (BB) and borderline tuberculoid (BT) leprosies. Strong CMI is the important feature of the polar tuberculoid patients. Conversely, the lepromatous patients, both BL and LL are characterized by a moderate and marked deficiency of cell mediated immunity, respectively. The different borderline leprosy manifestations are typically unstable and most frequently subjects to burst of increased cellular immunity called reversal reactions (RR) or increased immunity seen in lepromatous patients called erythema nodosum leprosum (ENL). ENL is believed to be a type III hypersensitivity reaction with vasculitis. These immunolo-pathological reactions result in various
sequelae, the most important being nerve damage. Figure 2.1 summarizes the salient features of the immunological spectrum of leprosy.

The main classification (Ridley and Jopling 1966) describes 5 categories (Figure 2.1) and is primarily for research use. Follow-up biopsies indicate that those patients in the borderline spectrum evolve towards either the lepromatous side or the tuberculoid side of the spectrum, if left untreated. Since a RR is an upgrade of cellular immunity, the consequence of a RR is often regarded as a shift to the left side of the spectrum (Figure 2.1) corresponding to a position in the spectrum closer to the tuberculoid pole.

Figure 2.1: Immunological spectrum of leprosy. This diagram summarizes the salient features of the spectrum of Leprosy as defined by Ridley and Jopling. TT = polar tuberculoid, BT = borderline tuberculoid, BB = borderline, BL = borderline lepromatous, LL = polar lepromatous.

Since there is so far no evidence of any genetic diversity by restriction length fragment polymorphism or other methods amongst *M. leprae* obtained from diverse geographic areas (Williams, Gillis et al. 1990; de Wit and Klatser 1994), it is currently believed that the leprosy bacillus is phylogenetically represented by one strain with no variation in pathogenicity. If so, then it is assumed that the clinico-pathologic and immunologic variations observed in Hansen’s disease patients may only represent the ability of the host to cope with the microbe. It is thought that differences in innate and acquired immunity are the key to the understanding of the leprosy spectrum.
2.1.7.1 CMI in Tuberculoid Patients

Tuberculoid leprosy is characterized by a few limited, well demarcated lesions in the skin containing well formed and organized granulomas resembling the granulomas in tuberculosis except for an absence of central necrosis. Epithelioid macrophages, which are thought to be strongly activated MΦ, are most common cell in the granuloma together with lymphocytes with a CD4:CD8 ratio of ~ 2:1 (Modlin, Hofman et al. 1983; Modlin and Rea 1994). In tuberculoid granulomas, immunohistochemistry from frozen biopsies shows that the CD4 positive cells are distributed in the center of the granuloma in close association with the macrophages and the CD8+ T cells are primarily distributed at the periphery. Double immunofluorescence labeling and flow cytometry show the large majority of CD4+ T cells to be CDw29+ and CD45RO+, suggesting that the CD4+ cells are of the activated/memory phenotype (Modlin, Melancon-Kaplan et al. 1988). The majority of CD8+ T cells are CD28 positive, a prime marker of positive co-stimulation following TCR engagement. CD28 is an important costimulatory marker necessary to up-regulate T cell activation upon T cell presentation. The antigen presenting cells (APC) in the epidermis, the Langherans’ cells, as assessed by the CD1 marker by immunohistochemistry, are numerous in the overlying epidermis as well as at the periphery of the granuloma (Modlin, Hofman et al. 1983). Numerous CD1 positive dendritic cells are also present in granulomas of patients suffering from Reversal Reaction.

In tuberculoid lesions, there are 100 times more lymphocytes capable of proliferation in response to M.leprae antigen than in the blood of the same patient, as assessed by T cell precursor frequency analysed by limiting dilutions of T cells (Modlin, Melancon-Kaplan et al. 1988). T cell lines generated from these lesions are mostly of helper phenotype (Modlin, Mehra et al. 1986) and they secrete a predominance of type 1 cytokines, independently of whether the cells are of CD4 and CD8 phenotype. The lesions have a strong ex vivo amplification signal for γ-IFN, IL-2 and lymphotoxin (TNF-β) after relative RT-PCR standardized on CD3-δ (Yamamura, Uyemura et al. 1991). T lymphocyte lines (T cell clones) extracted from these tuberculoid lesions have the same characteristics, that is γ-IFN, IL-2 and GM-CSF production, with little or no secretion of IL-4, IL-5 or IL-10 (Salgame, Abrams et al. 1991). Similar results are found in the blood, assessing M. leprae stimulated total PBMC as well as T cell clones, except that such a polarization and clear cut results are not found. Notably, half
of the tuberculoid patients has, directly or from T lymphocyte clones cultured from peripheral blood, co-expression of IL-4 and IFN\(\gamma\) messenger RNAs (mRNA) (Salgame, Abrams et al. 1991; Misra, Murtaza et al. 1995). Messenger RNAs encoding cytokines mainly secreted by macrophages, such as IL-1\(\beta\), TNF-\(\alpha\), GM-CSF and IL-6, were found at high levels in tuberculoid lesions. Notably, the highest expression of \(\gamma\)-IFN \textit{in situ} and \textit{ex vivo} has been found in reversal reaction (Cooper, Mueller et al. 1989).

### 2.1.7.2 CMI in Lepromatous Patients

Lepromatous leprosy is characterized by numerous poorly demarcated and sometimes nodular cutaneous lesions composed of sheets of foamy macrophages with ill-defined cell boundaries, containing myriads of acid fast bacilli. The lymphocytes are scattered throughout the lesion and are not statistically significantly in reduced numbers compared to TT lesions. The CD4:CD8 ratio is lower, however, in the range of 0.5:1 and the macrophages/lymphocytes ratio has not been examined and compared to tuberculoid lesions. The majority of CD8+ lymphocytes are devoid of the CD28 co-receptor of T cell activation, but express high levels of CTLA-4 (CD152) (Schlienger, Uyemura et al. 1998). This co-stimulatory molecule is transiently expressed in activated T cells, is believed to deliver a negative immuno-regulatory signal and is suspected to be able to induce anergy and acquired tolerance (recently reviewed by (Sayegh 1999). The proportion of memory/activated CD4+ T cells, as assessed by the CDw29+ and the CD45RO+ markers is lower than the one observed in the tuberculoid patients, with notably more CD4+ T cells devoid of CDw29 and expressing the isoform of CD45 (CD45RA) associated with naïve T cells (Modlin, Melancon-Kaplan et al. 1988). CD1-positive antigen presenting cells are infrequent in lepromatous patients (Modlin, Hofman et al. 1983; Sieling, Jullien et al. 1999). Recently, the discovery of CD1 mediated antigen presentation has been emphasized (Porcelli 1995) (Maher and Kronenberg 1997). CD1 is a family of non-polymorphic transmembrane proteins that associate with beta-microglobulin, resemble MHC class I molecules structurally and are prominently expressed on APCs. The unique feature of CD1 restricted presentation is the ability to present non-peptide, lipid and lipid-containing antigens to CD4 negative T cells (Porcelli, Morita et al. 1992; Grant, Degano et al. 1999; Sieling, Jullien et al.1999). This third lineage of presentation molecules (Grant, Degano et al. 1999) have been shown to play a significant role in immunity against mycobacteria and \textit{M. leprae} since (1) the CD1 molecules are down-regulated in lepromatous leprosy \textit{in vivo} (Sieling,
Jullien et al. 1999), (2) there is down regulation of CD1 on APC following in vitro infection with virulent M. tuberculosis, suggesting a new escape mechanism (Stenger, Niazi et al. 1998) and (3) there is abrogation of M. tuberculosis induced γ-IFN production by CD1 restricted T cell lines with CD1b monoclonal antibodies in vitro (Gong, Stenger et al. 1998). Lipid and glycolipids such as lipo-arabino-mannan (LAM) are typical antigens that are CD1 restricted and presented to αβ double negative CD4-, CD8-, T cells. It also appears that CD1 mediated presentation is rather broad and also involves presentation of non-peptide antigens from gram negative bacteria. It has already been shown for Haemophilus influenzae type B (Fairhurst, Wang et al. 1998).

The T cells in lesions of lepromatous leprosy have been shown to exhibit significant suppressive activity, as evidenced by the inhibitory effect of the addition of Dharmendra lepromin after lectin-induced proliferation (Modlin, Mehra et al. 1986). These suppressive activities were found mostly, but not exclusively, in CD8+ T cell lines. The lesions have a strong ex vivo amplification signal for IL-4, IL-5 and IL-10 after relative RT-PCR standardized on CD3-δ (Yamamura, Uyemura et al. 1991). In addition, after analysis of cytokine production profiles of isolated T cell clones, these CD8+ suppressor cells from lepromatous lesions have a strong expression of IL-4 and lower amounts of IL-5 (Salgame, Abrams et al. 1991). Following this landmark discovery, several experiments were designed to demonstrate the suppressive role of IL-4 in lepromatous lesions. Interestingly, IL-4 was shown to selectively augment the proliferative potential of PBMC from lepromatous patients, but not tuberculoid patients (Salgame, Abrams et al. 1991; Sieling, Abrams et al. 1993). These data together with other important findings of this important role of IL-4 will be developed in the next section (page 36). The other important player of the down modulation of CMI in lepromatous lesions turned out to be IL-10 and TGF-β which are mainly secreted by macrophages. Interestingly, M. leprae stimulated PBMC from patients and healthy donors secrete abundant IL-10. In this system, IL-10-blocking monoclonal antibodies significantly increased the secretion and proliferation of T cells. These T cells have augmented γ-IFN, TNF-α and GM-CSF (Sieling, Abrams et al. 1993). Lastly, and very importantly, recombinant cytokines such as IL-2 and IFNγ injected in the site of lepromatous lesions produced significant clearing of M. leprae bacilli and recruitment of large numbers of T cells, most of them being of "helper" phenotype, with minimal side effects (reviewed by (Kaplan and Cohn 1991). These
therapeutic trials are in any case fundamental because they indicate that the ‘primary defect’ of the specific anergic state and lepromatous leprosy research is not at the T lymphocyte level because the lesions are almost fully reversible if the right milieu is artificially recreated. The “defect” may rather be during antigen presentation or in an as yet not described deficient macrophage function (or a combination of defective functions). A very interesting opening in the direction of antigen presentation has been quite recently observed by Dr. Modlin’s group: they found a profound down regulation of B7-1 (CD80), a slight down-regulation of B7-2 (CD86) and a marked down-regulation of CD28 mRNA expressions in skin lesions of lepromatous patients, whereas CTLA-4 mRNA expression was up-regulated in lepromatous patients compared to tuberculoid patients, which had expressions of B7-1, B7-2, CTLA-4 and CD28 mRNA, in relatively similar levels. Moreover, in vitro, they showed that the B7-1 (CD80), but not CD28, is crucial for the maintenance of the CD4+ T cell response in tuberculoid leprosy (Schlienger, Uyemura et al. 1998). This finding points strongly towards APCs, on which the different types of B7 molecules are usually expressed at high levels. Importantly, the kinetics of the expression of the B7 molecules are important during an immune response. Elucidation of factors regulating the expression of B7-1 (CD80) and B7-2 (CD86) molecules over time on APC and macrophages in leprosy, and in particular indeterminate leprosy and evolving borderline leprosy might enable leprosy investigators to gain insight into differences in antigen presentation in this disease. Longitudinal studies following M. leprae inoculations in animal models might also be critical in that regard.

2.1.8 CONCLUSION ON CMI AND LEPROSY

In conclusion, there has been a tremendous effort in the last 20 years to carefully study and contrast the immuno-pathologic features of established leprosy, especially by Modlin and collaborators (Modlin and Rea 1994). Careful review of CMI in leprosy leads to formulate the two following statements and associate questions:

1 - Contrary to classic opinion, cell mediated immunity (CMI) mediated by T cells does exist throughout the leprosy spectrum, even though it is greatly reduced in the lepromatous pole of the spectrum, most probably contributing to the uncontrolled replication of the organism. In contrast, delayed-type hypersensitivity (DTH), is totally abrogated in lepromatous leprosy. The important question is then whether this CMI down-regulation is critical and at the origin of the initial development
of LL or accompanies and limits the consequences of a defect located elsewhere in the immune and defense system of the lepromatous patient.

Likewise, it is an established fact that CMI is relatively high in tuberculoid pole of the disease spectrum. This CMI is not completely efficient since the organism does persist. What is different between the CMI of an individual that rejected \textit{M. leprae} and never had the disease and a CMI of a tuberculoid patient? And is there any difference at all? These are the crucial questions that might be answered only through animal models. One can, however, propose that CMI is higher in asymptomatic exposed individual as compared with tuberculoid patients but this has not been demonstrated by TTL and LST tests and thus remains to be proven.

2.2 TYPE I AND TYPE II T LYMPHOCYTE HELP PARADIGM AND ITS APPLICATION IN LEPROSY

2.2.1 INTRODUCTION

This review focuses on the recent publications on the topic from leprosy research and other mycobacteria research and a few other diseases and animal models that can help to comprehend the consequences of the type 1 / type 2 T cell help paradigm in the understanding of the immune response to Leprosy, as Modlin and collaborators have done (Modlin 1994). Also mentioned will be the puzzles that still remain in this finely tuned network of cytokine cross regulations. Some important experimental results that are useful for the understanding of the research results published in this volume will also be developed and discussed.

2.2.2 OVERVIEW OF THE TH1/TH2 PARADIGM

Recently, it has been established that CD4 positive T-helper lymphocytes are committed to secrete certain sets of cytokines that have been originally characterized from cloned cultured T lymphocytes of murine origin (Mosmann and Coffman 1987) and have differing functions in immune regulation (Lucey, Clerici et al. 1996).

At the clonal level, T lymphocytes bearing the $\alpha \beta$ T cell receptor can be divided into at least 3 subsets with regard to the pattern of cytokine secretion, as initially defined by murine CD4+ T cell clones cultured in vitro: The T helper type 0 (Th0), Th1 and Th2 (Mosmann, Cherwinski et al. 1986). This finding was subsequently shown to apply to humans (Romagnani 1991) and other animal species, in particular non-human primates (Ansari, Mayne et al. 1994). T helper type 1 cells mainly secrete IFN-
γ, TNF-β (lymphotoxin –LT), GM-CSF and IL-2. They may also secrete under appropriate conditions low levels of IL-15. T helper 2 cells mainly produce IL-4, IL-5, IL-6, IL-10 and IL-13. Under appropriate conditions and depending on the species studied, Th1 cells may also produce IL-10 (Muraille and Leo 1998). Th0 can produce a combination of IL-4, IFN-γ and any of the cytokines mentioned above. In T lymphocyte cultures (T cell clones), it appears that the Th0 phenotype may be unstable and gradually evolves towards one pole or the other. Other researchers consider that unstable precursors of T cell clones subsequently mature to stable Th1, Th2 and stable Th0 clones. Differences between groups and species of mammals are possible (Lucey, Clerici et al. 1996).

After extensive studies of Th1 and the Th2 system applied in vivo and in vitro, several facts came to light: First, the most critical cytokines secreted by T lymphocytes with a defined, stable, "mature" cytokine pattern are γ-IFN and IL-4, for Th1 and Th2, respectively. Second, the Th1 and Th2 dichotomy also includes cells other than populations of CD4+ T lymphocytes, including also CD8+ T lymphocytes, termed Tc1 and Tc2 (Sad, Marcotte et al. 1995) and macrophages secreting IL-12 + IFNγ and IL10, respectively. However, whereas IL-12 is an early, critical cytokine shifting the response towards type 1 cytokine secretion, IL-10 is a late cytokine that down-regulates inflammation and does not influence early type 2 differentiation (Muraille and Leo 1998).

2.2.3 THE ANTAGONISTIC ACTIONS OF IL-4 AND IFN-γ

IL-4 and IFN-γ have antagonistic, but not symmetrical regulatory actions in the acquisition and maintenance of type 2 and type 1 CMI, respectively (Muraille and Leo 1998; Paludan 1998). Whereas IL-4 can drive the differentiation towards a type 2 response, IFN-γ appears be dependent upon the secretion of IL-12 to be secreted by T lymphocytes. Both IL-4 and IFNγ are then critical in the maintenance of their respective type of immune response and the inhibition of the other type of response. Whereas the trigger of secretion of type 1 cytokines in the innate stage of the immune response is now well established to be IL-12, in synergy with IL-18, secreted by the macrophages and APC, the early trigger of the type 2 response, believed so far to be early secretions of IL-4 is still ill-defined and subject to considerable debates (Haas, Falcone et al. 1998). Although it is suspected that mast cells and basophils, which are strong producers of IL-4, might be also early producers of IL-4 driving outbred mammals to mount type 2 immune responses in vivo, this has not yet been formally
demonstrated (reviewed in (Haas, Falcone et al. 1998). Another uncertainty is whether some subsets of antigen-presenting cells (APC), such as dendritic cells (DC), could be early producers of IL-4 or another, as yet not characterized, cytokine (i.e. the homologous of IL-12 for type 2 response).

Interleukin-4 and IFNγ can inhibit each other’s production quite strongly at the level of T cell clones, findings that are not true for any other cytokine such as IL-2 and IL-13, for example. IL-4 and IFNγ also have antagonistic actions on macrophages. IL-13 has down regulatory actions very similar to IL-4. However, in contrast to IL-4, which has its main action on T cells, IL-13 acts predominantly on monocytes and MΦ, where it has differing actions (de Vries 1996). In monocytes, IL-13 promotes differentiation towards a dendritic APC. In macrophages, it inhibits some key markers of macrophage activation after exposure to LPS, including as down modulation of CD14 and of the receptors of the constant region of immunoglobulins (FcγR) and reduction of effector functions such as the generation of nitric oxide (NO) (de Vries 1996). In the macrophage, these actions are closely similar to those seen with IL-4 and may contribute to the deletion by apoptosis of the incoming monocytes in inflammatory sites via down regulation of CD14 (Heidenreich, Schmidt et al. 1997).

The two key cytokines IL-4 and IFN-γ can activate widely different pathways of second messengers and transcription factors (recently reviewed by (Paludan 1998). Reviewing the fine molecular events responsible for the activation of specific sets of genes is beyond the scope of this review. It suffices here to say that, according to the present state of knowledge, there are no shared receptors or even second messengers and transcription factors between the IL-4 and the γ-IFN mediated activation pathway, which are through the signal transducer and activator of transcription (STAT) 4 and STAT 1, respectively.

2.2.4 THE DICHTOMY OF T Helper 1 AND T Helper 2 CYTOKINE SECRETION PATTERNS IS BEYOND THE T LYMPHOCYTES SUBSETS

2.2.4.1 Introduction

This grouping of T cell derived cytokines and other critical ones predominantly produced by other cell types such as APC and MΦ can be found in less stringent in vitro systems and in vivo systems. This corresponds to the preferential development of strong cell mediated immunity or a strong, predominant humoral immunity. From the observations made on in vivo immune responses came the concept of type 1 and type 2 nomenclature (Lucey, Clerici et al. 1996) and the important concept of
cytokine milieu. However, whereas cross regulation between Th1 and Th2 was clearly established at the cloned T cell level, the cross regulation of type 1 and type 2 cytokine expression in complex cellular systems and tissues turned out to be far more difficult to decipher than originally hoped (Muraille and Leo 1998). Nevertheless, this pattern of cytokine secretion corresponds to clinical and pathologic entities and fully establishes the model at a central pivotal position of up regulation (helper) and down regulation (suppressor) of the immune and inflammatory (i.e. immuno-pathologic) response.

2.2.4.2 Tentative Model of Cross Regulation of the Type 1 and Type 2 Cytokine Systems: Application to Immuno-Pathologic Conditions

Assuming that the T lymphocytes are the leaders in an immune reaction, what factors are driving T cells towards a “mature” pattern of secretion of cytokines? Does this dichotomy of cytokine secretion and trans-regulation exist in any other cell types, such as mononuclear phagocyte (MNP) system and the granulocytes? It may be hypothesized that MΦ and APC, notwithstanding of their suggested variations related to their tissue/organ homing, might be viewed as two different cell types, dendritic cell and activated epithelioid macrophages could represent type 2 and type 1 APCs, with a common precursor, the monocyte. Even though this is not firmly established, there are a few recent studies that suggest that it may be the case, at least in vitro (Mills 1999; Sato, Iwakabe et al. 1999). Likewise, eosinophils/ basophils/ mast cells on one side and neutrophils on the other side may be viewed as type 2 and type 1 cells, respectively. A more established theory is that the mast cell/basophils may drive type 2 response (Haas, Falcone et al. 1998) and the APC/DC/ MΦ drive the type 1 response (Lucey, Clerici et al. 1996).

The difficulty in describing an integrated model of Type 1 and Type 2 cross regulation is related not only to the different cellular levels of secretion, but also in the timing of various cytokine secretions and the up-regulation of their receptors that peak, wax and wane while an immune reaction takes place in vivo. This may be different from isolated cells and lymphocytes that proliferate in vitro in response to cytokines and other mediators. This timing has not been extensively investigated yet because of repetitive analysis required. However, as various technologies mature to measure levels of cytokines and other mediators more easily and reliably, more immunologic studies will be generated regarding this important parameter.
It is now well established that an early peak of IL-12 is secreted by macrophages and/or NK cells upon entry of a pathogenic microorganism and activation of recruited inflammatory cells. This peak is critical for the subsequent elevated sustained secretion of IFN-γ that will strongly drive appropriate CMI and inflammation (O'Garra and Murphy 1996). As with any strong agonist, down regulatory mechanisms must exist to avoid excessive CMI and tissue damage, both up-stream (tissue cells such as epithelial cells, mesenchymal cells, endothelial cells, etc...) and down-stream (lymphocytes). In this regard, in humans, IL-10 secreted by Th2 lymphocytes and MNP cells and TGF-β secreted by various cells from an inflammatory site may play an important immuno-regulatory and anti-inflammatory role. Also, it seems that IL-18, secreted mainly by MΦ, which is structurally like IL-1 and functionally acts as a strong co-inducer of IL-12 to drive the immune response towards a type 1 cytokine milieu (reviewed by Kohno and Kurimoto 1998), might play an important role. Recent results in CD3+ T lymphocytes and NK1.1 cells in mice indicate that IL-18 is a potent co-inducer of IL-2 for the secretion of IL-13, especially in the absence of IFN-γ (Hoshino, Wiltrout et al. 1999). Therefore, in a strong type 2 environment or in an uncommitted cytokine milieu with no or low levels of secretion of IL-12, IL-18 secreted by certain subsets of APC or MΦ, enhances a type 2 milieu, instead of driving towards IFN-γ production and a type 1 response. This appears to operate through IL-13 production that in turn promotes the differentiation of monocytes into dendritic APC and IgA and IgE production by B cells (Table 2.1).

Beside cytokines secreted by MΦ, the relative expression of co-receptor molecules on APC and MΦ seems also to play a role in the differential activation and / or the maturation of Type 1 versus Type 2 T helper lymphocytes in the development of immune responses. For example, one group showed recently that CD28 with B7-1 versus B7-2 controlled Th1 versus Th2 development based in the experimental auto-immune encephalitis in mice and in in vitro assays (Kuchroo, Das et al. 1995). However, this field is still young and apparent paradoxes exist. For example and to illustrate further this field of co-receptors, there are studies of transgenic and knockout mice that suggest that CD28 co-stimulation can promote the production of Th2 cytokines (Rulifson, Sperling et al. 1997).
Table 2.2: Type 2 and type 1 cytokine milieu/network elicited against invading micro-organisms, dissected by cell types, dose, antigen presentation and accessory molecules.

### Cytokine milieu

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Differentiation</td>
<td>Secretion</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>IL-3, GM-CSF Neutrophils?</td>
<td>?</td>
<td>IL-3, GM-CSF IL-5 eosinophil IL-4 basophils</td>
</tr>
<tr>
<td>APC/MΦ*</td>
<td>Monocytes IL-3, GM-CSF IL-12 LPS§</td>
<td>Macrophages IL-12 +++ TNF-α IL-18 IL-1</td>
<td>Monocytes IL-3, GM-CSF IL-4</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Naïve T cells IL-12</td>
<td>Th1 cells IFN-γ+++ IL-2 TNF-β (LT) GM-CSF</td>
<td>Naïve T cells IL-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Other stimuli (Ag presentation)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Differentiation</td>
<td>Secretion</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Peptide-MHC</td>
<td></td>
<td>High affinity</td>
<td>Intermediate affinity</td>
</tr>
<tr>
<td>Dose</td>
<td>low</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Accessory molecules APC*</td>
<td>Anti B7-2? B7-1(CD80)</td>
<td>B7-1(CD80) Anti-B7-1 B7-2 (CD86)?</td>
<td>CD28</td>
</tr>
<tr>
<td>Accessory molecules T cells*</td>
<td>CD28</td>
<td>CD28†</td>
<td>CD28</td>
</tr>
</tbody>
</table>

§Lipopolysaccharide; LPS also blocks differentiation of DC (Palucka, Taquet et al. 1999).
¶ Antigen presenting cells / macrophages
† In the differentiation columns, the cytokines/factors mentioned favor or increase the given cytokine milieu and CMI type.
* (Kuchroo, Das et al. 1995; Ranger, Das et al. 1996; Rulifson, Sperling et al. 1997; Schlienger, Uyemura et al. 1998).
†CD28 and CTLA-4 appear to be associated with up and down regulation, respectively, of CMI rather than favoring type 1 and type 2 response, respectively. The CTLA-4-dependent inhibition of cytokine production (both IFN-γ and IL-4) in all types of T cells in mice is mediated by TGF-β (Chen, Jin et al. 1998).

IL-10, initially shown to be highly produced by Th2 murine lymphocytes, and also by macrophages, has been proposed to contribute in the development of the type 2 immune reaction. However, in humans, both Th1 and Th2 cell clones can produce IL-10 (reviewed in (Muraille and Leo...
Since many of IL-10 actions are similar to IL-4, one may suggest that IL-10 is more a down regulator of the inflammatory response than a strong promoter of a type 2 immune response like IL-4, thus confirming the initial name of this latter cytokine, namely cytokine synthesis inhibitory factor (CSIF). IL-10 turns out to be a cytokine with complicated actions, probably inhibiting APC migration in mice through the down-regulation of TNF-α and of IL-1 production (Wang, Zhuang et al. 1999) (reviewed in (Wang, Amerio et al. 1999), down modulating the Mφ activation system and cytotoxicity (Nabioullin, Sone et al. 1994) while at least partially promoting lymphocyte responsiveness by the up-regulation of CD25, the α chain of the IL-2 receptor (IL2Rα) of T cell clones in vitro in humans (Cohen, Katsikis et al. 1994).

At another level, it is established that IL-2 shares one and two common receptor subunit(s) with IL-4 and IL-15, respectively, including the IL-2 receptor gamma (IL-2R γ or yγ) (de Jong, Famer et al. 1996). At the cellular level of T cells in vitro, as well as ex vivo in lesions, there are no notable differences between IL-2 and IL-15, especially concerning the function, even though the main in vivo cell sources are different: IL-15 is mainly secreted by epithelial cells whereas IL-2 is the prototypic lymphokine. This example, which is probably frequent in the cytokine network, may in part explain some in vivo results found using cytokine gene knockout mice, which suggest variable levels of redundancy in the functions of cytokines: compensatory mechanisms are often shown when cytokine genes are inactivated. Studies of human genetic defects predisposing them to severe infection suggest that receptors rather than mediators may be limiting factors for adequate immune response (Newport, Huxley et al. 1996; Altare, Durandy et al. 1998; de Jong, Altare et al. 1998). Differential expression of these receptor sub-units in a particular cell type in vitro and discrete populations of cells in an inflammatory site in vivo are often tightly regulated by the early cytokine milieu (O'Garra and Murphy 1996).

Sharing of one receptor sub-unit of IL-4 and IL-2 would suggest that engagement of these cytokines would potentially activate, with variable efficiency, similar sets of transcription factors in the cells. This is puzzling since, at first analysis, IL-2 and IL-4 should have opposite actions. This can probably be reconciled by considering that IL-2 is probably an early autocrine growth factor mainly secreted transiently at high levels in precursor T cells that are not still committed into going towards any
type of cytokine profile. In a sense, IL-4, since it is closely related structurally to IL-2 and is also a cytokine with a high autocrine up-regulation, may be considered as an evolution of IL-2 that may down regulate this latter, whereas IFN-γ is from a radically different group of mediators, the interferons. Actors of innate immunity such as NK cells and macrophages predominantly secrete this group of structurally mediated molecules and this must be kept in mind when analyzing the type 1 cytokine environment.

To summarize, in the type 1 cytokine milieu, MΦ and NK cells play a key role by secreting IL-12 and IFN-γ, respectively, whereas the type 2 milieu would be the responsibility of Th2 lymphocytes or mast cells and basophils rapidly secreting high levels of IL-4. Cytotoxic NK cells and properly activated MΦ secrete a significant amount of IL-12 and IL-18 to drive the helper T cells which in turn secrete strong, sustained Th1 cytokines, while MΦ/APC present antigens to T cells, another strong inducer of IFN-γ by T cells. However, in the mononuclear phagocyte (MNP) system, IL-18 (and IL-1) may be the equivalent of IL-2 in T cells, since IL-18, depending on the initial cytokine milieu, may subsequently enhance a type 1 or type 2 cytokine milieu. On the other hand, IL-12 can be proposed as being the equivalent of IFN-γ at the MNP level. Although IL-10, mainly secreted by macrophages, has been proposed to be a strong driver of the type 2 cytokine milieu, the data, at least in humans, do not support this hypothesis. Rather IL-4, by virtue of its autocrine mode of action, seems to be able to perform this function. However, it is still possible that an additional mediator, stimulating type 2 response, possibly secreted by the dendritic (DC) cells and other APC/phagocytic cells will be soon characterized.

With the large numbers of studies exploring the function, the cell source, the regulation of cytokines, it may sometimes be fruitful to try to delineate where and when a cytokine is not produced. For example, careful analysis of primary data on cytokine mRNA expressions ex vivo in leprosy skin tuberculoid lesions reveals that the type 1 milieu has a low but detectable amount of IL-4 and IL-10 as detected by relative (semi-quantitative) RT-PCR, albeit in much lower amount, as compared to lepromatous patients. Conversely, with the detection limits of RT-PCR, absolutely and uniformly no signal for IFN-γ and IL-2 has been detected in lepromatous patients (Yamamura, Uyemura et al. 1991; Modlin 1994). Knowing that this relative RT-PCR was standardized on the amount of CD36 cDNA,
even if we take into account differences of priming and hybridization efficiency between these primer pairs and probes (that have been checked by serial dilution of plasmid positive controls (Yamamura, Uyemura et al. 1991)), these differences may teach one about the type 1/type 2 cross regulation and leprosy disease. First it may be that IL-4 is a down regulator of γ-IFN and IL-2 in a type 1 lesion whereas the latter 2 cytokines may not be strong down regulator of the former in a type 2 milieu. In other words, it might be that type 0/type 2 milieu are the default, less effective immune response in intracellular parasite infections, including mycobacteria, if the APC/macrophages fail to secrete key cytokines early after encounter of the invading organism, such as IL-12, TNF-α, IL-18, and IL-1, by decreasing order of importance. In fact, in vivo data from inoculations of animal models by pathogenic microorganisms and parasites is now accumulating showing that this is the case (reviewed by (Lucey, Clerici et al. 1996; O'Garra and Murphy 1996). That is also supported by in vitro data showing Th1/Th2 cross-regulation in lymphocyte culture systems. IL-4 can down regulate Th1 cell differentiation whereas IFN-γ cannot (O'Garra and Murphy 1996). At the clinical level of the leprosy disease, this subtle feature of the type 1 cytokine milieu provides a basis to the observation of certain patients on the tuberculoid side (BT) going progressively to the lepromatous side of the spectrum, especially before multidrug therapy was available.

2.2.5 TYPES OF CYTOKINE SECRETION AND MYCOBACTERIAL DISEASES, WITH EMPHASIS ON LEPROSY

In intracellular infections with predilection to the mononuclear phagocyte system (MNP), such as leishmaniasis and mycobacteriosis [reviewed in: (Lucey, Clerici et al. 1996)]. Th1/type 1 immune responses are associated with protection against disease development whereas Th2/type 2 immune responses are clearly linked to inadequate handling of the pathogen and progresson to disease. However, the unique situation of leprosy as an example of mycobacterial disease is that the Th1/Th2 paradigm seems to exquistely match with the spectrum of the disease (Figure 2.2).

2.2.5.1 Type 1 Immune Response in Leprosy and Other Mycobacteriosis

Type 1 cytokines, such as IFNγ nd IL-2 secreted by the T lymphocytes and IL-12 secreted by MΦ correlate with usually strong inflammatory responses and CMI in mycobacterial diseases, including leprosy. Type 1 cytokines are typically found in patients in the tuberculoid side of the immuno-
pathological spectrum (Figure 2.2). Interestingly, these T lymphocyte responses are driven by IL-12, mainly secreted by cells of the MNP system.

![Diagram showing the Th1/Th2 paradigm and the immunological spectrum of leprosy]

Figure 2.2: Immunological spectrum of leprosy and Th1/Th2 paradigm. This diagram is similar to the one shown in figure 2.1., except that it shows the types of cytokine secreted at site of leprosy skin lesions along the spectrum. Th1/type 1 and Th2/type2 stand for the type 1 and type 2 cytokine milieu driven by type 1 and type 2 T cells, respectively.

In tuberculosis, tuberculous pleurisy, considered to be a response with favorable outcome in tuberculosis (Ferrer 1997), has high amounts of IL-12 in the inflammatory pleural fluid (Zhang, Gately et al. 1994). The factors that determine IL-12 secretion are still ill defined, but it likely follows phagocytosis and presentation of antigens from the leprosy bacillus. The dose and pattern of enzymatic antigen processing before antigen presentation appear to be important (Maekawa, Himeno et al. 1998; Power, Wei et al. 1998). It may be that overloading of the phagocytic cells impedes the proper mRNA up-regulation of IL-12 but this has not been proven. In addition to the secretion of IL-12, which seems to be the key early driver to the type 1 cytokine milieu, IL-18, TNF-α, and also to a lesser extent by, IL-1 seem to be important, or at least to participate in the promotion of a type 1 response (Garcia, Uyemura et al. 1999). At the same time of this appropriate cytokine environment secreted by the key actors of the innate immunity, cells from the adaptive immunity, mainly the CD3+ T lymphocytes, relay by secreting high levels of IFNγ and GM-CSF (O'Garra and Murphy 1996). Some kinetic studies indicate that the
recruitment of numerous lymphocytes before the monocytes may be critical for an early strong type 1 cytokine milieu and development of an adequate primary CMI, at least in leprosy (Lathrop, Scollard et al. 1996) as well as in other models of intra-cellular infection (Buchanan and Murphy 1997). In this regard, the role of IL-2 seems crucial in the amplification of the recruitment and multiplication of lymphocytes at sites of invasion of microorganisms. Type 1 cytokines and chemotactic cytokines (chemokines) such as the α-chemokine MIG (monokine induced by IFN-γ) and IFN-γ induced protein-10 (IP-10) (Kaplan, Luster et al. 1987) and β-chemokines such as monocyte chemotactic protein (MCP) and macrophage inflammatory protein (MIP) are also important in mycobacterial disease to recruit more activated lymphocytes at sites of invasion [reviewed by (Luster 1998) and (Schluger and Rom 1997)]. Type 1 cytokines also favor granuloma formation; IL-12 enhances survival in long term (10 day) cultures of adherent human monocytes (Estaquier and Ameisen 1997).

In summary, a type 1 cytokine milieu in leprosy favors strong CMI with low, often undetectable levels of particular subtypes of immunoglobulin (Ig). This strong CMI is reminiscent of, but not identical to DTH, the prototype of which is tuberculin test after purified protein derivative (PPD). This also exists to a lesser degree in tuberculoid patients after injection of lepromin and is called the Fernandez reaction (reviewed by (Harboe 1994)). CMI with a type 1 cytokine secretion is recognized as the most efficient immune response against intracellular parasites, notably mycobacteria.

2.2.5.2 Type 2 Immune Response in Leprosy and Other Mycobacteriosis

The second type of CMI, the type 2/Th2, generally observed when dealing with extra-cellular organisms, is not characterized by prominent granulomatous response (see previous section on CMI), but rather favors humoral immunity and is characterized by the secretion of alternate subtypes of Ig, including IgA and IgE (Lucey, Clerici et al. 1996). A type 2 response therefore limits the adverse consequences of marked granulomatous response. Type 2 immune response also favors mucosal immunity and is therefore very useful for protective immunity against several infectious agents. It also favors eosinophilic granulomatous responses, also useful against metazoal parasites.

In mycobacterial diseases, and in tuberculosis in particular, this type of T cell help is not associated with protection, and is at first analysis detrimental to the host. However, nobody knows exactly to what extent type 2/Th2 immune responses are detrimental in these diseases.
In leprosy, type 2 T cell help is found in the lepromatous/multibacillary side of the spectrum, where IL-4, IL-5 and IL-10 are more expressed than type 1 cytokines (Figure 2.2). In contrast to a few infectious agents where type 2 response may be associated with some degree of protection against disease, such as schistosomiasis (Lucey, Clerici et al. 1996), the type 2 cytokine milieu in mycobacterial diseases suggests a problem in handling the pathogen, such as in lepromatous leprosy, or in tuberculosis (Yamamura, Uyemura et al. 1991; Hernández-Pando, Orozcoe et al. 1996).

But the question remains why more IL-4 and other type 2 cytokines are produced in lepromatous patients. Is it due to an initial, vigorous and aberrant secretion of IL-4 shortly after encounter with *M. leprae*, as it may be the case after exposure to parasite nematodes such as *Schistosoma mansoni* eggs or filarial species (Haas, Falcone et al. 1998)? Or is IL-4 progressively and secondarily produced in maturing lesions evolving towards lepromatous lesions either after an initial adequate strong Th1 response (down-regulation hypothesis) or after an initial, weak, uncommitted Th0 milieu or a weak and delayed type 1 milieu? This question cannot be simply answered by assuming that some external source of IL-4 is responsible, such as from basophils, mast cells or neutrophils recruited at granuloma sites since that has not been observed in microscopic tissue sections of mature or indeterminate leprosy lesions (Job 1994). However the morphology of lesions in human leprosy after initial exposure is essentially unknown.

Important data have been recently published on the kinetics of cytokine mRNA expression of IL-4, IFNγ and IL-12 in a model of tuberculosis in susceptible BALB/c mice (Hernández-Pando and Rook 1994). This study showed that the initial cytokine response in this model is a Th1 environment with rapid high mRNA expression of both IFNγ and IL-12 within approximately 14 days after inoculation of *M. tuberculosis* in the lung. After 60 days, signals for IL-4 was detected and at high levels and increased thereafter, indicating the evolution towards a type 0 cytokine milieu at site of infection. Interestingly, the emergence of the type 0 cytokine milieu at site of disease correlated with progression from limited granulomatous pneumonia to disseminated, severe pneumonia (Hernández-Pando and Rook 1994). This kinetic study in a mouse model of pulmonary tuberculosis correlated with the situation in tuberculosis patients versus healthy PPD-positive individuals, where a Th2 versus a Th1, respectively, was observed (Sanchez, Rodriguez et al. 1994; Surcel, Troye-Blomberg et al. 1994)
reviewed in (Cooper and Flynn 1995). Therefore, these studies suggest that, in tuberculosis, the initial response is developed in a type 1 cytokine environment that may progressively shift to a type 0/type 2 milieu with development of pneumonia. Whether this scenario is also true or different in human patients after exposure to *M. leprae* when developing progressive lepromatous leprosy is still unknown.

Recent studies have focused on secreted proteins of *M. leprae* using molecular methods and have shown that these proteins were very close the proteins secreted by *M. tuberculosis* (Harboe and Wiker 1998). However, whereas several secreted proteins of mycobacteria, such as the heat shock protein 60 (hsp60), have been demonstrated to be major immunogenic antigens, there have been no studies exploring whether *M. leprae* or *M. tuberculosis* proteins or non-peptide antigens may elicit a Th2 response in mice or humans (Orme, Andersen et al. 1993). Even though it is likely that the type of cytokine response may be variable depending on the antigen presentation for same mycobacterial antigens, this whole area of research is worth exploring, especially in the light of recent studies indicating that CD1 antigen presenting molecules are not as polymorphic as MHC class I and class II molecules. This raises the possibility that the early human response to mycobacterial non-peptide antigens may be more stereotyped than the MHC-II-restricted response to protein antigens (Maher and Kronenberg 1997; Sugita, Moody et al. 1998).

There are no reports of studies exploring the cytokines secreted within hours days or weeks following an inoculation/exposure of *M. leprae* either in humans or in animal models. Differences in bacterial load and/or in the capability of MΦ to degrade and kill mycobacteria offer an alternate hypothesis to explain an initial absence of type 1 response or a slow shift from the type 1 to the type 2 cytokine milieu over long period of time. Experimentally, it is well established that dose, together with route and type of antigen administration, determine a mutually exclusive predominance of antibody or DTH response. This aspect of type 1/type 2 determination, besides the pre-existing cytokine milieu, has been reviewed several times recently (O'Garra and Murphy 1996; Constant and Bottomly 1997). Very few data are published from mycobacterial and leprosy research. However, two important studies have been reported recently, one on BCG and inoculation doses and route, the other on pre-existing cytokine environment. A landmark paper studying BCG infection and disease in susceptible mice (Balb/c mice) showed that the dose of *M. bovis* strain BCG was critical to the development of a type 1
and a type 2 cytokine response, independently of the route of administration of the organism (Power, Wei et al. 1998) (Table 2.1). High doses of inoculation are associated with a type 2 response whereas a low dose is consistently associated with a type 1 response, regardless of the route of infection. In leprosy, there are numerous epidemiological data demonstrating a higher risk to develop leprosy in healthy contact of lepromatous leprosy patients, which excrete higher numbers of leprosy bacilli (Doull, Guinto et al. 1945; Fine 1982). It is unfortunately more difficult to establish a link between a high exposure situation and subsequent development of higher rates of lepromatous leprosy as compared with tuberculoid leprosy (Doull, Guinto et al. 1945). Even though it would be foolish to confuse bacterial dose and bacterial load, augmenting bacterial load in lesions secondary to defective bacterial killing by MΦ might result in the same end-point as a high initial dose in an experimental setting (Power, Wei et al. 1998). Interestingly, the production of IL-4 by CD4+ T lymphocytes from already sensitized allergic patients is also down regulated when high dose of allergen is used (Secrist, De Kruyff et al. 1995), suggesting that the secondary high doses down-regulate the type 2 as well. From these results, it may be hypothesized that the already sensitized Th1 lymphocytes may behave similarly: if dose of antigen increase dramatically in the host, type 1 cytokines (IFN-γ and IL-2) may be down regulated, but this hypothesis has not been tested.

The other important studies concerning pre-existing cytokine milieu as predisposing factors to develop tuberculosis in children, the T cells of which have been shown to produce lower levels of IFNγ. Using high serum IgE as a marker of prominent Th2 response in a community with a high incidence of tuberculosis, it was shown that high IgE correlated with tuberculosis incidence (Beyers, van Rie et al. 1998). A similar study has been performed investigating any correlation between atopy, IgE serum levels and leprosy. Leprosy patients had significantly higher levels of IgE, regardless of whether they were atopic or not (Yong, Grange et al. 1989; Smith, Bahna et al. 1990). However, to our knowledge, no prospective study has been performed to assess the predictability of markers of high Th2 milieu in contacts and compare tuberculoid versus lepromatous patients in a region with high leprosy prevalence and incidence. One study found no significant difference between the mean serum IgE levels of tuberculoid and lepromatous leprosy patients (Saha, Dutta et al. 1975).
Another aspect that is of value in studying the skewing of cytokine secretion patterns is the study of antigen presentation. Two approaches have been very fruitful in understanding the contribution of APC in driving type 1 versus type 2 immune responses: the generation of "altered peptide ligands" (APL) and the generation of specific inhibitors of enzymes involved in antigen processing by the APC.

It is known that in MHC restricted presentation of small peptides, certain amino-acid (AA) residues are critical for successful TCR engagement and specific recognition. APL are peptides from antigens, the critical residues of which have been substituted by residues resulting in a lower affinity between the MHCII/peptide and the TCR. Results indicate that the MHC class II genotypes in mice were important in an in vivo immunization against a protein antigen (reviewed by Constant and Bottomly 1997). Low affinity between APL and MHCII favored a Th2 response whereas higher affinity resulted in a Th1 response (Table 2.1). An intermediate affinity result in T cells secreting a mixture of type 1 and type 2 cytokines as assessed by intracellular staining and flow cytometry. Using transgenic T cells bearing a single TCR and peptides differing in affinity for this particular TCR, it has been recently shown that the degree of affinity of the immunogenic peptides, the dose and the timing correlate with the initial stimulation levels and Th1 and Th2 differentiation. Over the short term, IFN-gamma-producing cells were induced by lower levels of stimulation than IL-4-producing cells, although optimal induction of both was seen with the same high level of stimulation. Over the long term, high affinity and high dose favor a high stimulation level of T cells and Th1 cell clones with type 1 cytokine differentiation, whereas moderate dose of high affinity peptides resulted in the development of Th2 cell clones. Low affinity at any concentration resulted in the failure to develop either Th1 or Th2 long term T cell clones. Therefore, dose and peptide-TCR affinity influence the level of signalling and the timing (i.e. the kinetic) of Th differentiation, which seem to be equally critical for successful Th1 and Th2 differentiation (Rogers and Croft 1999). These in vitro results differ from, but are not contradicting the results in the in vivo mouse study with BCG challenge (Power, Wei et al. 1998).

Another important result that is probably related to the APL findings concerns the effect of an inhibitor of cathepsin B, an enzyme involved in the processing of particulate antigens (Mackawa, Himeno et al. 1998). Cathepsin B is a lysosomal cystein protease involved in antigen processing. In a leishmania model of infection in mice, these authors showed that mice, which are normally susceptible
to the disease and secrete type 2 cytokines, treated with the inhibitor shifted to high production of IFN-γ and become resistant to the *L. major* infection (Maekawa, Himeno et al. 1998).

In summary, in addition of the cytokine environment such as the roles of IL-12 and IL-4 produced by cells of the inflammatory response and innate immunity, antigen presentation, antigen processing and other as yet poorly defined APC-T cell interactions are turning out to be very important for the evolution towards a type 1 or type 2 cytokine secretion and corresponding immune response. This may also be the case in leprosy.

**2.2.5.3 Application of the Th1/Th2 Paradigm to Studying the Healthy Leprosy Contacts.**

Recently, the cytokine response of circulating blood mononuclear cells (PBMC) stimulated by whole and recombinant proteins of *M. leprae* were studied in seven healthy volunteers who had been in contact with leprosy patients for over 3 years (Misra, Murtaza et al. 1995). The healthy contacts (HC) showed a pattern very similar to that of tuberculoid patients, with notably similar proportions of Th0/Type 0 and Th1/Type 1 responses, as well as anecdotal Th2/Type 2 (Table 2.2). Based on these limited results, the types of cytokine profile are not different from tuberculoid patients and support the idea that tuberculoid patients are closer to the disease-free status. However, there is at present no prospective study of the cytokine profile of *M. leprae* stimulated PBMC from a large number of healthy contacts, as a predictive indicator for subsequently development of Hansen’s disease as has been done for the lepromin test.

**Table 2.2: Comparison of the cytokine profiles of the healthy contacts and leprosy patients in the blood.** Number (percentage) of leprosy patients and healthy contacts, the stimulated PBMC of which exhibit a type 0 (co-expression of IFNγ and IL-4), a type 1 (expression of γIFN only) and a type 2 (expression of IL-4 only) cytokine profile as measured by relative RT-PCR. In the group secreting IL-4 and IFNγ, 2 lepromatous patients and one healthy contact had a higher level of IL-4 compared with IFNγ. Adapted from Misra, et al. 1995.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Contacts</th>
<th>Tuberculoid</th>
<th>Lepromatous</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ IFN and IL-4</td>
<td>3 (43%)</td>
<td>6 (46%)</td>
<td>8 (42%)</td>
</tr>
<tr>
<td>γ IFN</td>
<td>3 (43%)</td>
<td>4 (31%)</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>1 (14%)</td>
<td>1 (08%)</td>
<td>8 (42%)</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>2 (15%)</td>
<td>3 (16%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>7</strong></td>
<td><strong>13</strong></td>
<td><strong>19</strong></td>
</tr>
</tbody>
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2.2.6 General Conclusion on the Th1/Th2 Paradigm

Type 1 and type 2 cytokines provide differential help both in the magnitude of CMI and the magnitude and type of humoral immunity such as immunoglobulin isotype switching and the level of secretion of immunoglobulins by B lymphocytes.

The secretion of type 1 and type 2 cytokines by cells of adaptive immunity is determined, at least in part, by the type of cytokines secreted by different subsets of inflammatory cells of the innate side of immunity. The cytokines secreted by T cells, and probably also B cells, also reciprocally influence the differential priming and activation of cells of the innate side of immunity.

Similar concepts may apply to chemokines secreted at the site of inflammation or injury caused by an invading "non self element". The concept of type 1 and type 2 chemokine is now emerging (Boring, Gosling et al. 1997; Lukacs, Chensue et al. 1997; Schluger and Rom 1997; Luster 1998).

Finally, important parameters external to the cytokine milieu that are thought to play a role in determining the type of cytokine response include at least the amount of invading microorganisms (dose of inoculum) (Power, Wei et al. 1998) and the antigen presentation process (Maekawa, Himeno et al. 1998).

2.3 CMI, Th1/Th2 Hypothesis and Immuno-Suppressive Lentiviruses

The early, specific deficiency of CMI as indicated by DTH and LST assays in human immunodeficiency virus (HIV)-infected persons has led to the "Th1/Th2 switch" hypothesis to tentatively explain the progressive evolution towards Acquired immunodeficiency syndrome (AIDS). This part focuses on the experimental data from which the hypothesis has been developed and the results of some of the experiments that tested it.

2.3.1 Functional Defects in the Memory Side of CMI

2.3.1.1 In the Early Stages of the Disease (CDC stage II/III)

It seems logical to associate the loss of immunological competence in HIV infected individuals only with the concomitant decline of the CD4+ T lymphocytes. However, HIV infection elicits cell mediated immunity defects well before any abnormal CD4:CD8 ratio or any symptoms of advance disease are detected.
It is the important work of Frank Miedema and colleagues and the team lead by Gene Shearer that put forth evidence of an early, progressive crippling of CMI in asymptomatic HIV-1 infected individuals with normal CD4+ lymphocyte counts (Miedema, Petit et al. 1988; Clerici, Stocks et al. 1989). This loss of CMI was assessed by LST with PBL stimulated by CD3 monoclonal antibodies and an in vitro assay of IL-2 production following stimulation with PHA, alloantigens and two common recall antigens, tetanus toxoid and influenza. There is a progressive, sequential loss of response to these stimuli, starting with the loss of recall antigens, followed by the loss of response to alloantigens, and finally to lectin stimulation (PHA). These findings were independent of clinical staging and CD4+ counts (Clerici, Stocks et al. 1989). In fact, loss of T cell function in vitro predicts progression to acquired immune deficiency syndrome (AIDS) and a decrease of survival time (Dolan, Clerici et al. 1995; Roos, Miedema et al. 1995).

DTH skin test responsiveness to various products such as tuberculin PPD were also sometimes lost in healthy HIV+ individuals previously known to be responders. In one study, HIV-1 seropositivity was the strongest predictor of DTH anergy (Markowitz, Hansen et al. 1993). However, it is the use of antigens (Ag) having a high proportion of reactivity in the general population, such as Candida albicans Ag (98% responders), mumps Ag (83%) and tetanus toxoid (83%), that are most useful for assessing asymptomatic HIV+ individuals (Birx, Brundage et al. 1993). These studies indicate that partial DTH anergy (a few negative responses) is a strong, independent predictor of progression to AIDS in asymptomatic patients (Birx, Brundage et al. 1993; Blatt, Hendrix et al. 1993). In those conditions, combined loss of skin test reactivity is associated and declining CD4+ T cells, is a more accurate predictor of progression to AIDS than CD4+ T cell counts alone (Birx, Brundage et al. 1993).

2.3.1.2 In the Late Stages of the Disease (CDC stage IV-V)

In symptomatic patients, the immunodeficiency is more profound and less specific for CMI and the loss of the memory function. PBMCs are impaired in their proliferative response to lectins that activate both T and B lymphocytes. At those stages, anergy to the complete panel of antigens is often observed, and is a direct predictor of development of opportunistic infections such as thrush, for example. Complete anergy is also predictive of development of tuberculosis when compared with PPD-negative, non-anergic patients, but not with PPD+ non-anergic HIV+ individuals (Moreno, Baraia-Etxaburu et al. 1993).
2.3.2 THE 'TH1/TH2 HYPOTHESIS' IN HIV INFECTION
2.3.2.1 Why a Th1/Th2 Hypothesis in AIDS?

Cytokines are closely linked to the history of the discovery of HIV as the etiological agent of AIDS, since recombinant IL-2 was a necessary factor for HIV replication in cultures of lymphoblasts (Barre-Sinoussi, Chermann et al. 1983; Popovic, Sarnagadharan et al. 1984). This indicated that cytokine mediated lymphocyte stimulation is a necessary requirement for productive HIV replication in CD4+ T lymphocytes.

Also, some of the clinical features of recently HIV-infected persons and AIDS patients are believed to be cytokine mediated. For example, high serum IL-6 and TNF-α in HIV-infected patients without evidence of overt secondary opportunistic infection(s), in the AIDS related complex stage (ARC / CDC stage III) and terminal AIDS (CDC stage IV) may be at least partly responsible for the weight loss and other symptoms observed in these patients (Manetti, Annunziato et al. 1996).

The previous observations on reduced memory and CMI response early in the course of HIV infection led one group of investigators to propose that progressive HIV infection and subsequent crippling of the immune system is due to a progressive preferential absence of the type 1 T lymphocytes, leading to an impaired type 1 immune response and a shift to a Th2 ineffective response leading to the development of AIDS (Clerici and Shearer 1993; Clerici and Shearer 1994). This would be logically due to the crippling and death of the CD4+ Th1 clones, with dominance of type 2 T helper lymphocytes, as an ineffective compensatory mechanism. It would also take into account the differences between AIDS in developing countries and industrialized nations, since individuals in the former have higher levels of parasite infestation with a dominant Th2 milieu. This environment, in turn, would favor more rapid evolution towards immuno-deficiency and AIDS. This attractive theory would also account for the high level of immunoglobulins in the serum of HIV-infected patients, with occasional hyper-IgE syndromes that have been described previously (Paganelli, Scala et al. 1993; Raiteri, Sinicco et al. 1993).

2.3.2.2 Cytokines Secreted by Macrophages in HIV Infected Patients:

Several studies have shown an imbalance in the secretion of cytokines secreted by MΦ (monokines) during the course of HIV infection. Several monokines such as TNF-α, IL-1, GM-CSF and IL-3 have long been known to favor HIV replication in human monocytes and MΦ (reviewed in
In contrast, IFN-α and TGF-β would exert a suppressive activity on HIV replication in cells of the mononuclear phagocyte system. Also, it was found that IL-10 is produced constitutively (i.e. without stimulation) both in vivo and in vitro by HIV-infected macrophages. Subsequently, it was shown that PBMC from HIV-infected patients had defective secretion of IL-12, and this defect could be detected early, during the asymptomatic phase of HIV infection (Chehimi, Starr et al. 1994). By testing various cell culture systems of PBMC, monocytes and MΦ, including human hybridoma cell lines (Yoo, Chen et al. 1996), it was shown that upon exposure and infection by various strains and fragments of HIV, the envelope glycoprotein (gp) 120 evoked rapid (within 4 hours) and severe down regulation of IL-12 mRNA indirectly through up-regulation of IL-10 mRNA (Taoufik, Lantz et al. 1997).

In parallel with these findings in vitro, the study of identical monozygotic twins discordant for HIV infection revealed that epidermal APC (Langerhans’ cells) are not ability in their function to present recall antigens in HIV-infected individuals (Blauvelt, Clerici et al. 1995; Blauvelt, Chougnet et al. 1996). They are, however, impaired in their ability to generate a primary immune response, as assessed by proliferation following allogeneic stimulation (Blauvelt, Clerici et al. 1995). Also, even though Langerhans’ cells (LC) from HIV+ twins were able to induce IL-2 production comparable to LC from HIV- individuals when T cells from HIV- persons were added, IL-2 production was markedly decreased in cultures containing T cells from HIV+ patients. This points towards a defect in T lymphocytes themselves. However, since it is estimated that only ~ 1 % of these cells are infected by HIV and that the reservoir of HIV is believed to be in dendritic cells in the germinal centers of the lymph nodes, these findings should not be generalized. More studies are necessary to decipher the exact contribution of APC to cytokine dysregulation in HIV infected patients progressing to disease. The results of these studies are so far contradictory (Fan, Huang et al. 1997; Fidler and Rees 1999; Sapp, Engelmayer et al. 1999).

Addition of recombinant human IL-12 (rhIL12) enhanced endogenous Ag-specific IL-2 production by T cells from 2 patients with relatively high CD4+ counts (>600/µL). In contrast, blocking anti IL-10 monoclonal antibodies (mABs), but not rhIL12, enhanced IL-2 production by T cells in influenza virus stimulated cultures in patients with very low CD4 cell counts (<20/µL) (Blauvelt,
Chougnet et al. 1996). These results indicate that APC are important in modulating the immune response in HIV patients, and that the response to cytokines and cytokine blockers are variable, and probably related to the clinical phase of HIV disease. In fact, recent reports have indicated little benefit in terms of viral load of short-term treatment with rhIL-12 in rhesus monkeys chronically infected with virulent stains of SIV (Watanabe, Sypek et al. 1998). This result must be interpreted in light of the knowledge of the stage of SIV infection and the fact that even if rhIL12 is bio-active in rhesus monkeys, it may not be as potent in this species as in humans.

2.3.2.3 Cytokines Secreted by Lymphocytes During HIV Infection

The hypothesis of a possible Th1/Th2 switch during the evolution of HIV infection was extensively tested shortly after the theory had been put forth. Cytokine profiles in T lymphocyte populations from both peripheral blood and lymph node during the evolution of HIV infection and disease have been shown to be equivalent, without a of shift towards a strong or at least frank type 2 cytokine profile (Graziosi, Pantaleo et al. 1994; Maggi, Mazetti et al. 1994). Rather, there was an unquestionable shift to a predominant type 0 profile and a weakening of type 1 responses in T cell clones stimulated by PPD and Toxoplasma gondii antigens. Importantly, type 2 and type 0 T lymphocyte clones are more permissive to HIV-1 replication than type 1 T cell clones (Maggi, Mazetti et al. 1994). Careful analysis of data generated from these experiments on T cells reveal some features unique to HIV-infected patients as compared to HIV-negative individuals, such as impaired IL-2 secretion even though γ-IFN is elevated (Graziosi, Pantaleo et al. 1994). In vivo, the dominant cytokines present in the plasma, and constitutively at the m-RNA level in lymphocytes in HIV-infected patients are TNF-α, IFN-γ, IL-6 and IL-10, while IL-2 is consistently low (reviewed by (Oyaizu and Pahwa 1995). Since in most disease systems IFN-γ and IL-2 are classically considered to be dually and concomitantly regulated, deciphering the mechanisms of this difference could be of the great interest in understanding the immune dys-regulation in HIV infected individuals and AIDS patients. It might also be that, in contrast with strong Th1 and Th2 responses requiring robust T cell responses and fully competent CMI, HIV infection globally weakens the T helper response at the cytokine level, regardless of its type. This is shown very clearly in the report by Maggi (Maggi, Mazetti et al. 1994). It has also been very recently confirmed using the enzyme-linked immunospot assay (ELISPOT)(Byl, Gerard et al.
1999). Whereas the numbers of IFN-γ secreting cells were equivalent between HIV+ and HIV- cells stimulated with PHA, there was a significant decrease of both IFN-γ secreting cells and IL-4 secreting cells derived from HIV+ patient as compared with control when cells were stimulated with tetanus toxoid. PHA-induced secretion of IL-4 was also less frequent in HIV+ patients than controls. It was also shown that this decreased number of secreting cells was related to CD4+ T cell impairment of secretion (Byl, Gerard et al. 1999).

The situation has been further clarified recently following kinetic studies measuring the number of HIV-1 RNA copies in the blood with ultra-sensitive quantitative RT-PCR methods. These studies revealed detectable levels of HIV-1 in plasma during all stages of infection (Piatak, Saag et al. 1993). They also revealed a gigantic struggle between HIV replication and CD4+ T cells: a high rate of CD4+ cell death and regeneration occurs, with an insidious, slight advantage to the former (Ho, Neumann et al. 1995; Wei, Hosh et al. 1995).

Augmented T cell death, both in CD4 and CD8 positive lymphocytes, occurs early in HIV infection (reviewed by Oyaizu and Pahwa 1995), which is believed to involve gp120, up-regulation of Fas antigen (CD95) and Fas ligand and down regulation of the bcl-2 family of proteins. So where is the link with the cytokine world? Several investigators have independently shown that type 1 cytokines, such as IL-2, IFN-γ, and IL-12, can rescue from apoptosis resting and activating T cells from HIV+ patients and activated T cells from HIV- donors (Clerici 1994; Estaquier, Idziorek et al. 1995). IL-4 and IL-10 have no effect or are detrimental. Interestingly, IL-12 can restore or augment antigen-specific immune responses in vitro, such as against influenza and env-antigen (gp120), in asymptomatic HIV-infected patients (Clerici, Lucey et al. 1993). However, this restoration of responsiveness may be a double-edged sword since it is well documented that T lymphocyte activation during episodes of common viral infection(s) in vivo may be detrimental and favor progression to AIDS (reviewed by Copeland and Heeney 1996). Similarly, vaccinations, albeit not detrimental, do promote a transiently higher viral burden and direct cytolysis (apoptosis) of pre-activated T cells (Farber, Barath et al. 1996), but without a net loss of CD4 T cells (O'Brien, Grovit-Ferbas et al. 1995; Staprans, Hamilton et al. 1995; Stanley, Ostrowski et al. 1996). These in vivo results are also supported by in vitro results (Weissman, Barker et al. 1996). Since memory T cells are more prone to be activated, through
mechanisms still not entirely understood (see next section on memory T cells page 59), it is logical to assume that these cells are the preferential targets of direct cytolysis and/or apoptosis during an immune reaction. This fact would readily explain why the memory side of CMI is preferentially affected early, even in apparently healthy HIV-infected individuals.

More recent studies, analyzing type 1/ type 2 cytokines from specific sites, such as the saliva from HIV+ and HIV- individuals with or without oropharyngeal candidiasis, reveal a more clear cut deviation towards the type 2 milieu (Leigh, Steele et al. 1998). Similarly, mucosal vaccine studies in mice have indicated for the first time the importance of mucosal cytotoxic T lymphocytes and the adjuvant effect of IL-12 delivered with the vaccine. Interestingly, these vaccines were HIV peptide immunogen and HIV-1 gp160-expressing vaccinia live virus (Belyakov, Ahlers et al. 1998).

2.3.2.4 Dynamics of the Th1/Th2 Balance and Progression to AIDS

HIV kills the very cells that could support a thorough switch to a type 2 cytokines milieu. Th2 CD4+ cells preferentially support replication of HIV-1 (Maggi, Mazetti et al. 1994). HIV-1 requires certain types of chemokine receptors to gain entry in the cell (reviewed by (Levy 1996). CXCR4 and CCR5 are 2 chemokine receptors that have been shown to be necessary for HIV entry in lymphocytes and macrophages, respectively (Alkhatib, Combadière et al. 1996; Feng, Broder et al. 1996). More recently, it has been found that type 2 T lymphocytes producing high levels of IL-4 had up-regulation of the CXCR4 chemokine receptor, thereby favoring the preferential entry and replication of HIV in these cells (Galli, Annunziato et al. 1998; Wang, Harada et al. 1998). Very recently, Suzuki et collaborators showed that macrophage-tropic strains of HIV-1, which depend on CCR5 for virus entry, can replicate in IL-12 stimulated Th1 cell cultures from normal donors more efficiently than T cell lines. In contrast, T-tropic strains of HIV can more efficiently replicate in IL-4 stimulated Th2-type culture cells (Suzuki, Koyanagi et al. 1999). Taken together, these findings provide mechanisms responsible the switch from Th1 to Th0 cytokine profiles in CMI responses in HIV-infected patients.

If, then, the ongoing infection with HIV does not clearly switch CMI responses from a type 1 to a type 2 milieu, but rather to a weakened type 0 environment, what does a pre-existing polarized type 2 milieu due to ongoing parasitic infections before HIV infection? This would be especially detrimental for the lymphocyte population present in the mucosae, such as the gastro-intestinal (GI) tract. In fact, recent experiments in rhesus monkeys have indicated that very early during the acute syndrome, before
the time of sero-conversion, the worst loss of CD4+ T cells occurs among lymphocytes in the lamina propria of the GI tract (Veazey, De Maria et al. 1998). Similarly, studies of the viral and proviral load present in biopsies of the GI tract from HIV-infected patients revealed the GI tract to be a reservoir of viral production, as assessed by p24 ELISA (Fackler, Schafer et al. 1998). These latter findings provide a pathogenesis support to the following epidemiological observations. In individuals with an over-expression of type 2 cytokines due to genetic causes (asthmatic and atopic individuals) or due to environmental causes (mainly multicellular parasites infections), the likelihood to rapidly develop AIDS after HIV exposure is worse (Manetti, Annunziato et al. 1996). This hypothesis has been put forth to take into account the differences between rate of HIV infections and features of AIDS progression in developing countries such as Africa and AIDS in highly industrialized countries (Bentwich, Kalinkovich et al. 1995).

2.3.3 The Non-Progressor HIV-Infected Individuals

In recent years, a few individuals at high risk for exposure to HIV, but with non-detectable or very low viremia have been found. They have normal CD4+ counts, vigorous cell mediated immunity, mild humoral immunity (Haynes, Pantaleo et al. 1996) and present with extremely robust CMI against HIV antigens such as p24, as assessed by LST (Rosenberg, Billingsley et al. 1997) (reviewed by (Shearer and Clerici 1996)). They also have quite high responses to envelope antigens. This CMI is mainly mediated by CD4+ T lymphocytes that secrete high to very high levels of IFNγ. The p24-specific response was not associated with the production of IL-4 and IL-10 and was abrogated by the addition of recombinant human IL-10. Therefore, these rare individuals have evidence of protective immunity mediated by a very strong, specific, memory type 1 CMI response, indicating that HIV makes no exception to the model of protection by type 1 CD4 T lymphocyte-mediated cellular immunity. Moreover, in the same report, the study of CMI in 3 patients diagnosed with the acute HIV-1 syndrome (Stage 1 of HIV infection) and rapidly put on aggressive combination antiretroviral therapy, demonstrated that the lowering of plasma viremia was accompanied by the generation of a p24-specific proliferative response (Rosenberg, Billingsley et al. 1997). This suggests that, during primary infection, it is the very destruction of the protective type 1 helper T lymphocytes whose TCR are specific for HIV-1 antigens by direct virus-replication-cytolysis and/or activation-induced cell death by apoptosis, that is responsible for the viral persistence and possibly the progression to generalized immunodeficiency.
2.3.4 **CONCLUSION**

It appears therefore that there is no shift from a Th1 to a Th2 response because of a crippling of both Th1 and Th2 cell responses, although such a shift may indeed exist among the memory cells. There may be an even more profound crippling of T helper type 2 cells. A more permissive environment to HIV replication is found in type 2 T cells, probably due to a higher cell expression of the chemokine receptor CXCR4, which is one of the 2 co-receptor of entry of HIV-1 into the cells identified so far. This may be the basis for the observation that individuals with dominant Th2 responses, or merely a higher percentage of T cells involved in ongoing immune response at the time of HIV primary infection, are at risk of progressing more rapidly.

In vivo, it seems that this imbalance is more readily detectable in local mucosae than at the systemic levels, especially at sites of infections. Lastly, studies of recently infected HIV+ individuals show that an early, vigorous and specific type 1 CD4+ T cell response correlates at least with non-progression to AIDS, and possibly with protection.

2.4 **THE COMPLEX INTERACTION BETWEEN MYCOBACTERIOSES AND HIV/AIDS**

2.4.1 **DIFFERING INFLUENCE OF HIV ON RESPONSES TO DIFFERENT MYCOBACTERIA**

Compared to normal, immuno-competent individuals, the epidemiology of various mycobacterioses is quite different in the HIV-positive population. Whereas several species of mycobacteria are usually harmless in the general population, eliciting at worst a localized infection, several species, such as *Mycobacterium avium* and a few other relatively fast growing species are particularly dangerous in immunodeficient, HIV+ individuals and in immuno-suppressed patients (Benson and Ellner 1993; Race, Adelson-Mitty et al. 1998).

Tuberculosis, erroneously regarded in recent years as a receding disease thanks to improved hygiene and efficient antibiotics, has re-emerged as a potential secondary major threat due to the development of multi-drug resistance and to an increase in the number of particularly vulnerable persons. One major group is HIV+ individuals (Havlir and Barnes 1999), but this also includes cancer patients undergoing immunosuppressive chemotherapy (Bloom 1994).

However, one mycobacterial disease does not seem to be associated with any increased incidence and/or worsening of the clinical picture in HIV-infected immunodeficient patients: Leprosy (Pömminghaus, Mwanjasi et al. 1991) (Munyao, Bwayo et al. 1994) (Frommel, Tekle-Haimanot et al. 1995).
Since the first case of a dually infected person was described (Lamfers, Bastiaans et al. 1987), case reports from the literature so far have not mentioned any down-grading along the Ridley & Jopling immuno-pathologic scale (Faye, Mahé et al. 1996). HIV seropositivity does not appear to favor any particular type of leprosy. Relatively small but carefully controlled epidemiological studies have also failed to demonstrate any significant influence of HIV on leprosy (Frommel, Tekle-Haimanot et al. 1994). However, no one so far has carefully examined the influence of the type of leprosy on the evolution of HIV disease. For example, according to the type 1/type 2 theory discussed above, since lepromatous leprosy patients have a strong down regulation of CMI due to high expression of type 2 cytokines at least in the skin, one might anticipate that these patients, once infected by HIV, would have an accelerated evolution towards AIDS (Bentwich, Kalinkovich et al. 1995). This has also been suggested and tested for tuberculosis (Goletti, Weissman et al. 1996) (reviewed by (Havlir and Barnes 1999). However, in leprosy, it is believed that the immune activation in humans is minimal and mainly localized to sites of infection: the skin. Therefore it is possible that leprosy does not influence the course of HIV infection, but this has not been tested with epidemiological surveys or in animal models.

**2.4.2 CMI IN PERSONS INFECTED WITH HIV AND MYCOBACTERIA SPP.**

**2.4.2.1 Overview of CMI in Tuberculosis, and Comparison with CMI in Leprosy**

Tuberculosis, which is a major pathogen of humans, has several features of CMI that are quite similar to leprosy (reviewed by (Orme, Andersen et al. 1993). First, the majority (85-90%) of exposed individuals successfully manages the infection, developing a strong DTH reaction characterized by a type 1 cytokine milieu. Tuberculous pleuritis is an example of such a successful outcome (Ferrer 1997) (Barnes, Lu et al. 1993). Second, in the smaller fraction of infected persons developing progressive tuberculous pneumonia, there is an insufficient of CMI, probably related to a down regulation of initial strong CMI, characterized by a weakening of DTH, decreased LST against PPD antigens and a shift from a type 1 to a type 0 cytokine profile (Sanchez, Rodriguez et al. 1994; Hernández-Pando, Orozco et al. 1996) (reviewed in (Cooper and Flynn 1995). It rarely culminates in an anergic response to the purified protein derivative (PPD) test in a few persons in far advanced and disseminated disease. For example, one epidemiological study found PPD anergy in 0.4 to 31% (median 6.1%) of tuberculosis patients within 5 institutions (Johnston, Saltzman et al. 1960). Also, there is a fraction of infected
persons that, after a weakening of the memory side of CMI, have a late activation or re-activation of the
disease (Havlir and Barnes 1999). This may occur in the elderly (Stead, Lofgren et al. 1985) and this is
believed to be related to a weakening of the memory function, in human as well as in murine models
(Orme 1988).

In contrast, leprosy is a spectral disease, characterized by a significant proportion of patients
which develop a progressive infection related to a severely insufficient CMI, possibly corresponding to
a shift from a type 1 to type 2 cytokine milieu (Modlin 1994), culminating in lepromatous leprosy. The
alternate hypothesis is that a type 2 cytokine milieu develops initially in lepromatous patients, who may
be genetically predisposed to this disease. This possibility would then be different from the known
situation in tuberculosis, where the initial CMI response is considered to be set up in a strong type 1
environment, as it is the case in tuberculous pleurisy (Barnes, Lu et al. 1993). At the other end of the
spectrum, tuberculoid patients exhibit strong CMI (Harboe 1994) (Kaufmann 1994), similar in
magnitude to the CMI of PPD-positive healthy individuals exposed to \textit{M. tuberculosi}s.

The major difference in CMI between the two diseases could lie in the kinetics rather than the
magnitude of the CMI due to pathogens drastically different in terms of growth and toxicity. Adequate,
protective CMI may now be defined according to at least three factors: it must be rapid, strong enough
(a high frequency of responder T cells, high cytokine and chemokine secretion) and appropriate to the
features of the pathogen(type 1 for intracellular pathogen; type 2 CMI for extracellular parasites).

Another possibility is that CMI can be overwhelmed rather than initially inadequate/defective
in both diseases. In lepromatous leprosy, LL patients may have to down-regulate an adequate (i.e. type
1) response over time due to failure to control the growth of the organism, as in patients with
progressive primary tuberculosis, rather than initially failing to adequately respond to the first exposure.
Some reports suggest that healthy contact individuals with an absent Mitsuda reaction are at higher risk
to develop LL disease (Harboe 1994). This does not necessarily disprove the hypothesis of down-
regulation due to overwhelming \textit{M. leprae} infection, since there is no measure of exposure and early
infection in leprosy, and therefore leprosy without gross lesions and nerve deficits could be more
common than previously thought.
Alternatively, it may be that some patients, upon first exposure to either pathogen, have either an initial inadequate (i.e. Th2) CMI response, or an initial weak and delayed, but adequate (Th1) response culminating into disease development after widely different time intervals. These two hypotheses are not necessarily mutually exclusive, since an adequate but weaker and/or delayed CMI may be subsequently down-regulated.

In conclusion, both *M. leprae* and *M. tuberculosis* may elicit very similar patterns of immune response in the human host, but over very different setting of time intervals and possibly different initial strength. Microorganism virulence properties such as speed of multiplication, mode of entry in the host and host’s cells, and efficiency of intracellular multiplication may be determinants, but probably are not sufficient to explain all of the differences (Silver, Li et al. 1998).

2.4.2.2 Tuberculosis in HIV Infected Patients

Tuberculosis is highly prevalent in HIV infected patients and often has a much poorer clinical course (Barnes, Bloch et al. 1991; Daley, Small et al. 1992; Moreno, Baraia-Etxaburu et al. 1993) (reviewed in (Havlir and Barnes 1999)). Anti-microbial chemotherapy is also less efficacious in HIV-infected patients as compared with HIV- individuals (reviewed in (Shafer and Edlin 1996). Although numerous species of mycobacteria are considered to be opportunistic infections in HIV+ patients, it is not generally considered to be rigorously the case for tuberculosis for the following 3 reasons: 1) the disease may occur quite early in the course of HIV infection, before obvious immunodeficiency is apparent (Gallant and Ko 1996), 2) tuberculosis is prevalent in urban areas in certain groups of persons such as the drug abusers, where HIV is also highly prevalent and 3) *M. tuberculosis* can induce disease in immuno-competent individuals.

The rate of PPD anergy is higher in HIV+ individuals than in the general population and correlates with low CD4+ T cell counts in the blood (Markowitz, Hansen et al. 1993; Havlir and Barnes 1999). The PPD anergy often parallels anergy to other antigens such as those of *Candida albicans* and tetanus toxoid (Markowitz, Hansen et al. 1993). In HIV+, PPD anergic patients, a loss of lymphocyte proliferation was observed in response to *M. tuberculosis* antigens, and this loss was associated with deficient CD4 T cell helper activity, but was not correlated with the total number of circulating CD4 T cells (Zhang, Gong et al. 1994). More importantly, the HIV+ patients with tuberculosis had lower type 1 secretion and mRNA expression in PBMC cultures stimulated with *M. tuberculosis* antigens. Since
there was no difference in type 1 cytokine secretion among cultured, sorted CD4+ T cells between HIV+ and HIV- individuals, the diminished type 1 cytokine secretion was believed to be due to lower numbers of CD4+ T cells after culture, not to a diminished secretion of these cytokine on a per-cell basis (Zhang, Gong et al. 1994).

Since the number of CD4+ T cells able to respond strongly to *M. tuberculosis* were reduced in HIV+ patients, the response of other T cell subsets such as CD8+ and γδ CD3 T cells have been investigated. Many of these cells, such as γδ CD4-CD8- (DN γδ), are recruited early upon mycobacterial infection in man (Inoué, Yoshikai et al. 1991). Logically, defective HIV-infected or destroyed CD4+ T cells should not affect the responsiveness of CD4-negative γδ T cells. However, in HIV+ individuals, a subset of DN γδ cells, the Vy9V82 T cells, are reduced in number and reactivity to *M. tuberculosis* stimulation *in vitro* is very decreased or absent in the majority (~60%) of the HIV-1 infected individuals with advancing HIV disease (CDC stages III and IV). In contrast, there is polyclonal expansion of another γδ T cells subset, Vδ1+ cells (Boullier, Cochet et al. 1995). This subpopulation is known to contribute to immunity against mycobacteria through recognition of the phosphoantigen called TUBAg (Boullier, Poquet et al. 1999). This loss of reactivity was shown to be due to a deficiency of CD4+ T cells secreting a type 1 cytokine profile, since responsiveness could be restored *in vitro* with these latter cells (Wesch, Kabelitz et al. 1996). The γδ Vy9V82 T cells also elicit strong type 1 cytokine secretion in HIV+ responders characterized by the selective production of IFNγ and TNF-α. In contrast to CD4+ T cells specific for *M. tuberculosis* antigens from HIV+ patients (Zhang, Gong et al. 1994), HIV+ patient with the Vy9V82 T cells anergy did not have any significant secretion of type 1 or type 2 cytokines, even after stimulation with IL-12 and IL-15 (Boullier, Poquet et al. 1999). The mechanism of functional anergy of the Vy9V82 T cells from HIV+ patients is presently unknown. In summary, the results of these studies suggest a complex regulation of *Mycobacterium tuberculosis*-reactive γδ T cells in HIV+ patients and a role of CD4+ T cells in the adequate function of these cells.

2.4.2.3 Leprosy in HIV Infected Patients

Epidemiological studies so far have failed to show any increased incidence of leprosy, even in areas of rapidly increasing incidence of HIV seropositivity and AIDS in Africa (Leonard, Sangare et al. 54).
1990; Tekle-Haimanot, Frommel et al. 1991) (Pönninghaus, Mwanjasi et al. 1991) (Munyao, Bwayo et al. 1994) (Frommel, Tekle-Haimanot et al. 1994) (Lienhardt, Kamate et al. 1996), India (Sayal, Das et al. 1997) and South America (Machado, David et al. 1998). No similar study has been reported so far from southeastern Asia, where the epidemiology of leprosy is slightly different, with a higher proportion of lepromatous cases. One study from Tanzania has shown a slightly higher incidence rate of lepromatous leprosy in an urban cohort of HIV+ individuals (Borgdorff, van den Broek et al. 1993). These epidemiological studies on leprosy and HIV infection sharply contrast with the data concerning tuberculosis (TB), sometimes found in the same African survey (Pönninghaus, Mwanjasi et al. 1991).

Likewise, the only immuno-pathological study so far in humans did not reveal any difference between HIV+ and HIV- patients in the borderline tuberculoid or the borderline lepromatous area of the spectrum (Sampaio, Caneshi et al. 1995). Similar percentages of CD4+ and CD8+ T lymphocytes were present in lesions of HIV+ and HIV- borderline tuberculoid patients. Even though cytokine mRNA expression was not assessed in the lesions in this study, examination of gamma interferon mRNA expression from non stimulated and M. leprae sonicate (MLS) stimulated peripheral blood mononuclear cells were similar or higher in HIV+ individuals (Sampaio, Caneshi et al. 1995). Likewise, histopathological studies have failed to demonstrate any augmentation of the incidence of lepromatous leprosy or downgrading of leprosy from tuberculoid to lepromatous disease in humans (study and review in (Faye, Mahé et al. 1996). However, since no reliable test can be performed to assess the ratio of the individuals suffering from lepromatous/ multibacillary leprosy to the individuals exposed (infected) to the leprosy bacillus without disease (leprosy disease rate: leprosy infection rate) in an HIV-infected versus HIV-negative population, the question of whether HIV infection and/or disease favors the development of lepromatous leprosy after exposure to the pathogen remains largely unanswered and difficult to address. A few reports suggest an increased immunopathological (i.e. reversal) reactions in HIV+ individuals as compared with HIV- leprosy patients (Bwire and Kawuma 1994).

Experimentally controlled studies using rhesus monkeys inoculated with large doses of M. leprae and concurrently infected with SIV have shown higher proportion of animals that develop persistent M. leprae infections compared with SIV- animals (Gormus 1989) (Gormus, Murphey-Corb et al. 1998). Rhesus macaques, which are considered as the best available model of HIV infection and
disease (Simian AIDS) may react differently than humans. Alternatively, careful analysis of the timing of SIV inoculation and *M. leprae* inoculation suggests that the inoculation schedule may not be representative of what occurs in nature in humans co-infected with *M. leprae* and HIV. Numerous carefully controlled epidemiologic studies about *M. leprae* indicated that exposure to *M. leprae* in endemic areas occur early during childhood (Doull, Guinto et al. 1945; Fine 1982). In contrast, HIV infection is sexually transmitted and therefore individuals are exposed during early adulthood, aside from the vertically transmitted cases. Therefore it can be inferred that, in most cases, leprosy exposure precedes HIV infection. This inoculation schedule, that is leprosy being inoculated several months before SIV has not been reported in rhesus macaques. The higher proportion of rhesus macaques that developed persistent *M. leprae* infection was seen in animals that were inoculated with *M. leprae* at the same time, or 8 to 10 months after inoculation with SIV and were animals often with advanced SAIDS (Gormus 1989) (Gormus, Murphey-Corb et al. 1998). The very large doses of *M. leprae* used to inoculate these animals constitute another discrepancy between what is observed in experimental leprosy in SIV+ rhesus macaques and natural leprosy in HIV+ humans. Extrapolation of these findings to humans must therefore be made with caution.

2.4.3 THE INFLUENCE OF MYCOBACTERIA AND OTHER SECONDARY INFECTIONS ON THE COURSE OF HIV INFECTION

2.4.3.1 Antigen Challenge and Levels of Lentivirus Replication

Since the discovery of HIV, it has been known that the production of HIV-1 virions *in vitro* requires activated lymphoblasts, as initially achieved by addition in the culture medium of recombinant human IL-2 (Barré-Sinoussi, Chermann et al. 1983). HIV would replicate more efficiently in mitogen stimulated peripheral lymphocytes (Folks, Kelly et al. 1986) and this feature was not merely a consequence of dividing cells, since HIV, unlike many retroviruses, can infect and produce low levels of virions in non-dividing cells (Heinzinger, Bukrinski et al. 1994).

In fact, this decade has witnessed a series of studies, both *in vivo* and *in vitro*, linking immune activation during common, rather benign viral and bacterial infections to the progression of HIV-infected individuals from the asymptomatic stage to AIDS related complex and AIDS (reviewed in (Copeland and Heeney 1996) and (Bentwich, Kalinkovich et al. 1995)) (Weissman, Barker et al. 1996) (Stanley, Ostrowski et al. 1996) (Staprans, Hamilton et al. 1995) (O'Brien, Grovit-Ferbas et al. 1995).
Asymptomatic HIV-infected individuals receiving common vaccination protocols such as influenza vaccination and/or booster dose of tetanus toxoid have transiently higher HIV titers in the blood and lymph nodes, corresponding to a boost in HIV replication (Stanley, Ostrowski et al. 1996) (Staprans, Hamilton et al. 1995) (O'Brien, Grovit-Ferbas et al. 1995). In a cell culture system, similar results were found in APC pulsed with tetanus toxoid and CD4+ cells from HIV+ donors during the immune activation of an in vivo immunization. With this system, during Ag-specific immune activation, 100 times fewer HIV virions are necessary to initiate a productive infection in vitro (Weissman, Barker et al. 1996). These immunologists have suggested that, in addition to or instead of a shift from Th1 to Th0/Th2, a high state of immune activation characterizes AIDS in developing countries and may readily explain the differences of clinicopathologic and epidemiologic features between the western industrialized world and Africa, for example (Marlink 1987) (Bentwich, Kalinkovich et al. 1995). Interestingly, several at-risk groups of the industrialized world have also a higher rate of chronic infections and diseases (Marlink 1987) (Quinn, Piot et al. 1987). Future work will be aimed at trying to decipher the mechanisms of the increased HIV replication associated with immune activation in order to attempt to counteract this component of HIV pathogenesis without using drugs that induce immunosuppression (Greene 1996).

2.4.3.2 Do Active Tuberculosis and Other Mycobacterioses Precipitate the Evolution of HIV Disease?

As with other potentially persistent infections, such as cytomegalovirus and other herpes viruses, it is conceivable that a focus of M. tuberculosis infection may chronically and repeatedly stimulate the immune system and favor the progression of the HIV disease. In fact, tuberculosis is not always a disease of full-blown terminal AIDS as was observed with M. avium and other opportunistic mycobacteria (Ellner 1990; Gallant and Ko 1996; Shafer and Edlin 1996; Havlir and Barnes 1999), but has been shown to be associated with rapid deterioration of the clinical status of HIV-1 infected patients toward AIDS (Whalen, Horsburgh et al. 1995). The basis of this action is multiple and include increased replication of HIV in activated lymphocytes at the time of the acute phase of the disease (Goletti, Weissman et al. 1996) and macrophages when they phagocytize mycobacteria and/or secrete TNF-α (Shatton, Friedland et al. 1994)
2.4.3.3 Leprosy and AIDS in Developing Countries

As pointed out in the introduction contrasting the epidemiology of different species of mycobacteria in HIV infected persons, there are essentially no data dealing with the influence of *M. leprae* infection and leprosy on the susceptibility and evolution of newly acquired HIV infection.

In addition to epidemiological studies in areas where both diseases are highly endemic, the use of different sequences of SIV and *M. leprae* dual infections in different species of monkeys, such as for example the sooty mangabey (*Cercocebus atys*), the cynomolgus macaque (*Macaca fascicularis*) and the rhesus macaque (*Macaca mulatta*) could be highly informative. Investigations in controlled experimental conditions have already been performed (Gormus, Murphey-Corb et al. 1998) after the fortuitous finding that rhesus monkeys accidentally infected with SIV of sooty mangabey origin at the time of *M. leprae* inoculation would develop disease in a higher proportion as compared with SIV-controls (60% of SIV+ versus 21% of SIV- developed leprosy) (Gormus 1989).

2.4.4 CONCLUSION

Reviewing the literature, it is puzzling to note the difference of susceptibility and prognosis between tuberculosis and leprosy in HIV+ individuals. Tuberculosis is more frequent and has a poorer prognosis in HIV+ individuals and AIDS patients. Tuberculosis also has been demonstrated to be a negative prognosis factor for development of AIDS. In contrast, leprosy is not more frequent in HIV positive individuals or AIDS patients. A quite similar proportion of clinico-pathologic presentations of leprosy exists in HIV+ and HIV- persons.

2.5 THE MEMORY STATUS OF THE IMMUNE RESPONSE

2.5.1 Definition of a Memory T Lymphocyte

The theoretical definition of a memory T lymphocyte seems quite easy. It is a T lymphocyte that was involved (or at least the lineage of which was involved) in an immune response. This T cell, or one of its predecessors, escaped down-regulating homeostasis, most often apoptosis, and possibly reverted to a non-activated state with an absence of mitoses and low metabolic activity. This cell should have a lower trigger of re-activation compared with a naïve T cell and should express a set of adhesion molecules and chemokine receptors that favor the rapid recruitment of these cells in the tissue where the primary immune reaction took place. In practice, this cell should elicit vigorous and rapid response to recall antigen stimulation and survive treatment with drugs that destroy activated, multiplying T lymphocytes, such as the antimitotic drugs. The first practical definition of a memory T cells is the...
cyclophosphamide-resistant T cell population that can confer DTH and protection within 2-3 days after adoptive transfer (Orme 1987; Orme 1988), developed in laboratories dealing with tuberculosis research.

"However, given the fact that the memory T cell is the primary target in the development of new antituberculous vaccines, it is unfortunate that very little is really known about this population of cells and, because of the great expense incurred in maintaining infected animals over the long periods necessary, few laboratories have any interest in actively studying it." Ian M. Orme et al, 1993.

This simple, very efficient definition of T cell memory is applicable only in well-controlled rodent experiments where adoptive transfers may be easily performed. For a long time now, simple surface markers of memory T cells have been sought (reviewed in (Cerottini and MacDonald 1989) with variable success.

Memory T cells are circulating lymphocytes that are said to perform immunosurveillance because they are present in the blood stream. Their frequency can be estimated using appropriate limiting dilution micro-culture systems and determining the frequency of effector cells precursors, such as cytotoxic T lymphocytes (CTLp) or of CD4+ helper T cells producing cytokines and capable of proliferation (Varga and Welsh 1998; London, Perez et al. 1999). Also, memory cells are characterized by a higher density of adhesion molecules and surface cofactors, and usually produce higher amounts of cytokines (Cerottini and MacDonald 1989). Lastly, memory T cells are suspected to differ qualitatively and quantitatively in the requirements for activation; they have a lower threshold for activation. Recently, important insights in the understanding of this last property have been made in the analysis of the common leukocyte antigen (CLA), also called CD45, discussed below. Qualitative difference in activation requirements of memory T cells is also illustrated by the findings that memory T cells, but not naïve T cells, are proliferating upon exposure to cytokines, such as IFNα, IFNβ and IL-15 for CD8 memory T cells, and IL-2 and IL-4 for CD4 T cells, during an infection (Ahmed 1996; Tough, Borrow et al. 1996; London, Perez et al. 1999).

2.5.2 POTENTIAL MEMORY MARKERS OF T LYMPHOCYTES

2.5.2.1 The Short Isoform of the Common Leukocyte Atigen (CLA): CD45RO

In 1988 and 1989, Matthias Merkenschlager and collaborators showed that CD45RO was preferentially expressed in both memory CD4+ and CD8+ T cells, that is cells responding to recall antigens in stimulation tests in vitro (Merkenschlager, Terry et al. 1988) (Merkenschlager and Beverley

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More recently, the intracytoplasmic domains of CD45 molecules have been shown to have tyrosine phosphatase activity (Trowbridge 1994). This phosphatase activity modulates the tyrosine kinase activity following TCR engagement and T cell activation (Chui, Ong et al. 1994; Ong, Chui et al. 1994) (reviewed by (Trowbridge 1994). By integrating this two findings, it may be proposed that CD45RO, which may require a lower activation threshold, contributes to the memory phenotype of T lymphocytes. However, the lower activation threshold of CD45RO isoforms have not been directly proven.

CD45 is a family of alternatively spliced proteins, ranging in size from 180 kDa to 220 kDa, expressed on all leukocytes, exclusively upon cells of the hematopoietic system. The alternative splicing of 3 exons, which encode sequences near the amino-terminus of the molecule, generates 8 isoforms. For example, when the 3 exons 4, 5, and 6 are spliced out, the isoform is termed CD45$\alpha$. When the 3 exons are present or only exon 6 is lacking, the isoforms are termed CD45$\alpha$ and CD45$\beta$, respectively (reviewed by (Trowbridge 1994).

To identify these isoforms, a set of 4 monoclonal antibodies (MoAb) has been generated against human leukocytes expressing common leukocyte antigens, termed CD45R. The A, B and C MoAb recognize protein epitopes corresponding to protein isoforms, the genes of which contain exon 4, 5 and 6, respectively. Hence, CD45$\alpha$ can be identified because a cocktail of CD45RA, CD45B and CD45RC will bind the isoform. The CD45RO MoAb, corresponding to the clone UCHL-1 in humans, binds to an epitope of the protein isoform CD45$\theta$. This epitope corresponds to an amino-acid sequence, the encoding gene of which includes exons 3 and 7 without the three intervening exons 4, 5 and 6 that had been spliced out during transcriptional maturation.

All leukocytes, including T cells, express variable exons at different maturation stages, but at least in T cells this expression is neither unidirectional nor irreversible in long term human T cell lines (Rothstein, Yamada et al. 1991). Moreover, transfer of CD45R+ or CD45R- allotype-marked CD4 T cells into athymic nude rats demonstrated that function followed phenotype, not parentage (Bell and Sparshott 1990). So, in contrast to other markers such as CD4 and CD8 in the thymus, CD45R are not maturation markers but rather functional markers. It can be stated that, in humans, CD45RO binds up-regulated CD45$\theta$ isoforms that are abundant in activated cells in vitro. Recent experiments have shown

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that the memory phenotype was not exclusively found in cells expressing high densities of CD450 isoforms (CD45ROhi cells), but could be found also in cells with higher molecular weight isoforms in rats (Hargreaves and Bell 1997) and humans (Richards, Chapman et al. 1997).

Therefore the studies linking the increase of CD45RO+ T cells associated with progression towards AIDS in HIV+ subjects (Bofill, Mocroft et al. 1996; Mahalingham, Pozniak et al. 1996; Benito, Zabay et al. 1997), which were apparently contradicted studies of memory cell functional loss (Miedema, Petit et al. 1988; Clerici, Stocks et al. 1989), were in fact indicative of increased CD4 T cell activation. A previous study also exploring the activation markers HLA-DR and CD38 reached similar conclusions (Kestens, van Ham et al. 1994).

2.5.2.2 L-Selectin (CD62L)

High levels of L-selectins are present in naïve T cells but not in memory T cells (reviewed in (Dutton, Bradley et al. 1998). As with CD45R0 expression detected by CD45RO MoAb, recent experiments suggest that the down regulation of L-selectin accompanies T cell activation rather than T cell memory (London, Perez et al. 1999). The implication that an adhesion molecule is differentially expressed between naïve and memory function is interesting and should be expended by differential expression of other critical adhesion molecules. In fact, it has been shown that the β integrin LFA-1 detected by the marker CD11b are also higher and associated with activated/memory T cells. However, again, costimulatory molecules are definitively more associated with activation than memory status.

2.5.2.3 The Surface Marker CD44

In a situation opposite to L-selectin, it was shown that CD44hi was present in memory T cells, whereas CD44lo is usually characteristic of naïve T cells (reviewed in (Dutton, Bradley et al. 1998)).

2.5.3 CONCLUSION

Two theories of lymphocytes memory are currently put forth, that are not necessarily excluding each other: 1) "dormant" memory, recirculating long lived T lymphocytes and 2) periodic re-stimulation of memory lymphocytes, either through retained Ag in APC with frequent/reCURRENT presentations of persistent antigen fragments to T and B lymphocytes, or frequent re-stimulation of memory cells with cytokines secreted during intermittent infections, since these latter cells do not require co-stimulations (Dutton, Bradley et al. 1998). This latter mechanism (i.e. periodic re-stimulation) is particularly attractive to explain the selective loss of memory T cell pool in HIV infection, since the virus may
replicate preferentially in activated T cells, and experimental data suggest that memory T cells have much less stringent requirements for activation than naïve T cells during benign infections (Trowbridge 1994).

Non naïve T and B-lymphocytes can be defined by markers but it is currently difficult to differentiate between activated naïve and activated memory T lymphocytes. There are no established markers between species. Some markers are established markers of cell activation (such as CD45 RO) with uncertainties about their status/role as memory cells. However, the most convincing demonstration that CD45RO may be an adequate marker of T cell memory in addition to be an established marker of T cell activation lies in the fact that ex vivo CD45RO+ cells are augmenting instead of diminishing in type 1 cytokine secreting CD4 T cells during influenza vaccination (Kallas, Gibbons et al. 1999) (McElhaney, Pinkoski et al. 1995).

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3 SORTING OF CD4 AND CD8 T CELLS FROM LYMPH NODE CELL SUSPENSIONS OF Rhesus Macaques Using Antibody-Coated Magnetic Microbeads: A Preliminary Study

3.1 INTRODUCTION

Many immunological studies rely on the functional and phenotypic analysis of key cell subsets of lymphocytes, macrophages and natural killer cells. In addition to cell cultures in vitro, inflammatory and immune cells from an inflammatory site and draining regional lymph nodes (LN) can now be sorted and isolated to near purity ex vivo prior to be either cultured in vitro or analyzed for phenotypic properties or cytokine secretions (Orfao 1996). The most precise technique for isolating cell subsets that exhibit specific surface molecules is the fluorescent activated cell sorting (FACS) (Orfao 1996), which exhibits good performance even in rare cell subsets (McCoy 1991; Rosenblatt 1997). Ninety five percent to 99% purity of cells with a specific surface cell marker is common with this technology with high recovery, even using markers with dim fluorescent signal (Orfao 1996). However, this technique is relatively slow (McCoy 1991), requires to use reagents such as monoclonal antibodies (MoAb) that may alter the cells, may not allow the isolation of cells in sterile conditions (Orfao 1996). In addition, it must be performed on live cells and may generate aerosols, a potential source of hazards to laboratory workers when isolating cells that potentially harbor dangerous infectious agents.

Alternative technologies have been developed using columns and beads, taking advantage either of the magnetic field (Miltenyi, Muller et al. 1990) (Lea, Vartal et al. 1988) (reviewed in (Battye 1991)) or of antigen-antibody affinity as the separation principle (Tanke 1993).

Our long-term goal is to isolate the memory/activated fraction of CD4+ T lymphocytes present in the regional LN draining skin inoculation sites in order to study the ex vivo cytokine mRNA expression of this important T cell subset after a second inoculation of fresh Mycobacterium leprae in rhesus macaques (Macaca mulatta). The cell surface marker chosen for the memory/activated fraction was CD4SR0, specifically binding the short isoform CD4SR0 of the common leukocyte antigen family of surface molecule. This marker has been shown to be more expressed on the cell surface of human activated/memory T lymphocytes (Akbar, Terry et al. 1988; Merkenschlager, Terry et al. 1988; Akbar.
It has also been shown that the CD45RO isoforms play a modulating role in the ease and magnitude of T cell responsiveness (Chui, Ong et al. 1994) (reviewed by Trowbridge 1994).

In a preliminary effort to determine the feasibility to isolate the cell subsets present in healthy and SIV+ rhesus LN (rLN), we chose to use the magnetic cell sorting (MACS) technique to purify memory/activated T lymphocytes. Results indicate a performance of this method similar to human peripheral blood mononuclear cells (hPBMC) in purifying CD4 and CD8 T lymphocytes. However, only partial enrichment of memory/activated CD4 T lymphocytes could be achieved using the MACS technique in both hPBMC and rLN. The implications of this are discussed.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Cell Isolation and Cell Yields

Heparinized blood was collected from 2 healthy human volunteers. Lymph nodes were collected from one healthy and one SIV infected (SIV+) rhesus monkey (*Macaca mulatta*) housed at the Delta (Tulane) regional primate center under sedation or prior to euthanasia. Human PBMCs were isolated using density gradient centrifugation (Ficoll-paque®, Pharmacia,Upsalla, Sweden) at a ratio of 3 ml of Ficoll for 10 ml of blood. Lymph nodes (LN) were excised from the inguinal area, minced under sterile conditions, filtered through a 67 μm-wide metallic filter and suspended in RPMI medium with 10% fetal calf serum. Cells were subsequently adhered to plastic petri dishes for 1 hour at 37°C to deplete B cells and macrophages from the starting LN cell suspensions (LNCS) and the starting hPBMC suspensions. A range of 50 to 100 millions hPBMC and were isolated. Rhesus LNCS had a total yield varying from 2.4 $10^7$ (SIV+ animal) to 3.4 $10^8$ cells (SIV- animal).

#### 3.2.2 Isolation of CD4, CD8, CD45RO and CD4+CD45RO+ T Lymphocytes Using MACS

Columns of 1.5 ml capacity (Mini-columns™ type MS+/RS+ for Mini-MACS®), with a sheath of iron wool and were obtained from Miltenyi Biotec Inc, Auburn, CA. Magnetic beads coated with fluorescein isothiocyanate (FITC) and magnetic beads coated monoclonal antibodies (MoAb) were used. The reagents and sources were the following: anti-CD4-FITC isomer 1 (clone OKT4 – Ortho, Raritan, NJ) used with anti-FITC isomer 1 microbeads (Miltenyi), anti-CD8-microbeads (clone SK1, Miltenyi) and anti-CD45RO-microbeads (clone UCHL-1, Miltenyi) monoclonal antibodies.

One parameter (single) and 2-parameter (double) magnetic cell sorting were performed according to manufacturer’s instructions (Miltenyi Biotec Inc, Auburn, CA). The linkage element of the
Iron microbeads to the anti-FITC MoAb is composed of a dextran polymer. The detachment necessary before the second sort is accomplished by incubation for 10 minutes with dextranase, an enzyme that catalyses the dextran bridge lysis.

Sorted fractions were kept at 4°C on ice until a sample was prepared for three-color flow cytometry using a FACScan™ (Becton Dickinson immunocytometry systems, San Jose, CA). Cells were incubated with the following MoAb: CD4-FITC and CD4-phycocerythrin (PE) (clone OKT4 – Ortho), CD8(Leu2a )- peridinin chlorophyll protein (perCP) (clone SK1, Becton Dickinson), mouse anti-human CD3-PerCP (clone SK7, Becton Dickinson), mouse anti-monkey CD3-FITC (clone FN-18, Serotec, Oxford, UK), CD45RO-PE (clone UCHL-1, Dako, Carpinteria, CA). Incubation conditions, washing and fixation were performed under standard conditions: The MoAb were incubated at room temperature in the dark for 20 minutes, washed once with saline and fixed with 1% paraformaldehyde. The proportion of CD4+ macrophages in hPBMC and rhesus LNCS were assessed using CD14-FITC (clone TUK4, Dako).

3.2.3 PANNING OF B CELLS

Rhesus LNCS or rhesus PBMC were obtained from SIV- rhesus macaques and were depleted of B lymphocytes and macrophages using plastic surfaces coated with rabbit anti-human immunoglobulins (Accurate, inc., Westbury, NY –cat. # AXL230), according to standard protocol with slight modifications (Lewis and Kamin 1980). Two concentrations, 1.2 mg/ml (1:10 dilution) and 0.6 mg/ml (1:20 dilution) were tried to pan B cells or Petri dishes and 25 cm² cell culture flasks were compared for relative performance (figure 3.6). The system selected was plastic surfaces of 25 cm² cell culture flasks coated overnight at 4 °C with immunoglobulins at a concentration of 0.6 mg/ml. Cells were added at a concentration of 1.5 10⁷ cells/ml and incubated for 2 times for 45 min. at 37°C in humidified 5%CO₂ incubators was then used to deplete both macrophages and B cells.

Non adherent (non-adh) cells were collected by pipetting and stained with mouse anti-simian CD3 (FN18), mouse anti-human CD4 (OKT4) and analyzed by flow cytometry. The total CD3 percentage is obtained from CD3 versus CD4 scatter plot quadrants of a 2 color flow cytometry analysis.
3.3 RESULTS AND DISCUSSION

3.3.1 CD-4 POSITIVE SELECTION AND CD8 NEGATIVE SELECTION

Performance was similar between hPBMC and rLN cells with regard to CD4 single sorting (positive selection), consistently yielding fractions with >95% purity (Fig. 3.1). Similar results of 95% to 98% yields were obtained using CD8 single sorting of rLN (Fig. 3.2). Immunomagnetic sorting technology can be used in rhesus monkey lymphocytes to yield near-pure CD4 and CD8 cell fractions usable for further analysis with commercially available columns. This has also been performed in a few other transplantation studies using rhesus monkeys using CD8 and CD3 (clone FN18) negative selection (Thomas, Carver et al. 1991) and cynomolgus monkeys with CD8 (Voss, Nick et al. 1992). However, to our knowledge, there is no report describing the successful positive selection with high purity yields using immunomagnetic beads and CD4 marker (clone OKT4 – Ortho). The advantages of MACS over FACS is the possibility of performing near 100% purification of cell subsets with light equipment that can easily be placed in a common biohazard containment hood if cells are infected with hazardous agents.

3.3.2 TWO PARAMETER CD4CD45RO DOUBLE SORT FAILS TO ENRICH CD4+CD45RO+ T LYMPHOCYTES IN BOTH HUMANS AND RHESUS MONKEY CELLS USING MAGNETIC SORTING

The second sorting of CD45RO cells from CD4+ purified fraction was not successful. No enrichment was obtained either from human PBMC or rhesus LN cells using 2-parameter (double) sort with (Fig. 3.1). This failure may be due to an incomplete detachment of the magnetic beads attached to the anti-FITC MoAb necessary for the first sort. The CD4 positive fraction was not passed through an additional column to remove any cell with persisting microbeads so the percent release could not be determined.

Recently, purification of CD45RO and CD45RA cell subsets has been described from hPBMCs after CD4 and CD8 positive selection using detachable beads (O'Brien and Kemeny 1998). They used large quantities of blood (100ml), a large magnet and negative selection to enrich at > 90% purity CD45RO and CD45RA using mouse anti-human CD45RA (clone B-C15) and anti-human CD45RO (clone UCHL-1). Interestingly, purity of CD45RO was not as good as purity with CD45RA (92 versus 98%). In another experiment assessing sorting and functional properties of cryo-preserved hPBMCs, positively selected CD4+ T cells with detachable magnetic microbeads were depleted of
CD45RO and CD45RA using the same strategy as the study mentioned above and enrichment of at least 90% were obtained (Sleasman, Leon et al. 1997).

3.1a

3.1b

Figure 3.1: Double sorting of (a) human PBMC and (b) SIV+ rhesus macaque's lymph node cell suspension (LNC) for CD4 and CD45RO. The bars represent the proportion (%) in a given cell fraction analyzed by flow cytometry. There was very good separation of CD4+ T cells using OKT4 MoAb but an absence of separation in the second sort for CD45RO in cells from both species.

The data depicted in (a) are the best results of 3 experiments to double sort human PBMCs. The data presented in (b) was from a single experiment. The total percentages of CD4+ cells (TOTCD4) and the total percentages of CD45RO+ cells (TOT RO) were obtained by adding the appropriate 2 quadrants from CD45RO versus CD4 scatter plots acquired from 3 color flow cytometry analysis. The percentages of double positive (DP) CD4+CD45RO+ cells among the total CD4 percentages (%CD4+RO+) are obtained by dividing the percentage of CD4+CD45RO+ DP cells (upper left quadrant) by TOT CD4 and multiplying the ratio by 100.
Figure 3.2: Single sort of CD8+ cells T cells from one SIV- rhesus monkey’s lymph node cell suspension. From the non-adherent cells (non-adher) collected after panning, there is a 146.25% enrichment of CD4+ T cells in the negative fraction (CD8 neg) and 262.42% enrichment of CD8+ T cells in the positive fraction (CD8+ pos). The total percentages of CD4+ cells (TOTCD4) and the total percentages CD8+ cells (TOT CD8) are obtained by adding the appropriate 2 quadrants from CD8 versus CD4 scatter plots acquired from 3 color flow cytometry analysis.

In addition, in both rhesus and humans, only the cells having a high concentration of CD45RO epitopes (CD45ROhi) are retained by the magnetic field when percolating through the magnetic column. In fact, in both human and rhesus monkeys, CD45RO is characterized by a continuous band-like cloud of points, varying from low to high expression, when viewed on a flow cytometry scatter plot (Fig. 3.3a) whereas CD4, CD3 and CD8 have discrete populations of frankly positive and frankly negative cells. This is due to the molecular biology of the CD45 isoforms that are often co-expressed on a particular cell (Trowbridge 1994). Interestingly, in hPBMCs, there were 2 populations of CD4+ lymphocytes, each representing approximately 45% of the gated cells, one with bright CD4+ cells and the other with dim CD4+ cells (Fig. 3.3b). Ninety % of these latter cells, whether of lymphoid or macrophagic lineage, were in also CD45RO positive. When these cells were positively selected by the CD4 first sort, they retained a high proportion of CD45RO. These cells, however, were not brighter with CD45RO marker than the bright CD4+ cells and would not have probably contributed to a better enrichment with CD45RO.
Figure 3.3: a,b - Flow cytometry scatter plot of the acquired events physically gated on lymphocytes using the CD4 positive fraction from (a) SIV+ rhesus macaque LNCS and (b) PBMCs from a healthy individual. These scatter plots show the continuous distribution of the CD45RO+ fluorescence. The X-axis (FL1) is CD4-FITC; the Y-axis (FL2) CD45RO-PE.

c-f - Flow cytometry scatter plots showing the two distinct subpopulation of double positive CD4+ CD45RO+ cells in human PBMC (starting cell suspension). Region R1 is composed of the bright CD4+ T cells (CD4hi) that are also 53% CD45RO positive. Region R2 is composed of the dim CD4+ T cells that represent 41% of the acquired events and are in great majority (90%) also CD45RO positive. These cells may be interpreted as monocyte/macrophages and/or lymphoblasts. CD3 cytofluorometric analysis was not performed. SSC=side scatter; FSC=forward scatter.
However, a higher concentration of CD45RO monoclonal antibodies used to label non-adherent rLN cells permitted detection of a higher proportion of positive CD45 RO cells, as assessed by a scatter plot with the same quadrant cut-off (Fig. 3.4a). In hPBMCs, there was a minimal augmentation (Fig. 3.4b). Together, results indicated a continuously varying concentration of CD45RO markers from low to high on the surface of CD4+ T lymphocytes that might have been detrimental to an efficient sorting using this marker. The microbead-coated CD45RO monoclonal antibodies were used according the manufacturer’s recommendation, with no attempt to increase their concentration.

![Figure 3.4](image)

**Figure 3.4:** Effect of adhesion to plastic and augmenting the concentration of CD45RO for labeling the starting suspensions (start susp) and the non-adherent fractions (non-adher) of (a) rhesus LNSC and (b) hPBMCs. The proportions are on the y axis and the flow cytometry (FACS) analysis cell types (x axis) are identical to the definitions of figure 3.1.

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3.3.3 CD45RO SINGLE SORT SLIGHTLY ENRICHES CD4+CD45RO+ T LYMPHOCYTES IN BOTH HUMANS AND RHESUS MONKEY CELLS

Increased percentages of CD45RO+ cells, as assessed by flow cytometry, were obtained from the positive fraction after CD45RO single sort from non adherent rhesus LN (Fig. 3.5a). Similar results were obtained with negatively selected, enriched CD4+ cells after CD8 single sorting, also from rhesus LN from the same animal (Fig. 3.5b). The efficiency of the sorting was, however, much lower for CD45RO single sort than CD4 and CD8 single sorts: 120% enrichment versus 483% (fig. 3.1b) and 326% (fig. 3.2) enrichment, respectively, as defined by ratio of the percentage of yield over the percentage of starting cells suspension (% yield:%start ratio). The retention rate is defined by the following formula:

\[
\text{Retention rate} = \frac{(\% \text{ positive cells in original fraction}) \times (\% \text{ negative cells in negative fraction})}{(\% \text{ negative cells in original fraction}) \times (\% \text{ positive cells in negative fraction})}
\]

The retention rate of CD45RO single sorting, using the adherent cells as the original fraction, and of CD45RO single sorting after CD8 negative selection, using the CD8 negative fraction as the original fraction, were 1.53 and 3.74, respectively. A retention rate is considered adequate when the retention rate is between 6 and 8, according to the manufacturer’s instructions.

Lastly, whereas CD4+CD45RO+ were absent in SIV+ rhesus PBMCs and present in very low numbers in SIV+ rhesus LNSC (Figure 3.1b), they were present in more appreciable numbers in SIV-LNSC (Figure 3.5a and 3.5b), of the same order than hPBMC (figure 3.1a). There has been conflicting results as to whether the UCHL-1 clone is reactive in rhesus macaques (Polacino-Firpo, Axberg et al. 1992; Jacobsen, Aasted et al. 1993; Reimann, Waite et al. 1994) and no immunoprecipitation studies have been done to clarify this. A recent study found weak staining (Sopper, Stahl-Hennig et al. 1997), like the preliminary study presented here. This suggests either a lower affinity of the UCHL-1 monoclonal antibody to the correct epitope, or alternatively, the existence of a cross-reacting irrelevant epitope in rhesus monkeys.
3.3.4 ENRICHMENT OF CD3+ T LYMPHOCYTES AFTER PANNING OF RHESUS PBMCs WITH RABBIT ANTI-HUMAN IMMUNOGLOBULINS

Since results of enrichment of CD4+ and CD4+CD45RO+ cells by depletion of CD8 cells were encouraging, additional enrichment of CD4+ cells was attempted by depleting the residual B cell population not adherent to plastic. The enrichments for CD4+ and CD4+CD45RO+ were 155% and 87% respectively, as shown in Figure 3.5.

Figure 3.5: CD45RO single sorting from (a) non-adherent cells and (b) CD8 depleted, CD4 enriched cell fraction of lymph node cell suspensions of SIV-rhesus macaques.

A slight enrichment of CD45RO+ CD4+ T cells is obtained (120%) similar in the 2 different attempts. The flow cytometry analysis of cells is according those defined in figure 3.1 and 3.2. In panel b, the relatively high percentage of CD8+ cell in the CD8-/RO+ fraction can be explained by the relative numbers of cells sorted during the CD45RO sort: from 16.3 x 10^6 cells used, only 1.3 x 10^6 were sorted in the CD8-/CD45RO+ fraction. If it is assumed that ~ all residual CD8+ cells of the CD8-negative fraction were preferentially retained in the CD8-CD45RO+ cell fractions and ~ none were retained in the CD8-CD45RO- fraction, then this result is readily explained.
130%, respectively (Figure 3.5b). The principle is that B lymphocytes and some sub-populations of macrophages bind to the immunoglobulins bound to plastic, mainly through their Fc receptors (FcR). The non-adherent cells should then be mostly T lymphocytes.

Using Petri dishes and flasks with the modified protocol, reproducible enrichments in the range of 2 to 3 times the initial CD3+ T cell proportions were achieved (Figure 3.6). This first step, combined with CD8 sort to obtain highly enriched CD4 populations, will permit to perform CD45RO enrichment if the proportion of CD45ROhi is sufficient among the rhesus LNCS to be studied.

![Figure 3.6: Panning of rhesus PBMCs with rabbit anti-human Ig enriches in T lymphocytes. There is 231.5% and 277.3% enrichment of the initial CD3+ T cell pool, when analyzing the non-adherent (non-adh) cell fraction, using 1.2 mg/ml (1:10 dilution) and 0.6 mg/ml (1:20 dilution), respectively. The graph presents representative results from one out of 2 experiments.](image)

3.4 CONCLUSION

We obtained very good sort results using anti-CD4 (OKT-4) and anti-CD8 (SK1) monoclonal antibodies, reagents known to cross react between humans and rhesus macaques. The results obtained with CD45RO sorting are probably at least in part reflective of the lower affinity of the UCHL-1 clone monoclonal antibody for the homologous epitope of the CD45RO isoform expressed on simian leukocytes. The pattern of CD45RO reactivity that presents with a progression from CD45ROlo and CD45ROhi expression could also account for the poor enrichment obtained after CD45RO single sorting of rhesus lymph node cell suspensions. The absence of enrichment obtained in the double sort of both human PBMCs and rhesus LNCS may be accounted for by an inadequate cell:CD45RO ratio or a
non-complete cleavage of the microbeads coating the MoAb present on the positively sorted fraction of the first sort.

These preliminary studies determined the following protocol to be optimal: 1) panning on IgG-coated plastic to remove B cells, 2) CD8 magnetic sorting to remove CD8+ cells and 3) magnetic sorting of the CD45RO cells from the CD4 enriched, CD8 negative fraction to obtain an enriched CD4+CD45RO+ cell fraction.

3.5 LITERATURE CITED


4 ANALYSIS OF RHESUS MACAQUE LEUKOCYTES ISOLATED FROM INCOMPLETE FREUND'S ADJUVANT INTRA-DERMAL INJECTION SITES: A PRELIMINARY STUDY

4.1 INTRODUCTION
Several methods have been developed to attempt to isolate and study mononuclear inflammatory cells at site of granulomatous response against an infectious agent, including blister formation (Kenney, Rangdaeng et al. 1987; Scollard, Suriyanon et al. 1990), use of gelatin sponges (Buchanan and Murphy 1997) and use of type IV collagenase from site of lesions (Sacco, Jenson et al. 1996). However, since at early time points after inoculation of \textit{M. leprae}, it can be anticipated there will be an absence of well formed granulomas, the use of type IV collagenase may not be necessary. Moreover, since type I collagenase damages many cell surface molecules, jeopardizing phenotypic studies, it was decided to attempt to isolate lymphocytes from skin inoculation sites simply by fine mincing for the main study (see chapter 5).

In an effort to predict lymphocyte average yields a few days after \textit{M. leprae} inoculations in the skin, we assessed the feasibility of collecting leukocytes present in skin lesional sites after injection of incomplete Freund's adjuvant (IFA). Results indicated an adequate number of leukocytes collected, permitting flow cytometry analysis without density gradient purification, reverse transcriptase polymerase chain reaction (RT-PCR) from skin cell suspensions, and cytospin cytology. This sampling strategy is intended to be used for the study of \textit{M. leprae} skin inoculation sites in naive animals and a second challenges in previously inoculated monkeys, as will be presented in subsequent chapters.

4.2 MATERIALS AND METHODS
The animals used in this pilot study were housed in Tulane’s Regional Primate Research Center, Covington, LA, in accordance with established standards of the United States Federal Animal Welfare Act, the American Association for Accreditation of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. The procedures described below were performed on a group of 6 rhesus monkeys (\textit{Macaca mulatta}) previously used in an unrelated study and scheduled for euthanasia.
Two healthy rhesus monkeys each were sedated and inoculated with 0.1 ml of Incomplete Freund’s Adjuvant (IFA) (Sigma immunochemicals, St Louis, MO) in the dermis of the forearm at 2, 5 and 7 days prior to sampling of inoculation sites. The inoculation sites, corresponding to a slightly thickened, red ~ 5-15 millimeter wide area, were collected as a 2.5 cm long wedge biopsy in all six monkeys the same day immediately after euthanasia, as well as heparinized peripheral blood. Margins of the biopsies were trimmed off to reduce the inoculation sites to approximately 6-10 millimeters in diameter. One half of each inoculation site was immediately immersed in buffered neutral 10% formalin. The other half of the each biopsy site was finely minced, and debris and clumps were filtered out using a 64-μm metallic screen (Fisher, Pittsburgh, PA).

4.2.1 Cell Yield Determination
Skin inoculation site cell suspensions (Skin CS) were diluted and maintained on ice in 10 ml of RPMI culture medium with 10% fetal calf serum until counted using an hemocytometer. Viability was assessed by Trypan blue dye exclusion. Aliquots of cells were sampled for flow cytometry, mRNA isolation and for cytospin in order to check for the relative qualitative composition of the Skin CS and a differential leukocyte count.

4.2.2 Flow Cytometry Analysis
Three parameter flow cytometry was performed using a FACScan™ (Becton Dickinson, Mountain View, CA) using the following monoclonal antibodies (MoAb): CD4-fluorescein isothiocyanate (FITC) (clone OKT4 – Ortho, Raritan, NJ) and CD45RO-phycocerythrin (PE) (clone UCHL-1, Becton Dickinson), CD8 (Leu2a)-peridinin chlorophyll protein (PerCP) (clone SK1, Becton Dickinson). The proportion of CD4+ macrophages in blood and skin CS were assessed using CD14-FITC (clone TUK4, Dako, Carpinteria, CA) and CD4-PE (clone OKT4 – Ortho) by two-color flow cytometry. Mouse anti-human δ2 -FITC (Endogen, Woburn, MA) and mouse anti-human CD16 (Leu-11c)-PE (clone B73.1, Becton Dickinson) MoAb were also used to assess the numbers of γδ T cells and NK cells, respectively. Incubation conditions, washing and fixation were performed under standard conditions. The MoAb were incubated at room temperature in the dark for 20 minutes, washed once with phosphate buffered saline and fixed with 1% paraformaldehyde.
4.2.3 RNA ISOLATION AND REVERSE TRANSCRIPTION AND cDNA AMPLIFICATION BY PCR

For RNA isolation, 1-5 10⁶ cells were lysed using RNA STAT-60 lysing solution (Tel-test"B", Friendswood, TX) and immediately frozen at -70°C. Subsequently, the lysates were thawed and total RNA was isolated using guanidinium thiocyanate according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Nucleic acid content determination and purity was assessed by measuring the 260 nm absorbency and the 260nm:280nm ratio, respectively, using a GenQuant™ (Pharmacia Biotech Biochrom Ltd, Cambridge, England, UK) spectrophotometer. The isolates were then reverse transcribed (RT) using Superscript II™ (Gibco Life Technology, Gaithersburg, MD) according to manufacturer’s specifications, with the addition of 1 U.I. of recombinant RQ1Rnase inhibitor proteins (Rnasin™, Promega, Madison, WI). Complementary DNA (cDNA) for rhesus β-actin was subsequently amplified by PCR for 30 cycles using primers designed to amplify human β-actin (Clontech, Palo Alto, CA). The melting-annealing-extension temperatures were: 95°C-63 °C-72 °C, each for 1 minute, using a Perkin-Elmer 480 thermal cycler (Perkin Elmer- applied biosystems, Norwalk, CT). PCR conditions were as follows: 1X PCR buffer II (PE applied biosystems), 20 nanomoles of forward and reverse primers, 150 or 200 nanomoles of MgCl₂, 80 nanomoles of deoxynucleotide tri-phosphate mixture (NTPs mix), and 1.25 units of Taq polymerase (PE Biosystems) in a 100 µl reaction volume. Since human β-actin primers may amplify genomic DNA, resulting in a product of similar size, PCR from the RNA isolates without RT were also performed on experimental. Deoxy-ribonuclease I (DNase I) treatments were performed at room temperature for 15 minutes at a ratio of 0.5:1 of Dnase I (units):RNA isolate (µg) (Gibco Life Technology) prior to RT. One sample with abundant β-actin as seen by PCR (K967 - 2nd replicate) was tested for genomic DNA (gDNA) contamination (Fig. 4.3b, see also Appendix 2).

4.2.4 CYTOLOGICAL EVALUATION OF SKIN CELL SUSPENSIONS (CS)

At least 100,000 cells were used for the cytopsins and prepared in a Shandon cytocentrifuge using standard procedures. Slides were stained with rapid modified Giemsa and according to the Papanicolaou procedure to evaluate the amount of contaminating epithelial cells. Amount of collagen fibers and other debris were qualitatively assessed as minimal (grade 0), slight (grade + ), moderate (grade ++) and abundant (grade +++).
4.2.5 HISTOPATHOLOGY
Formalin-fixed skin inoculation sites were embedded in paraffin, cut at 4 μm and stained with Harris' hematoxylin and eosin (H&E) following standard procedures.

4.3 RESULTS
4.3.1 YIELDS OF CELLS WERE IN THE RANGE OF 1 TO 70 x 10⁶ VIABLE CELLS
The total number of collected cells ranged from 2.6 x 10⁶ to 68.6 x 10⁶, beyond what was initially expected. Data obtained from hemocytometer cell counts are presented in Table 4.1. There was no apparent difference of cell yields with regard to the number of days post inoculation (DPI). Trypan blue exclusion revealed <5% dead cells from Skin CS. A field representative of cell and debris present in cytospins is presented in Figure 4.1. Operator to operator variability in mincing and collection of cells may partially explain the important variability observed between each of the two skin CS at day 2 and day 7-post injection. Cells were counted by one investigator, without trying to exclude the granulocytes from the count.

Figure 4.1: Cytospin from the skin inoculation site cell suspension (skin CS) of rhesus monkey number L129 5 days after intra-dermal injection of 0.1 ml of Incomplete Freund's Adjuvent (IFA). Notice the minimal contamination with collagen debris (arrow), numerous red blood cells and the presence of moderate numbers of granulocytes among the SICS. Bar = 75 μm, rapid modified Giemsa method.
See also Appendix 2 page 210.
Table 4.1: Total cell yields and density of collagen fibers and other debris from skin cell suspensions from incomplete Freund's adjuvant (IFA) injection sites.

<table>
<thead>
<tr>
<th>Monkey #</th>
<th>DPI</th>
<th>total count</th>
<th>collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>K282</td>
<td>2</td>
<td>2.6 x 10^6</td>
<td>+</td>
</tr>
<tr>
<td>K967</td>
<td>2</td>
<td>42.5 x 10^6</td>
<td>+</td>
</tr>
<tr>
<td>K131</td>
<td>5</td>
<td>51.0 x 10^6</td>
<td>+</td>
</tr>
<tr>
<td>L129</td>
<td>5</td>
<td>55.5 x 10^6</td>
<td>0</td>
</tr>
<tr>
<td>K966</td>
<td>7</td>
<td>8.0 x 10^6</td>
<td>++</td>
</tr>
<tr>
<td>L132</td>
<td>7</td>
<td>68.6 x 10^6</td>
<td>+</td>
</tr>
</tbody>
</table>

DPI: Days post inoculation of 0.1 ml of IFA. Collagen amount grading is defined in material and method.

4.3.2 FLOW CYTOMETRY ANALYSIS

The acquired events from the skin were gated on the physical properties of lymphocytes, a population either visible on the side versus forward scatter plots (fig. 4.2) or, when a discrete population of lymphocytes was not readily visible, extrapolated from the gate obtained when analyzing blood from the same animal. Isotype fluorescent MoAb controls showed that the non-specific staining due to debris and dead cells in the gated population in the skin varied from 5% to 15% (Fig. 4.2a,b). It was possible to obtain interpretable results of CD4, CD8 and CD45RO by 3 color flow cytometry (shown for CD4 and CD8 - Fig. 4.2c,d). Unfortunately, possibly due to high numbers of granulocytes, mainly eosinophils, that might have been activated to degranulate or degenerating and damage the mononuclear cells, most of the acquired samples had low total number of events and often insufficient numbers of events gated on lymphocytes for optimal analysis (Table 4.2).

There were high proportions of γδ T cells amongst the gated lymphocytes (Average ± standard deviation: 37.82 ± 21.78%). This proportion was 64% of the percentage of CD4 and CD8 cells CD8 single positive cells (Average ± standard deviation: 59.23 ± 9.50%). These results could not be confirmed because mouse anti-monkey CD3 (clone FN18) was not used in this experiment to assess the proportion of CD4-CD8-CD3+ γδ T cells, and hence the potential proportion of non-specific staining. In blood, the proportion of γδ T cells were less than 1% (Average ± standard deviation: 0.25 ± 0.11%). Importantly, the proportion of CD4+ T cells in the skin CS was significantly greater than the proportion of CD4+ T cells in the blood whether the double positive CD4+CD8+ T (DP) cells were included or not (p=0.01, one tail paired Student's t test). The proportion of CD8+ cells in the skin, including the DP cells, are also significantly different from the one in the skin (Table 4.2). However, the proportion of
CD8+ T cells was not significantly different when excluding the double positive CD4+CD8+ cells (p=0.542, two tails paired Student’s t test). Taken together, proportions of lymphocytes in IFA injection sites were statistically significantly different (p<0.05, paired t test on combined CD4 and CD8 cells), which provides good evidence that the lymphocytes collected were not from the blood. Proportions of contaminating circulating lymphocytes cannot be ruled out, however. Natural killer cells as assessed by CD16 were not detected, suggesting that the marker was not binding any rhesus monkey cells.

Table 4.2: Gamma-delta, CD4 and CD8 percentages in the IFA skin injection sites and blood

<table>
<thead>
<tr>
<th></th>
<th>total events</th>
<th>cells gated</th>
<th>cells analyzed</th>
<th>% of γδ cells</th>
<th>% of CD4*</th>
<th>% of CD8*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Skin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVG</td>
<td>3445.8</td>
<td>288.7</td>
<td>7.7%</td>
<td>37.82</td>
<td>54.70</td>
<td>39.29</td>
</tr>
<tr>
<td>STD</td>
<td>2840.8</td>
<td>305.3</td>
<td>3.7%</td>
<td>21.78</td>
<td>15.51</td>
<td>10.22</td>
</tr>
<tr>
<td>SEM</td>
<td>579.9</td>
<td>62.3</td>
<td>0.8%</td>
<td>8.89</td>
<td>6.33</td>
<td>4.17</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVG</td>
<td>10000.0</td>
<td>2219.1</td>
<td>22.2%</td>
<td>0.25</td>
<td>24.83</td>
<td>20.97</td>
</tr>
<tr>
<td>STD</td>
<td>0.0</td>
<td>931.5</td>
<td>9.3%</td>
<td>0.11</td>
<td>5.84</td>
<td>9.22</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0</td>
<td>190.1</td>
<td>1.9%</td>
<td>0.05</td>
<td>2.38</td>
<td>3.77</td>
</tr>
<tr>
<td><strong>T-TEST</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.020</td>
<td>0.007</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* Percent of CD4+ and CD8+ cells including CD4+CD8+ double positive cells
AVG = sample average (mean).
STD = sample standard deviation
SEM = standard error of the mean
T-TEST = two-tailed paired Student’s t test

4.3.3 RNA ISOLATION FROM SKIN CS IS FEASIBLE
A total of two aliquots of 5 x 10⁶ cells were removed from Skin CS from each animal, which had been lysed in 0.5 milliliter RNA-STAT60 and kept at −70°C, were studied, except for the 2 isolates from which insufficient numbers of cells were isolated (Monkeys # K282 and K966), where 0.5 million and 1 10⁶ cells were lysed in 0.5 ml, respectively. Nucleic acid determination of the RNA isolate replicates by spectrophotometry revealed the isolates to be composed of a median of 23 μg (range 12-76 μg) of total nucleic acids with a median 260:280 ratio of 1.452 (range 1.089 to 1.612). The low ratio of some isolates is attributed to possible hemoglobin and protein contamination secondary to a significant amount of red blood cells and dead cells, respectively. One μg of each replicate of the RNA isolates was
reverse transcribed and amplified by PCR and bands at the expected size were found in 8 out of 12 samples (Fig. 4.3a).

Figure 4.2: Scatter dot plots from the skin CS (incomplete Freund’s adjuvant injection site) of rhesus monkey K282. Events of each suspension of skin cells were physically gated on the lymphocytes (a,c). Percentages of each quadrant of fluorescent scatter plots are represented in tables on the right side (b,d).

- a, b - Isotype controls to assess non-specific staining. A total of 5.65 % of the gated events exhibited non-specific staining (Total events: 5080; events gated: 708 (13.94%).
- c, d - CD8-PerCP (y-axis) versus CD4-FITC (x-axis) analysis. A significant proportion of events (*) from the upper right (UR) quadrant (double positive CD4+CD8+) and a minimal proportion of the upper left quadrant (CD8+) are non-specific events. Total events: 9520; events gated: 838 (8.80%).
Performing direct PCR without RT showed that significant and often-abundant genomic β-actin DNA contaminations were present in rhesus monkey samples from these experiments (Fig. 4.3b - lane 4). Subsequent samples from rhesus monkeys, including rhesus, but not human, MLR cultures, using the same lysing solution under similar conditions of RNA isolation also occasionally contain genomic DNA contamination. Dnase I treatment succeeded in entirely digesting genomic DNA contaminants, as assessed by 31 cycles of amplification using the β-actin human primers (Fig. 4.3b - lane 3).

4.3.4 HISTOPATHOLOGY

The IFA injection sites had consistently high content of inflammatory cells (Fig. 4.4). In particular, small lymphocytes and lymphoblasts were numerous around capillaries and venules at day 2, but waned thereafter. At day 2-post inoculation, the mononuclear cells: granulocytes ratio was close to 1, increasing to close to 3 at day 5 and falling to ~2 at day 7 post injection (Table 4.3). At all time points, in the panniculus, macrophages and eosinophils were numerous with fewer lymphocytes. Lymphocytes were numerous in the deep dermis and subcutaneous panniculus at day 5 post inoculation. Well formed eosinophilic granulomas with epithelioid macrophages and a few multinucleate giant cells were seen at day 7 post injection.

Figure 4.3:

a: β-actin RT-PCR from Skin CS. Two μg of total RNA isolate were reverse transcribed in a 40-μl volume. 5% (2 μl) of the RT product (equivalent to 0.1 μg of initial total RNA isolate) was used for 30 thermal cycles of PCR with β-actin primers. Lanes: L: 100 bp ladder, A: K282, 2 DPI; B: K967, 2 DPI; C: K 131, 5 DPI; D: L129, 5 DPI; E: K966, 7 DPI; F: L132, 7 DPI. panel 1: 1st replicates, panel 2: 2nd replicates. Column G, lane 2: negative control (distilled water). Lane H: panel 1=positive control at 1:100 dilution; panel 2=undiluted positive control.

b: testing genomic cDNA contamination from Skin cell suspension: Total RNA isolated from CS of monkey # K967 – 2nd replicate. 2 μg of total RNA isolate were used for Dnase I treatment in a 20 μl volume. Half of the Dnase I treated product (1 μg) was reverse transcribed in a 40-μl volume. Template from lane 1 was 0.1 μg of the RT-only product, lane 2 was 0.1 μg of the DnaseI+RT product, lane 3 was 10 % = 0.2 μg of Dnase I only product and lane 4 was 0.66 μg of the initial total RNA isolate, which were used to perform 31 thermal cycles with 20 nmoles of β-actin primers PCR. (fig. cont.)
Table 4.3: Leukocyte percentages in sections of skin sites injected with 0.1 ml of IFA.

<table>
<thead>
<tr>
<th>DPI*</th>
<th>% granulocytes</th>
<th>% monocytes</th>
<th>mono/gran§</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>45.13</td>
<td>54.87</td>
<td>1.22</td>
</tr>
<tr>
<td>2</td>
<td>54.37</td>
<td>45.63</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>22.16</td>
<td>77.84</td>
<td>3.51</td>
</tr>
<tr>
<td>5</td>
<td>25.11</td>
<td>74.89</td>
<td>2.98</td>
</tr>
<tr>
<td>7</td>
<td>36.36</td>
<td>63.64</td>
<td>1.75</td>
</tr>
<tr>
<td>7</td>
<td>29.58</td>
<td>70.42</td>
<td>2.38</td>
</tr>
</tbody>
</table>

The counts were performed from H&E-stained, paraffin embedded sections. More than 200 cells were counted for each biopsy.

*DPI: Days post inoculation
§ mono/gran = ratio of mononuclear cells : granulocytes.

4.4 DISCUSSION

The injection of incomplete Freund's adjuvant (IFA) in the skin of rhesus macaques elicited a focal inflammatory response that enabled the collection of inflammatory cells for flow cytometry analysis of CD4 and CD8 and γδ lymphocytes, and cells for RNA isolation at 2, 5 and 7 days post-
injection. The histopathological features of IFA injection sites, with abundant edema and eosinophils and dense lymphocytic infiltrates, correlated with the cell yield and quality as assessed by cytospins. This experiment was designed to determine the feasibility of a model for sampling the skin after *M. leprae* inoculation in the skin, and to optimize the methods.

However, early sampling after inoculation of *M. leprae* into the skin is anticipated to be more challenging because there may be less highly cellular lesions at the earliest time points. This will be especially true if there are no granulomas locally from which to extract large numbers of lymphocytes using collagenase to degrade reticulin fibers, such as type III and Type IV collagenase, at early time points (see chapter 6). Investigations at early time point are limited to retrieving the recruited perivascular mononuclear cells after fine mincing only. In leprosy, this technique has been successfully performed to obtain a small number of cells for cloning from fully developed lesions, yielding cell suspensions from leprosy lesions of the range of 5 \(10^5\) to 2 \(10^6\) lymphocytes after purification by gradient sedimentation (Modlin, Mehra et al. 1986). In the preliminary study reported here, the total numbers of mononuclear cells ranged from 2.6 to 68.6 \(10^6\) without density gradient purification after injection of 0.1 ml of incomplete Freund’s adjuvant.

One of the most encouraging findings with this approach was the strong evidence that the lymphocytes analyzed by flow cytometry are indeed recruited into the injection site, and were not merely circulating blood lymphocytes collected at the time of sampling, since CD4+ and \(\gamma\delta\) T cells percentages from the blood and skin of the same monkey are significantly different. The high to very high proportion of \(\gamma\delta\) T cells can be accounted for by the recent finding of an over-representation of T cells, the \(\gamma\delta\) T cell receptor of which may recognize lipid and complex lipid (e.g. lipoglycans, lipoproteins) antigens in the context of CD1c (Porcelli, Brenner et al. 1989) (reviewed by (Sugita, Moody et al. 1998). To our knowledge, there is no study reporting high numbers of \(\gamma\delta\) T cells recruited at sites of IFA injection in rhesus macaques.

Abundant \(\beta\)-actin mRNA was obtained from 8 out of 12 RNA isolates, showing the feasibility and the difficulties of this approach. Low or absent \(\beta\)-actin cDNA correlated with low 260 nm optical density (O.D.) and low purity as assessed by 260nm:280nm OD ratio, and was presumably due to inadequate isolation of nucleic acids. However, with the technology employed (Chomczynski and

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Sacchi 1987), we had consistent genomic DNA (gDNA) contamination. If variable amounts of gDNA are present in any of specimens, a competitive setting will be present during amplification between gDNA and complementary DNA (cDNA) that can further diminish the value of semi-quantification using relative RT-PCR, because the amount of cytokine mRNA would be under-estimated. Dnase I treatment before RT is necessary in such specimens to accurately standardize the samples for the housekeeping gene β-actin to perform relative ("semi-quantitative") RT-PCR.

Numerous granulocytes, a majority of which were eosinophils, were seen in the cutaneous IFA injection sites. Whether these cells may have been detrimental to optimal flow cytometry analysis by releasing their eosinophilic granules, for example, is unknown. It would be desirable to eliminate granulocytes by density gradient centrifugation, especially if high mononuclear cell yields can be obtained, as in this study. However, for the collection of inflammatory leukocytes from sites of injection of a less irritating inoculum, such as Mycobacterium leprae in buffered saline, lower numbers of leukocytes with minimal numbers of granulocytes may be retrieved, Skin CS analysis without density gradient centrifugation may be satisfactory for flow cytometry. The lower number of leukocytes with a minimal number of granulocytes can be anticipated, since M. leprae is minimally toxic by itself (Job 1994)

Based on the results of this preliminary study, the following method will be used in the main study: 1) two 8 mm punch biopsies will be taken. 2) one will be swabbed with a sterile gauze to reduce blood contamination and be finely minced and filtered to obtain a Skin CS for FACS analysis only. 3) The other will be cut in two halves, one to be fixed in neutral buffered zinc-formalin and the other frozen for mRNA and immunohistochemistry.

4.5 LITERATURE CITED


5 A VARIABLE CELL-MEDIATED IMMUNE RESPONSE IS PRESENT IN Rhesus Macaques (Macaca mulatta) AFTER A SECOND INOCULATION OF Mycobacterium leprae, WHICH CORRELATES WITH PERSISTENT INFECTION, BUT NOT THE SIV STATUS

5.1 INTRODUCTION
The main objective of this study was to assess any difference in the timing, the magnitude and the adequacy of the immune response to Mycobacterium leprae between SIV+ and SIV- rhesus macaques, in the context of documented demonstration of impaired immunity against most mycobacteria in SIV+ animals [Levy, 1996 #236]. Also evaluated was the correlation between phenotyping by flow cytometry and the levels of cytokine mRNA expression with the development of persistent M. leprae infection. The association between grossly visible inoculation sites and SIV status or persistent M. leprae infection was also examined. The necropsy findings at the closure of the study 13 months after second M. leprae inoculation will be summarized. Performance of sorting of lymph node cell suspensions will be presented.

5.2 MATERIALS AND METHODS
5.2.1 ANIMALS
The animals studied have been described previously [Gormus, 1998, #126][Gormus, 1998 #125]. They were housed in Tulane’s Regional Primate Research Center, Covington, LA, in accordance with established standards of the United States Federal Animal Welfare Act, the American Association for Accreditation of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. Eight female and one male rhesus macaques (Macaca mulatta), that did not develop any sign of leprosy after intravenous (IV) and intradermal (ID) inoculation 26 months earlier with M. leprae from nine-banded armadillo (Dasypus Novemcinctus, Linn.) were studied. This primary inoculation consisted of a total of 1.075 \(10^9\) per animal, as previously described [Gormus, 1998 #126]. Two weeks before the initial M. leprae inoculation, 3 of them (N264, N356 and N976) were inoculated with the sooty mangabey-derived delta J943 strain of simian immunodeficiency virus (SIV).

One to 2 weeks before the M. leprae challenge, a biopsy of lymph node was taken from each animal as a reference control for baseline proportions of lymphocytes and baseline cytokine mRNA
expressions. No skin from a non-inoculated area was taken at any time point, the normal skin being assumed to have negligible infiltrates with lymphocytes and resident histiocytes and an absence of detectable cytokine mRNA expression. Main signalment features and inoculation schedule of the animals used in this study are presented in table 5.1.

Table 5.1: Signalment, inoculation schedule and euthanasia age of the rhesus macaques used for re-challenge with *M. leprae*. Also shown are the main clinical signs before euthanasia. On the first column from left is shown each monkey’s identification number.

<table>
<thead>
<tr>
<th>SIV</th>
<th>DOB</th>
<th>Sex</th>
<th>Age at inoculation (years)</th>
<th>Age at euthanasia (years)</th>
<th>Interval (year) after 1st ML inoculation</th>
<th>Clinical signs</th>
<th>Nasal smear AFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIV J943</td>
<td>1st ML</td>
<td>2nd ML</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N264</td>
<td>+ 05/21/92 female</td>
<td>3.2</td>
<td>3.3</td>
<td>5.4</td>
<td>6.7</td>
<td>3.4</td>
<td>None -</td>
</tr>
<tr>
<td>N356</td>
<td>+ 06/03/92 female</td>
<td>3.2</td>
<td>3.2</td>
<td>5.4</td>
<td>5.9</td>
<td>2.7</td>
<td>Diarrhea +</td>
</tr>
<tr>
<td>N976</td>
<td>+ 04/05/93 female</td>
<td>2.4</td>
<td>2.4</td>
<td>4.5</td>
<td>5.2</td>
<td>2.8</td>
<td>Diarrhea -</td>
</tr>
<tr>
<td>M726</td>
<td>- 03/30/92 female</td>
<td>3.4</td>
<td>5.6</td>
<td>6.8</td>
<td>3.4</td>
<td>None -</td>
<td></td>
</tr>
<tr>
<td>M736</td>
<td>- 03/31/92 female</td>
<td>3.4</td>
<td>5.6</td>
<td>6.8</td>
<td>3.4</td>
<td>None -</td>
<td></td>
</tr>
<tr>
<td>M849</td>
<td>- 04/17/92 female</td>
<td>3.4</td>
<td>5.5</td>
<td>6.8</td>
<td>3.4</td>
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<td></td>
</tr>
<tr>
<td>M879</td>
<td>- 04/21/92 female</td>
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<td>5.5</td>
<td>6.8</td>
<td>3.4</td>
<td>None -</td>
<td></td>
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<tr>
<td>M879</td>
<td>- 04/17/92 female</td>
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<td>5.5</td>
<td>6.8</td>
<td>3.4</td>
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<tr>
<td>N209</td>
<td>- 05/13/92 female</td>
<td>3.3</td>
<td>5.4</td>
<td>6.7</td>
<td>3.4</td>
<td>None -</td>
<td></td>
</tr>
<tr>
<td>P282</td>
<td>- 05/09/93 male</td>
<td>2.3</td>
<td>4.4</td>
<td>5.7</td>
<td>3.4</td>
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<td></td>
</tr>
<tr>
<td>AVG</td>
<td>07/17/92</td>
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<td>3.1</td>
<td>5.3</td>
<td>6.4</td>
<td>3.3</td>
<td></td>
</tr>
</tbody>
</table>

SIV: sooty mangabey-derived simian immunodeficiency virus chronic infection status (strain J943)
DOB: date of birth
ML: Mycobacterium leprae
AVG: average
AFB: acid fast bacilli

5.2.2 CHALLENGE

Fresh *M. leprae* from three nude (nu/nu) mouse footpad lepromas were collected aseptically 6 months after inoculation, as previously described [Sibley, 1988 #149]. They yielded a total of 13.5 $10^9$ bacilli with a typical bacterial viability for *M. leprae* as assessed by $^{14}$CO2 catabolic radiorespirometry measured at 1, 2 and 3 weeks post-culture medium inoculation, using Bactec460TB system® (Becton Dickinson, Sparks, MD) (Fig 5.1) as previously described [Franzblau, 1988 #151] [Franzblau, 1989 #152].

The challenge consisted of 12 intra-dermal inoculations per animal in the thighs and arms (i.e. 3 sites for each limb), each with $10^7$ *M. leprae* bacilli suspended in 100µL of phosphate buffer saline. The total dose per animal was $1.2 \times 10^8$ bacteria. Typical inoculation sites are shown in Figure 5.2. The inoculation sites were not tattooed to avoid of non-specific inflammatory responses to that procedure.
Instead, they were topographically mapped relative to anatomical landmarks such as bony processes of the elbow and knees.

Figure 5.1: Evaluation of *M. leprae* inoculum viability using $^{14}$C radiorespirometry. A total of 8 aliquots were evaluated at time of leproma collection, 1, 2 and 3 weeks later. The evaluation system is the following: The scale is from 0 to 999. $0 < \text{Growth index (GI)} \leq 300$: low activity; $300 < \text{GI} \leq 600$: medium activity; $600 < \text{GI} < 999$: high activity.
5.2.3 SAMPLING AND SAMPLE USES
The schedule of sampling is summarized in figure 5.3. Sampling of the inoculation sites were not at random, but matched the sampling of lymph node biopsy for a particular time point (fig. 5.4). The schedule for skin sampling was as follows: two 8-millimeter diameter punch biopsies were taken at days 2, 5, 8, 12, 26 and 62. One of these biopsy specimens sampled at each time point was immediately swabbed of excess blood, finely minced in RPMI culture medium with 30% fetal calf serum (RPMI-30), filtered through a nylon gauze and collected in a 15 ml centrifugation tube, and kept on ice until counted with a hemocytometer and processed for flow cytometry 2-4 hours later. The optimization of this sampling technique on inoculation sites has been tested on a model of intra-dermal injection of incomplete Freund's adjuvant, the results of which are presented in the chapter four.

The other skin biopsy was bisected and one half was immediately embedded in OCT compound (Sakura, Torrance, CA) and snap-frozen in liquid nitrogen for CD4 and CD8 immunophenotyping by immunohistochemistry and ex vivo cytokine mRNA expression by relative reverse transcriptase-polymerase chain reaction (RT-PCR). The other half was immersed in 4% neutral buffered zinc-formalin for standard histopathological assessment of inoculation sites.
The axillary or inguinal lymph node (LN) biopsies were taken at day -14/-7 (baseline), day 5, day 26 and day 62 after inoculation, with care to have them match the timing and location of the skin inoculation site biopsies (Fig. 5.4). Approximately 15-20% of the LN biopsy was immersed in 4% neutral zinc-buffered formalin for standard histopathological assessment of possible disease and/or mycobacteria spreading. Another ~15-20% was snap-frozen in liquid nitrogen. The remaining tissue was finely minced, passed through a 67-μm mesh and collected in RPMI-10, to obtain a lymph node cell suspension (LNCS). A sample of the LNCS was utilized for flow cytometry cell surface marker studies, another sample (5 x 10⁶ cells) was processed for mRNA cytokine expression studies and the rest panned to remove B cells and sorted immuno-magnetically into CD8 and non-CD8 cell fractions (described in chapter 6).

Four ml of heparinized blood was collected intravenously at days -7/-14 (baseline), day 5, day 26 and day 62 for immunophenotyping by flow cytometry. At day 5 and 96, an additional 15 ml of blood was drawn to collect PBMC by density gradient centrifugation in order to perform in vitro lymphocyte functional studies (lymphocyte proliferation assay).
1, 2: day 2
3, 4: day 5
5, 6: day 8
7, 8: day 12
9, 10: day 26
11, 12: day 62

Figure 5.4: Schematic drawing of the skin inoculation sites with regional lymph nodes studied. The numbers refer to the sequential succession of skin and lymph node biopsies. Skin and LN sampling match anatomically. All the inoculations, surgery procedures and sampling were performed by a trained veterinary surgeon. B = Baseline sampling 1 to 2 weeks before inoculation (-7 or -14 DPI).

5.2.4 DATA ACQUIRED BY COLLABORATORS

5.2.4.1 Evaluation of Grossly Visible Inoculation Sites
At the time of biopsy performed by a veterinary surgeon (Dr Marion Ratterree), the animals were evaluated for the presence or the absence of grossly visible inoculation sites, information which was recorded on each animal's medical history record. When a grossly visible site was noted, it was distinguished between 1) frankly visible, defined as an easily visible redness or an induration easily palpable and 2) barely visible, corresponding to slight, minimal gross changes noted at the inoculation site. Association with SIV status and outcome with regard of persistent *M. leprae* infection (see next section) were tested using a $\chi^2$-square test.

5.2.4.2 Post-Mortem Evaluation of Disease and Infection Status
When SIV+ animals developed SAIDS-associated untreatable disease necessitating humane euthanasia (see Table 3.1), or 15 months after the second inoculation of *M. leprae*, the animals were sacrificed by overdose of pentobarbital and a complete necropsy with sampling for microscopic evaluation was performed by Dr Gary Baskin, from the Tulane’s delta primate research center. Nerves, including the peroneal and sciatic nerves, several areas of the skin, including the inoculation sites, and nasal mucosa were systemically sampled and examined for persistent *M. leprae* infection and disseminated lesions. Persistent *M. leprae* infection was defined as the presence of one or more lesions.
characteristic of leprosy in the peripheral nerves, skin areas remote from inoculation sites or nasal mucosa.

5.3 RESULTS

5.3.1 GROSS FINDINGS IN THE SKIN OF Rhesus Macaques

Grossly visible lesions were discrete, small and often barely visible. At early time points, they were characterized by redness and slight elevation. At later time points (day 26 and 62), they were 0.5 to 1-cm slightly indurated areas (Fig 5.5).

Figure 5.5: Grossly visible inoculation sites on the right arm of a SIV-rhesus monkey (N209) at day 26 post inoculation. This animal did not develop persistent M. leprae infection. The sites were red, slightly raised and indurated (arrows). Also shown is a healing biopsy site from day 12 (arrowheads).

The proportion of animals grouped by SIV status and persistent M. leprae infection outcome (leprosy status), as assessed at post-mortem examination, exhibiting grossly visible inoculation sites, are presented in figure 5.6.
Grossly visible inoculation sites were first seen at day 2 in SIV- animals and at day 5 in SIV+ animals. SIV+ animals had more frankly visible lesions at day 62, suggesting more persistent inflammation at site of inoculation. Frankly visible inoculation sites at early time points, most evident at day 5, were significantly associated with resistance to persistent \textit{M. leprae} infection (p<0.05).

Interestingly, the animals that did not show any lesions were not randomly distributed. For example, the SIV+ animal that did not show any gross lesion from day 5 to day 62 was consistently the monkey number N356. It corresponded to an animal with minimal to no recruitment of lymphocytes as assessed by histology and immunohistochemistry (see below chapter 6). Moreover, the Fite stain showed histiocytes filled with acid fast bacilli (AFB) at day 5, indicating that the inoculation site was indeed biopsied. This particular animal (N356) developed disseminated leprosy (LL-like disease) 6 months after the last sampling (62 DPI), as indicated by a positive nasal smear for AFB before euthanasia (Table 5.1) and microscopically by the presence of granuloma composed of histiocytes and numerous AFB in the lesions in the nerves, skin and nasal mucosa (Table 5.2).

5.3.2 \textbf{Study End-point: Post Mortem Findings}

Two SIV+ animals developed incurable diarrhea by 2 and 4 months. Due to the rapid deterioration of their clinical conditions, euthanasia was performed at 6 months (N356) and at 8 months (N976) after the second challenge with \textit{M. leprae}. All the other animals remained apparently healthy and were scheduled to be sacrificed 15 months after the second inoculation, at an average of 6.63 years of age, and 3.4 years after the first \textit{M. leprae} inoculation (Table 5.1). Except for the two SIV+ animals that had clinical signs associated with gastro-intestinal (GI) disease, all the monkeys were clinically healthy and did not have any gross skin lesions similar to what is seen in humans with leprosy. At the time of euthanasia, no skin change suggestive of early leprosy was observed, except the SIV+ monkey N356 that had multifocal areas of hyperkeratosis in the skin, corresponding microscopically to widespread lesions in the skin and the nose. In this animal, the right superficial peroneal nerve and many cutaneous nerves were heavily infiltrated with histiocytes, which contained globi and numerous
<table>
<thead>
<tr>
<th>Rhesus #</th>
<th>SIV</th>
<th>Gross lesions²</th>
<th>Leprosy status¹</th>
<th>localization</th>
<th>AFB in nerve</th>
<th>other diagnosis (dx.) 1</th>
<th>other dx. 2</th>
<th>other dx 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>N264</td>
<td>+</td>
<td>-</td>
<td>none</td>
<td>nerve</td>
<td>-</td>
<td>mild pneumonia (P. carinii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N356</td>
<td>+</td>
<td>+&quot;*</td>
<td>LL</td>
<td>+ + +</td>
<td>3+</td>
<td>pneumonia (P. carinii)</td>
<td>MAI infection?</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>N976</td>
<td>+</td>
<td>-</td>
<td>none</td>
<td>nerve</td>
<td>-</td>
<td>Enteritis (cryptosporidiosis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M726</td>
<td>-</td>
<td>-</td>
<td>BL</td>
<td>+ + +</td>
<td>2+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M736</td>
<td>-</td>
<td>-</td>
<td>BL</td>
<td>+ +</td>
<td>1+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M849</td>
<td>-</td>
<td>-</td>
<td>BL</td>
<td>+ +</td>
<td>3+</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>M879</td>
<td>-</td>
<td>-</td>
<td>BL</td>
<td>+ + +</td>
<td>3+</td>
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<tr>
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<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P282</td>
<td>-</td>
<td>-</td>
<td>none</td>
<td>nerve</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹in the skin only. ²multifocal areas of hyperkeratosis, corresponding to leprosy skin lesions microscopically.
Figure 5.6: Proportions of rhesus monkeys exhibiting grossly visible skin inoculation sites. Graphs (a) and (c) show the frankly visible skin inoculation sites. Graphs (b) and (d) include the barely visible ones. Proportions are grouped by SIV status (a, b) and persistent *M. leprae* infection (c, d). Grossly visible inoculation sites were first seen at day 2 in SIV- and at day 5 in SIV+ animals. SIV+ animals had more frankly visible lesions at day 62, suggesting more persistent inflammation at site of inoculation. Frankly visible inoculation sites at early time points, most evident at day 5, were significantly associated with resistance to persistent *M. leprae* infection (p<0.05). Statistically significant results are marked by (*).
AFB. The skin of the nose, ears, arms and hands were also infiltrated and contained abundant AFB, as did the nasal septum. This monkey was therefore classified in the lepromatous leprosy (LL) part of the Ridley and Jopling spectrum (Ridley, 1966) based on these microscopic criteria because of widespread dissemination of the infection and the nature of the granulomatous infiltrate, even though no gross lesions such as lepromas were seen. This is most probably related to the short time range between the second *M. leprae* inoculum and euthanasia due to SAIDS opportunistic diseases (Table S.1 and table 5.2).

5.3.3 **Quality Control of the Skin Sampling by Examination of the Formalin Fixed and Frozen Skin Biopsies**

It was discovered retrospectively that some sites at day 5, 8 and 12 had been partially or completely missed when grossly visible lesions were observed adjacent to sampling scars at later time points, and confirmed microscopically on fixed and frozen biopsies. Those data were deleted from the flow cytometry (FC) database and not further processed for cytokine mRNA expression by RT-PCR. All data from day 8 was dropped from flow cytometry analysis for this reason (4 documented missed biopsies for flow cytometry) and cytokine analysis from frozen biopsies (3 missed sites confirmed on immunohistochemistry). Documented non-missed sites from day 8 were stained by immunohistochemistry (IHC) for CD4 marker only (see chapter 6). Day 5 and 12 had 2 confirmed missed sites on frozen biopsies that were not further processed and 2 suspected missed sites for flow cytometry that were deleted from the database.

5.3.4 **Performance of the Immuno-Magnetic Sorting of Lymph Node Cell Suspension**

The performance of the CD8 positive selection by Immuno-Magnetic Sorting (MACS) could not be evaluated due to the inability of CD8+ lymphocytes coated with anti-CD8 beads to be adequately labeled with anti-CD8-PerCP. We did not have any monoclonal antibodies tagged with PerCP or other fluorescent dye directed against magnetic beads, preventing performance analysis of the CD8 during the main study. The results in chapter 3 on magnetic sorting optimization indicate a very high performance of this sorting (consistently >95% purity). Results of descriptive statistics of the performance of this negative selection of CD4+ cells in the CD8 single sort are shown in Table 5.3. The standard deviation is higher than for a positively selected fraction. These results are better than those obtained during the preliminary attempts from rhesus LNCS reported in chapter 3 (see figure3.2 page).
Table 5.3: Performance of the CD4 enriched fraction from lymph node cell suspensions.

<table>
<thead>
<tr>
<th></th>
<th>% total CD4*</th>
<th>% CD8</th>
<th>% DP2</th>
<th>% total CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>74.22</td>
<td>9.53</td>
<td>6.63</td>
<td>16.16</td>
</tr>
<tr>
<td>std deviation*</td>
<td>17.69</td>
<td>15.97</td>
<td>8.32</td>
<td>22.48</td>
</tr>
<tr>
<td>median</td>
<td>74.65</td>
<td>2.20</td>
<td>1.75</td>
<td>3.56</td>
</tr>
<tr>
<td>minimum</td>
<td>43.70</td>
<td>0.19</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>maximum</td>
<td>94.14</td>
<td>60.95</td>
<td>29.19</td>
<td>80.05</td>
</tr>
</tbody>
</table>

* average of measures from 2 tubes, one tube having replicates measures (n=108)
\(^i\) DP=double positive CD4-CD8 lymphocytes
\(^*\) std = standard

5.3.5 THE MONKEYS SHOW EVIDENCE OF CELL MEDIATED IMMUNITY AGAINST *M. leprae* ANTIGENS

In the initial study design, lymphocyte stimulation tests would have been performed on day 5, day 26 and 62. However, day 5 cultures were contaminated. Day 26 and 62 were not done for logistic reasons. Rather, blood was collected at 96 DPI. Lower counts than expected were obtained with PHA stimulation, for unknown reasons. A statistically significant response was obtained in both ML-S stimulated cultures as compared with the non-stimulated cultures (p<0.05). An increased response was obtained with the higher concentrations of ML-S [ML-S] tested, that was statistically different from the lower [ML-S] when the natural logarithm of the counts per minute (lncpm) instead of cpm was used as the dependant variable in the ANOVA analysis (p<0.05) (Table 5.4).

Table 5.4: Stimulation indices of *M. leprae*-sonicate stimulated with rhesus PBMC at 96 days following the second challenge with *M. leprae*.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>None(^i)</th>
<th>ML-S(^\psi)</th>
<th>PHA(^\delta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dose</td>
<td>NA(^*)</td>
<td>5 µg/ml</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Lncpm(^\xi)</td>
<td>4.63</td>
<td>5.21</td>
<td>5.43</td>
</tr>
<tr>
<td>SI(^**)</td>
<td>1.00</td>
<td>1.78</td>
<td>2.23</td>
</tr>
</tbody>
</table>

\(^\xi\) Medium only
\(^\psi\) *M. leprae*-sonicate
\(^\delta\) Phytohemagglutinin
\(^*\) NA = non applicable.
\(^\xi\) Lncpm = natural logarithm (Neperian) of the average counts per minutes
\(^**\) SI = stimulation index

5.4 DISCUSSION

The primary goal of this immuno-pathological study was to compare the local and regional response to skin inoculations of *M. leprae* between SIV [+] and SIV [-] rhesus macaques, the best known model of HIV infection and AIDS in man. Our goal was not, therefore, to determine whether the
Rhesus macaques represent the best animal model of human leprosy. The strength of this model is that, with large quantities of sooty-mangabey-derived, nude mouse-derived and/or armadillo-derived *M. leprae* inoculations, different forms of leprosy can be produced within a few years in a small proportion of rhesus macaques, with occasional nasal excretion of leprosy bacilli, thereby mimicking the situation in humans (Gormus, Xu et al. 1998). Human-derived immunological reagents are also widely available. The weakness is that there are usually no gross cutaneous lesions in the animals developing the persistent infection, except in some severely immunodeficient, SIV-infected animals (Baskin 1990). The long incubation time before evidence of persistent infection is also a disadvantage, and the classic tuberculoid forms are not produced as in humans (Gormus, Xu et al. 1998). Upon gross examination of the skin inoculation sites at the time of sampling, no significant difference was observed between SIV+ and SIV- animals, in terms of both the intensity of the reaction and the proportions of animals showing visible lesions after inoculation. However, when comparing animals that later developed persistent *M. leprae* infection with those that did not, grossly visible inoculation sites were observed soon after inoculation, within 5 days, in the latter group.

Upon re-inoculation, 5 out of 9 monkeys developed persistent *M. leprae* infection, which is substantially higher than the proportion reported in previous studies (Gormus, Xu et al. 1998). The results in this study need to be taken with caution due to the low number of animals. They suggest, however, that among animals that apparently handled the infection without clinical consequences, some will develop persistent infection. An unsettled issue in this study that might be worth testing in further experiments would be to know whether those animals were already harboring *M. leprae* prior to re-inoculation or were without persistent foci of *M. leprae* until the second inoculation. This is an important point to consider in interpreting the cytokine data, notably from lymph node cell suspensions (see chapter 7).

These results also raise the question concerning repeated exposure to *M. leprae*. The classical view regarding immunity to bacteria and mycobacteria is that once an animal has been challenged with a dose of virulent organism and developed immunity against it, disease should not develop upon the second challenge. In light of these results, however, the matter of repeated *M. leprae* inoculations to experimental animal models is worth exploring. Lastly, the same number of animals that did not
develop persistent *M. leprae* infection was observed in both SIV- and SIV+ animals, suggesting that the rhesus macaques from the group of slow progressors towards SAIDS can handle a second challenge with *M. leprae*. It might be that there is a dichotomy of reaction in SIV+ animals with regard to primary and secondary responses to *M. leprae*. Upon primary infection, slightly more animals developed persistent infection (Gormus 1989; Gormus, Murphey-Corb et al. 1998). However, it is possible that the SIV+ animals that successfully limit the primary infection will better handle a second challenge. This would in any case be an hypothesis to explore with larger numbers of rhesus macaques and no firm conclusions should be drawn from this limited number of animals.

The "typical" exposure dose of *M. leprae* from a multibacillary human patient to contacts is unknown. Also the exact route and mode of exposure remains unknown in humans (Fine 1982). The transmission from the environment to abraded skin has recently been the subject of a renewed epidemiological interest (Abraham, Mozhi et al. 1998) in leprosy. These authors showed that the cooler and unprotected areas of the body were more likely to be site of the first detected leprosy lesion in children in Southern India. These results are compatible with findings from other investigators (reviewed in (Abraham, Mozhi et al. 1998), supporting the hypothesis that this route is likely in human leprosy, in addition to the alternative, but also unproven, nasal route (Rees, McDougall et al. 1974). Studies from nasal secretions from lepromatous patients have revealed an excretion rate of the order of 10 millions (10^7) per day (Davey and Rees 1974). Infective doses of other pathogens were extensively studied in the past and were found important in terms of development of cellular versus humoral immunity (reviewed by (Constant and Bottomly 1997). Recently, it has been shown that dose can influence the cytokine response to a mycobacterial infection in susceptible mice (Power, Wei et al. 1998).

The dose used in this study was appropriate based on the above considerations. Whether this particular dose of *M. leprae* might have favored a TH2/type 2 over a TH1/type 1 response in non-naive, *M. leprae*-infected monkeys in a manner similar to high dosed BCG-infected mice is unknown (Power, Wei et al. 1998). This possibility deserves to be investigated in future experiments.

### 5.5 LITERATURE CITED


6 CD4+ CELL RECRUITMENT AFTER A SECOND CHALLENGE WITH *M. LEPRAE* IS DELAYED IN SKIN INOCULATION SITES IN RHESUS MONKEYS WITH SUBSEQUENT PERSISTENT *M. LEPRAE* INFECTION

6.1 INTRODUCTION

The epidemiology of various mycobacteriosis is quite different in human immunodeficiency virus (HIV)-infected individuals than in immunocompetent individuals. Whereas Mycobacteria such as *M. avium* and other mycobacteria such as *M. kansasii* and *M. marinum* elicit only benign self-limiting infections at site of inoculation in most individuals, they have a devastating impact on acquired immuno-deficiency syndrome (AIDS) patients. Also tuberculosis is a more serious condition among HIV-infected persons, usually having a harsher evolution and worse prognosis. However, there are no reports suggesting an increase in incidence or a worsening of the evolution of leprosy in HIV-infected individuals and AIDS patients (Frommel, Tekle-Haimanot et al. 1994; Sampaio, Caneshi et al. 1995; Faye, Mahé et al. 1996; Machado, David et al. 1998). Studies are therefore needed to investigate the basis of this differential susceptibility in the immuno-compromised host to mycobacteria.

The rapid recruitment of primed, antigen-specific helper and cytotoxic CD4+ T lymphocytes at sites of invasion of *Mycobacteria* is believed to be critical for the outcome of mycobacterial infection (Reviewed in (Orme, Andersen et al. 1993). Whereas in tuberculosis the invasion site is usually the lung, it is believed that the portal of entry of the leprosy bacillus, *Mycobacterium leprae*, is through abraded skin and/or nasal mucosa (reviewed by (Fine 1982). The early pathogenic mechanisms involved in leprosy after natural exposure to the organism remain poorly understood because of a lack of well established models of the human infection that would enable studies of the early and probably critical events following the exposure to the organism. In humans, it is believed that single lesions of the indeterminate type (Harboe 1994; Job 1994; Job 1997) may represent the earliest manifestations of the disease, but the time interval after infection is not known. The only way to examine the early events in response to *M. leprae* exposure is to perform longitudinal studies in animal models such as the non-human primates (reviewed by (Meyers, Gormus et al. 1994). It is possible that the primary lesion and
initial immune response is polymorphic and predictive of the outcome, either healing or development of disseminated disease. In addition, it may be that the primary lesion and immune response at the site of *M. leprae* inoculation might be different in HIV+ patients compared with HIV- individuals.

To examine these early events, we re-inoculated *M. leprae* into the skin of 9 rhesus monkeys previously inoculated by *M. leprae*, which remained free of leprosy disease for 26 months: three animals that were simian immunodeficiency virus (SIV) positive and six were SIV negative monkeys. The doses of *M. leprae* inoculated corresponded to the average number of bacilli excreted by nasal secretions of lepromatous humans (10^7 viable bacilli per day) (Davey and Rees 1974).

The main hypothesis of this *in vivo* study was that the SIV positive rhesus macaques mount an inadequate cell mediated immunity against recall inoculations of *M. leprae* in the skin, as compared with SIV negative animals. In particular, we tested the hypothesis that SIV+ rhesus monkeys recruit inadequate proportions of recruited CD4+ T lymphocytes and insufficient numbers of activated/memory CD4+CD45RO+ T cells at site of skin inoculation.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 ANIMALS

The animals studied have been described previously (Gormus, Murphey-Corb et al. 1998; Gormus, Xu et al. 1998). They were housed in Tulane’s Regional Primate Research Center, Covington, LA, in accordance with established standards of the United States Federal Animal Welfare Act, the American Association for Accreditation of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. Eight female and one male rhesus macaques (*Macaca mulatta*), that did not develop any sign of leprosy after intravenous (IV) and intradermal (ID) inoculation 26 months earlier with *M. leprae* from nine-banded armadillo (*Dasypus Novemcinctus*, Linn.) were studied. This primary inoculation consisted of a total of 1.075 \times 10^9 per animal, as previously described (Gormus, Xu et al. 1998). Two weeks before the initial *M. leprae* inoculation, 3 of them (N264, N356 and N976) were inoculated with the sooty mangabey-derived delta J943 strain of simian immunodeficiency virus (SIV).

One to 2 weeks before the *M. leprae* challenge, a biopsy of lymph node was taken from each animal as a reference control for baseline proportions of lymphocytes and baseline cytokine mRNA expressons. No skin from a non-inoculated area was taken at any time point, the normal skin being
assumed to have negligible infiltrates with lymphocytes and resident histiocytes and an absence of detectable cytokine mRNA expression.

6.2.2 CHALLENGE
Fresh *M. leprae* from three nude (nu/nu) mouse footpad lepromas were collected aseptically 6 months after inoculation, as previously described (Sibley and Krahenbuhl 1988). They yielded a total of $1.35 \times 10^9$ bacilli with an typical bacterial viability for *M. leprae* as assessed by $^{14}$CO$_2$ catabolic radiorespirometry measured at 1, 2 and 3 weeks post-culture medium inoculation, using Bactec460TB system® (Becton Dickinson, Sparks, MD) as previously described (Franzblau 1988) (Franzblau 1989).

The challenge consisted of 12 intra-dermal inoculations per animals in the thighs and arms (i.e. 3 sites for each limb), each with $10^7$ *M. leprae* bacilli suspended in 100μL of phosphate buffer saline. The total dose per animal was $1.2 \times 10^8$ bacteria. Typical inoculation sites are shown in Figure 5.2. The inoculation sites were not tattooed to avoid of non-specific inflammatory responses to that procedure. Instead, they were topographically mapped relative to anatomical landmarks such as bony processes of the elbow and knees.

6.2.3 SAMPLING AND SAMPLE USES
The schedule for skin sampling was as follows: two 8-millimeter diameter punch biopsies were taken at days 2, 5, 8, 12, 26 and 62 from arms and thighs. One of these biopsy specimens sampled at each time point was immediately swabbed of excess blood, finely minced in RPMI culture medium with 30% fetal calf serum (RPMI-30), filtered through a nylon gauze and collected in a 15 ml centrifugation tube, and kept on ice until counted with an hemocytometer and processed for flow cytometry 2-4 hours later. The optimization of this sampling technique on inoculation sites has been tested on a model of intra-dermal injection of incomplete Freund’s adjuvant, the results of which are presented in the chapter four.

The other skin biopsy was bisected and one half was immediately embedded in OCT compound (Sakura, Torrance, CA) and snap-frozen in liquid nitrogen for CD4 and CD8 immunophenotyping by immunohistochemistry and *ex vivo* cytokine mRNA expression by relative reverse transcriptase-polymerase chain reaction (RT-PCR). The other half was immersed in 4% neutral buffered zinc-formalin for standard histopathological assessment of inoculation sites.
The axillary or inguinal lymph node (LN) biopsies were taken at day -14/-7 (baseline), day 5, day 26 and day 62 after inoculation, with care to have them match the timing and location of the skin inoculation site biopsies. Approximately 15-20% of the LN biopsy was immersed in 4% neutral zinc-buffered formalin for standard histopathological assessment of possible disease and/or mycobacteria spreading. Another ~ 15-20% was snap-frozen in liquid nitrogen. The remaining tissue was finely minced, passed through a 67-µm mesh and collected in RPMI-10, to obtain a lymph node cell suspension (LNCS). A sample of the LNCS was utilized for flow cytometry cell surface marker studies, another sample (5 x 10⁶ cells) was processed for mRNA cytokine expression studies and the rest panned to remove B cells and sorted immuno-magnetically into CD8 and non-CD8 cell fractions.

6.2.4 MICROSCOPIC EVALUATION
6.2.4.1 Histopathology
Half of one of the two biopsies was fixed in 4% buffered zinc formalin overnight. They were then dehydrated and embedded using standard procedures. Standard Hematoxylin and Eosin and standard Fite Faraco stains were performed on all biopsy specimens. The inflammation was graded as follows: Perivascular mononuclear infiltrates were graded as mild (+), moderate (+++) or severe (++++) when one row, 1-5 and >5 rows, respectively, of mononuclear cells were present around the vessels. The magnitude (size) and nature (aggregates of monocytes/histiocytes versus epithelioid macrophages) of the granulomas, first apparent at day 5, were also evaluated. These reading was performed before the outcome data was available.

6.2.4.2 Immunohistochemistry
Halves of eight-millimeter punch skin biopsies were covered with OTC compound (Tissue-Tek®, Sakura Finetek, Torrance, CA), snap frozen in liquid nitrogen immediately after excision. Frozen sections (4 µm) were cut using a Jung 4800 freezing microtome (Leica, Nußloch, Germany) and immediately fixed for 10 seconds in cold acetone (-20 °C) and then allowed to dry. They were either stored at - 70° C until processed (usually the next day) or directly forwarded to immunohistochemistry using the following protocol:

The sections were dehydrated for 5 min in cold acetone and washed twice in phosphate buffered saline (PBS). The majority of processed slides were pre-incubated for 20 minutes at 42 °C in avidin solution, then for 20 minutes at 42 °C in 2 % biotin solution (Dako, Carpinteria, CA) to reduce
the non-specific binding of antibodies to collagen bundles of the skin. They were then incubated with primary antibodies for 1 hour at 42 °C at the following working concentrations: mouse anti-macaque CD3 (clone FN-18, Serotec, Raleigh, NC) monoclonal antibodies: 1/50; mouse anti-human CD8 (clone DK25, DAKO, Carpinteria, CA): 1/50 and anti-CD4 (clone OKT4/Fischer, Houston, TX): 1/25. Due to the often weak CD4 immunostaining, an amplification technique, based on the local deposition of biotinyl tyramide at site of specific primary binding (Raikow 1998), was optimized and used according to manufacturer’s instructions (TSA™-indirect, NEN™ life science products, Boston, MA). Performance were similar or better using a CD4 MoAb at a dilution of 1:100 with tyramide signal amplification as compared with the previous protocol, using OKT4 antibodies diluted at 1:25.

For generating the IHC data, no minimum number of cells counted was adopted, due to the minimal inflammation in some animals, especially, but not only, at early time points (up to day 8 PI). The criterion for inclusion as a positive cell was a complete peripheral staining of the cytoplasm. Granular cytoplasmic staining was rare and was considered as non-specific (fig. 7.8 c and d). Areas such as perivascular infiltrates and granuloma were individually counted as units and then the average between the areas was done to generate the definitive average percentage of CD4, CD8 and CD3 positive cells. Comparison of CD4 IHC slides with and without biotinyl tyramide (BT) showed similar results, BT amplified slides having slightly higher proportions of CD4+ cells, as expected.

6.2.5 FLOW CYTOMETRY

Cell suspensions from the first skin biopsy and an aliquot of the LNCS were used for a 3-color flow cytometry. The cell suspensions obtained from the skin and lymph nodes were counted using an hemocytometer. The suspension of cells from each skin biopsy were equally divided between 4 polyethylene tubes. Approximately 0.05 x 10^7 cells from the starting LNCS and the sorted fractions were used for flow cytometry. Skin and LNSC aliquots were incubated for 20 minutes in the dark with the following monoclonal antibodies linked to fluorescent dyes, shown in Table 6.1. The cells were subsequently washed in PBS, fixed in 4% paraformaldehyde and stored at 4º C prior to FACS analysis.
### Table 6.1: List of flow cytometry monoclonal antibody reagents

<table>
<thead>
<tr>
<th>Tube 1:</th>
<th>Marker</th>
<th>Clone</th>
<th>IgG Isotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype CTRL-FITC</td>
<td>X40</td>
<td>IgG1</td>
<td>BD</td>
<td></td>
</tr>
<tr>
<td>Isotype CTRL-PE</td>
<td>X39</td>
<td>IgG2a</td>
<td>BD</td>
<td></td>
</tr>
<tr>
<td>Isotype CTRL-PerCP</td>
<td></td>
<td>IgG1</td>
<td>BD</td>
<td></td>
</tr>
</tbody>
</table>

| Tube 2:          | CD4-FITC | OKT-4 | IgG2a | Fischer Scientific |
| CD45RO-PE       | UCHL-1   | SK-1  | IgG1  | BD                  |
| CD8-PerCP       | TC 81    | SK-1  | IgG1  | BD                  |

| Tube 3:          | γδ T cells-FITC | TC δ1 | IgG2a | Fischer Scientific |
| CD4-PE          | OKT-4       | SK-1  | IgG1  | BD                  |
| CD8-PerCP       | TC 81       | SK-1  | IgG1  | BD                  |

| Tube 4:          | CD14-FITC  | TUK-4 | IgG1  | Dako             |
| CD16-PE         | Leu11c     |       | IgG1  | BD                |

| CTRL = control |
| IgG = immunoglobulin G |
| BD: Becton Dickinson Immunocytometry systems, San Jose, CA. |
| Houston, TX, Carpinteria, CA, Woburn, MA |

Data were acquired using a FACScan (Becton Dickinson, San Jose, CA) and analyzed using a Lysis-II software version 1.1 (Becton Dickinson). The acquired events were gated on the lymphocyte population, that was still identifiable in the skin, by forward and side scatter (FSC and SSC, respectively). Then two dimensional dot displays evaluated fluorescent subsets of the gated population.

The percentage (%) of CD4+ and CD8+ cells present in the skin, lymph node and blood, called 'totCD4' and 'totCD8' respectively, were obtained by adding the % of double positive cells CD4+CD8+ to the % of CD4+CD8- and CD8+CD4-, respectively. The CD4:CD8 ratio was similarly obtained by dividing 'totCD4' by 'totCD8'. The percentage of CD45RO+ cells among CD4+ and CD8+ T cells was obtained by dividing the % of double positive quadrant CD4+CD45RO+ and CD8+CD45RO+ respectively, with 'totCD4' and 'totCD8', respectively. In the skin, corrected values for the double-positive quadrants (CD4+CD8+, CD4+CD45RO+ and CD8+CD45RO+) were obtained by subtracting any percentage obtained with the isotype controls in the matching upper right quadrant for each tube analyzed in the same animal (see figure 6.3 page 137).

#### 6.2.6 Sorting of LN Cell Suspensions

The optimal protocol was developed after preliminary experiments with peripheral blood mononuclear cells (PBMCs) and lymph node cell suspensions from humans and rhesus monkeys and
the different approaches and results are presented in chapter 3 of this volume. The protocol used in the main study of this work is the following:

6.2.6.1 Panning of B Cells

Starting, freshly minced rhesus LNCS were depleted of B lymphocytes using 25 cm² cell culture flasks with the bottom plastic surface coated with rabbit anti-human immunoglobulins overnight at 4°C (Accurate, inc., Westbury, NY - cat. # AXL230), according to standard protocol (Lewis and Goodman 1978; Lewis and Kamin 1980), at a concentration of 0.6 mg/ml (1:20). They were incubated for 2X 45 min. at 37°C in humid 5%CO₂ incubators to deplete both macrophages and B cells. The obtained post-panning cell fraction was counted and a part of it (5 x 10⁶ cells) used for mRNA cytokine expression by RT-PCR.

6.2.6.2 Isolation of CD4, CD8, CD45RO and CD4+CD45RO+ T Lymphocytes Using MACS

Columns of 1.5 ml capacity (Mini-columns™ type MS+/RS+ for Mini-MACS®), with a sheath of iron wool, were obtained from Miltenyi Biotec Inc, Auburn, CA. The reagents and sources of magnetic beads coated with monoclonal antibodies (MoAb) were the following: anti-CD8-microbeads (clone SK1, Miltenyi) and anti-CD45RO-microbeads (clone UCHL-1, Miltenyi) MoAb were used according to manufacturer’s specifications (Miltenyi Biotec Inc, Auburn, CA). Because they were not already coated with the appropriate fluorescent dye, purity evaluation by flow cytometry was performed by staining with the standard 3-color staining, using a cocktail of CD4 (OKT4)-FITC, CD45RO-PE and CD8 (SK-1) PerCP.

Prior to sorting, the percentage of CD4+ and CD45RO+ cells was determined by flow cytometry. Two successive, one-parameter, magnetic cell sorts (2 “single” sorts) were performed according to manufacturer’s instructions. The first sort used CD8-coated micro-beads and was performed on all LNCS. The negatively selected, CD4-enriched fraction bearing no beads was then sampled for RNA isolation (Fraction 1) and, if sufficient cells were present with at least 5% CD45RO+ cells in the starting LNCS, were further processed. The second sort used CD45RO-coated micro-beads. The CD4-enriched (CD4+) CD45RO- were then first collected (Fraction 3) and then the CD4+ CD45RO+ fraction (Fraction 4). Sorted fractions were kept on ice until they were processed for RNA isolation and until three-color (1st sort) or two-color flow cytometry (2nd sort) was used to evaluate for
purity and to determine the proportion of CD45RO+ cells among the first sort. Five (5) million cells and
of CD8 sorted fractions (Fraction 1 and 2) and $1 \times 10^6$ cells from CD45RO sorted fractions (Fractions 3
and 4) were further processed for RNA isolation. The fraction 4 (CD4+CD45RO+) usually had a low
yield, with often $< 1 \times 10^6$ cells sampled for RNA isolation.

6.3 RESULTS

6.3.1 LYMPHOCYTES COLLECTED DIRECTLY FROM MINCED SKIN BIOPSIES AT
*M. lepra*e inoculation sites can be analyzed by flow cytometry
Based on the preliminary study on skin inoculation with incomplete Freund’s adjuvant (see chapter 4
page 97), infiltrating inflammatory cells were isolated directly from biopsy samples of *M. lepra*e
inoculation sites without gradient purification and collagenase treatment and the entire collection of skin
inoculation site cell suspensions was used (SkinCS). As few as 2.7% of the first 2 tubes analyzed per
monkey (n=108) could not be analyzed, nor could 14.4% of the entire set of samples acquired (n=234).
Large numbers of total events were acquired, in most cases: 80% and 70% were above 5,000 and 10,000
total acquired events, respectively (Fig. 6.1c). However, a frequent concern was the relative low number
of events gated on lymphocytes: 11% of the tubes analyzed had less than 500 events gated (Fig. 6.1a)
and 28% had less than 1000 events gated on lymphocytes on forward scatter and side scatter plot
displays (Fig. 6.1b). The cell acquisition of less than 100 events gated on lymphocytes (4 tubes - 2%)
were not considered for further analysis.

Figure 6.1: a - Distribution frequencies of the acquired events gated on
lymphocytes (gated events) from 183 skin cell suspensions. The average of
gated events (M) ± standard deviation (SD) was $2579 \pm 1731$ events. The
sum (percent) of the aliquots of skin cell suspensions of less than 400 events
analyzed, corresponding of values beyond M - 1.25 SD is 20 (11%).
b - Plot of the acquired events gated for lymphocytes from skin cell
suspended: On the x-axis are the events acquired for each tube. The y-axis
expresses the number of events gated on the small lymphocyte window. The
tubes were sorted according to increasing number of events. Four (2%), 11
(6%) and 51 (28%) tubes have less than 100, 200 and 1000 events gated on
lymphocytes, respectively. They were further analyzed when the events
were > 100.
c - Plot of the total acquired events from skin cell suspensions. The tube
order matches the sorted tubes in Fig. 5.1b. Total event acquisitions of
>5,000 and 10,000 events represent 146 tubes (80%) and 128 (70%),
respectively.
(fig. cont.)
Acquired events - skin

FACS tubes (N=183)

Acquired events gated on lymphocytes

FACS tubes (N=183)

FREQUENCY

6.1c

6.1d

6.1e
The skin cell suspensions were composed of a majority of mononuclear cells, with rare keratinocytes and granulocytes, and relatively abundant collagen bundles and debris (Fig. 6.2). The cytospins from *M. leprae* skin inoculation site cell suspensions contrasted in terms of amounts of granulocytes and collagen debris with the cytospins of the incomplete Freund’s adjuvant skin injection site cell suspensions (Fig 4.1). Total yields for skin were between 0.02 and 3.24 million cells [average (AVG) ± standard deviation (SD) is $0.61 \pm 0.78 \times 10^6$]. Significantly higher cell yields ($p<0.01$-Student’s t test) were obtained at day 5 compared with day 2 and day 8. There was no difference of total cell numbers between the SIV+ and the SIV- animals. In the lymph nodes, there were between 11.8 and 438 million cells (average ± standard deviation is $11.61 \pm 10.07 \times 10^7$).

Figure 6.2: Photograph of a cytospin of skin cell suspension from *M. leprae* inoculation site. Note the abundance of collagen fibers (arrow). This concentration is over-estimated due to the deposition of the collagen fibers at the bottom of the centrifuge tubes, the culot of which has been sampled for cytospin smear. Bar= 75 μm. Giemsa stain.
In the analysis of CD4/CD8/CD45RO, 52 tubes from all animals could be analyzed; 2 tubes could not be analyzed and 3 tubes had fewer than 400 events gated. Data corrected with the isotype controls were considered for further analysis (Fig. 6.3) and resulted in %CD4+CD45RO+ data with lower standard deviation and hence lower standard error, enabling the validation of the model with regard to parametric statistics (p<0.05).

Figure 6.3: Representative flow cytometry scatter plots from the skin of a SIV-negative rhesus monkey (N209). a - Scatter dot-plot of events physically gated on lymphocytes, stained with fluorescent isotype controls (tube 1) on a semi-logarithmic scale, shows a diagonal trail of non-specific staining. b - Matching scatter plot stained with CD4 (FITC) and CD8 (PerCP) (tube 2). This case is representative of average data quality obtained from skin cell suspensions from finely minced inoculation sites, gated on lymphocytes. There was a diagonal-shaped area of non-specific staining after gating on lymphocytes. This artifact was consistent and did not or minimally impede data analysis. These artifacts are interpreted as non-specific staining from dead cells and/or small collagen debris.
6.3.2 Flow Cytometry CD4+ Cell Percentages Correlate with Immunohistochemistry in the Skin

The percentages of CD4 and CD8 cells present in skin cell suspensions analyzed by flow cytometry and recruited in the skin by immunohistochemistry (IHC) were compared (Fig. 6.4). The findings in flow cytometry (FC) in the skin were confirmed by the observations made after determination of the proportions of recruited CD4+ T cells in skin inoculation sites by IHC. The proportions of CD4+ T lymphocytes at day 2, 5 and 62 were similar from one animal to the next, although consistently higher by IHC as compared with FC. At day 12 and 26 post inoculation, there were a higher % CD4+ T cells in IHC than by flow cytometry (Fig. 6.4).

Correlation between the 2 technologies was adequate to good for several time points (Table 6.2). For example, good correlations of %CD4 cells are noted at day 2 and day 5. Overall, however, we cannot confirm that the two approaches can replace one other. Despite the time consumed and the imprecision of manual counting, immunohistochemistry enables the evaluation of the relative composition of lymphocyte subsets in different structures such as the granulomas and the lymphocyte-rich perivascular infiltrates. Flow cytometry is however promising for ex vivo lymphocyte analysis from an inoculation site because it is characterized by a lower variance, is more automated and standardized and enables the evaluation of at least 3 surface markers.

<table>
<thead>
<tr>
<th>Table 6.2: Spearman's correlation coefficients on ranked data with associated probability that the parameter observed with both technologies are not correlated:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4%</strong></td>
</tr>
<tr>
<td>Day 2</td>
</tr>
<tr>
<td>Number (N)</td>
</tr>
<tr>
<td>Corr. Coeff.</td>
</tr>
<tr>
<td>Probability</td>
</tr>
</tbody>
</table>

| **CD8%**                                                     |
| Day 2 | Day 5 | Day 8 | Day 12 | Day 26 | Day 62 | Day 2+5 | Overall |
| Number (N) | 5 | 6 | 0 | 5 | 7 | 8 | 11 | 31 |
| Corr. Coeff. | 0.0000 | 0.2571 | NA | -0.8018 | 0.7143 | -0.3333 | **0.5960** | 0.1302 |
| Probability | 1.0000 | 0.6228 | 0.1041 | 0.0713 | 0.4198 | >0.05 | 0.4850 |

NA = not done

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6.3.3 The proportion of CD4+ T lymphocytes in the skin, lymph nodes and blood is consistently lower in SIV+ animals by FACS during the first month post-inoculation

By flow cytometry analysis, the proportion of infiltrating CD4+ T lymphocytes in the skin was consistently and significantly lower in SIV positive animals as compared with SIV- rhesus monkeys at all time points (day 2, 5, 12, and 26) (p<0.05) except at day 62 (p>0.78) (figure 6.5a). The difference between the two groups was most profound at day 5-post inoculation (p<0.001) because of an early
peak of the %CD4+ T lymphocytes in the SIV- population. This peak is apparent on median CD4+ percentages of the SIV- group (Fig. 6.5a). Similarly, the CD4: CD8 ratios were consistently different between the 2 groups at all time points (p<0.005) except at day 62 (p>0.09) and the ratios were most different at day 5 (p<0.0002) for the same reason. The increased mean CD4 percentage observed at day 5 post challenge was not statistically significant, whether the entire group (n=9) or the SIV- group (n=6) was considered.

The proportions of CD4+ T cells were significantly lower in the blood (p<0.002) and lymph nodes (p<0.001) of SIV+ animals, at all sampling time points, compared with SIV- monkeys (Fig. 6.5b and 6.5c). The same finding was noted with CD4: CD8 ratio. Patterns of CD4+ percentages over time in the blood and LN were similar in both groups, with a slight trend of slightly increased %CD4+ cells at day 5 in the LN and a slight tendency towards reduced %CD4+ cells in the blood (both phenomena statistically non-significant).

In the skin, analysis of IHC data indicates that the recruitment of CD4+ T cells in the SIV+ and SIV- animals was very similar until day 12 (Fig. 6.6b). IHC results consolidated the trend, without confirming it, that the first maximum median value of %CD4 suggestive of a recruitment peak may be around day 5 in SIV- animals (Fig. 6.6a) and around day 12 in SIV+ animals (Fig 6.6b). With both techniques, at day 26, SIV+ animals have significantly lower percentages of CD4+ cells than SIV- monkeys (p<0.01), with no overlapping %CD4 values between the two groups in IHC (Fig 6.6a and 6.6b) and FC (Fig. 6.5a). However, independent of the SIV status, IHC demonstrated high (>50%) %CD4+ cells (Fig. 6.6b) at day 2 in 3 animals, which also showed vigorous perivascular accumulation of almost exclusively CD4+ T cells at day 2 (Fig 6.8). Interestingly, the flow cytometry data showed the 3 highest percentages of CD4+ (>30%) in the same 3 animals. These 3 animals are the ones that remained free of persistent M. leprae infection 12 months after the last sampling day. The fourth animal that remained free persistent M. leprae infection (N976) had a vigorous CD8 accumulation as assessed by flow cytometry and IHC.
Figure 6.5: The proportion of CD4+ lymphocytes (a) at sites of *M. leprae* cutaneous inoculation, (b) in regional lymph nodes and (c) blood.  
**Graph a:** percent CD4+ lymphocytes in SIV+ monkeys do not augment at day 5 like the SIV- animals. There is significantly higher percentage of CD4+ lymphocytes in *M. leprae*-only infected monkeys compared to co-infected animals, except at day 62 (p<0.05).  
**Graphs b and c:** patterns of CD4+ percentages are similar in the blood and lymph nodes in both groups, with a slight increase in the regional LN and a slight drop in the blood at day 5 (not statistically significant).  
(Fig. Cont'd)

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Figure 6.6: Comparison of percentages of CD4+ in the skin of SIV+ and SIV- animals as determined by (a) flow cytometry and (b) immunohistochemistry. Comparison of both graphs at early time points (up to day 12) indicate that a higher proportion of CD4+ T cells is present (a) at day 5 by flow cytometry in SIV- and (b) at day 12 in SIV+ animals by immunohistochemistry (non statistically significant). Results are concordant by both technologies in day 26 and 62.
6.3.4 **In the Skin, the Proportion of CD45RO+ Cells Was Consistently Higher Among the CD4+ T Cells in SIV+ Animals**

In SIV infected animals (n=3), there is a higher percentage of CD45RO+ among the CD4+ cells in the skin and lymph nodes than in SIV- animals (Figure 6.7a and 6.7b). This finding was statistically significant in the skin only at day 5 and day 62 (p<0.05), where the general linear model using the corrected percentage of CD45RO+ cells among CD4+ T cells was validated (p=0.048). The %CD45RO corrected on isotype control background was normally distributed. In the lymph node, a trend towards higher percentages of CD45RO+ cells among CD4+ T cells was observed at day 5 and day 26. However the differences in median values were not statistically significant. At baseline and at day 62 PI, median values were almost identical. Although no firm conclusion can be drawn from the LN data, these results in the skin and LN suggest that CD4+CD45RO+ T cells are recruited in the regional lymph node at day 5 (Fig. 6.7b) in response to *M. leprae* challenge.

6.3.5 **The Early Recruitment of Numerous CD4+ T Cells Correlate with Protection, Regardless of the SIV Status**

The three animals having the highest numbers of perivascular lymphocytes, with a proportion of >50% of CD4% T cells at day 2, did not develop persistent *M. leprae* infection 12 months after re-challenge whereas the animals that developed persistent *M. leprae* infection had minimal recruitment (Fig. 6.8 - See also Appendix 4 page 212 and Table 6.3). The exact same pattern was observed with CD3 staining (data not shown). This early dichotomy of response was no longer evident at day 5, where higher percentages of CD4% cells were observed in animals that developed persistent *M. leprae* infection. Similar results, albeit less clear cut, were seen by FC, supporting these in situ observations.

The fourth SIV+ animal, that did not develop persistent *M. leprae* infection 12 months after re-challenge, is unique and showed an in situ marked recruitment of CD8+ lymphocytes at day 2 and day 5, both by FC and IHC. Examples of strong reactions and weak reactions are shown in Figure 7.8. The other monkeys developed substantial infiltrates only after day 8 or later by IHC.
Figure 6.7: The proportion of CD4+CD45RO+ lymphocytes (a) at sites of *M. leprae* cutaneous inoculation and (b) in regional lymph nodes. There was no significant difference between percentages of CD4+ CD45RO+ lymphocytes obtained from any site of the SIV-positive and SIV-negative groups of *M. leprae* inoculated monkeys, except in the skin at day 62 (7.6a). In lymph nodes at day 5 and 26 (7.6b), there was a trend towards a higher percentage of CD4+ CD45RO+ in SIV+ monkeys as compared with SIV- animals.
Clearance of acid fast bacilli (AFB) was similar for all animals. However, there was a trend towards fewer AFB as early as day 8 in animals which had had vigorous lymphocyte recruitment at day 2 and 5. At later time points, histopathological analysis and immuno-histochemistry revealed persistence of high lymphocyte cellularity with numerous CD3+ T cells and CD4+ cells in the group of monkeys that had high proportions of CD4+ and CD3+ T cells at early time points (Figures 6.9a and 6.10a). Specifically, more small lymphocytes were present at 62 DPI in animals that were devoid of evidence of *M. leprae* infection at the termination of the study (Fig. 6.9a and 6.9b) compared with the animals that presented with persistent infection (Fig 6.9b and 6.10b) and this was observed in both the SIV+ (Fig. 6.10) and the SIV- group (Fig. 6.9). Granuloma morphology was also an indicator of protection, with more organized granuloma in resistant animals, especially at early time points.

<table>
<thead>
<tr>
<th>Rhesus SIV §Outcome</th>
<th>day2</th>
<th>day 5</th>
<th>day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Persistent infections</td>
<td>Periv. inflitr.</td>
<td>%CD4</td>
</tr>
<tr>
<td>N264 pos 0 LL 0</td>
<td>4  12.66</td>
<td>0</td>
<td>36.14</td>
</tr>
<tr>
<td>N356 pos LL 0</td>
<td>4  26.43</td>
<td>++</td>
<td>49.28</td>
</tr>
<tr>
<td>N976 pos BL ++</td>
<td>4  27.42</td>
<td>++</td>
<td>49.28</td>
</tr>
<tr>
<td>M726 neg BL ++</td>
<td>4  35.44</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M736 neg BL ++</td>
<td>4  30.43</td>
<td>+</td>
<td>57.89</td>
</tr>
<tr>
<td>M849 neg BL ++</td>
<td>4  30.43</td>
<td>+</td>
<td>57.89</td>
</tr>
<tr>
<td>M879 neg BL ++</td>
<td>4  30.43</td>
<td>+</td>
<td>57.89</td>
</tr>
<tr>
<td>N209 neg 0 ++</td>
<td>4  50.89</td>
<td>++</td>
<td>46.15</td>
</tr>
<tr>
<td>P282 neg 0 ++</td>
<td>4  51.76</td>
<td>++</td>
<td>46.15</td>
</tr>
<tr>
<td>AVG</td>
<td>32.66</td>
<td>38.01</td>
<td>25.82</td>
</tr>
</tbody>
</table>

*Periv. Inflitr. = perivascular infiltrates; ++ = marked, ++ = moderate, = mild, = minimal/absent perivascular infiltrates. Evaluation on hematoxylin & eosin (HE) stained, formalin fixed sections. %CD4 = % CD4+ T cells.

Ψ: Evaluation from frozen biopsies immunostained for CD4 (OKT4, Fischer).
ND* = insufficient numbers of cell to count CD4+ T cells on IHC sections.
ND = missed sites. AVG = average of the % CD4 cells.
§ Outcome (leprosy in nerves ± nasal mucosa and skin): LL = disseminated lepromatous leprosy; BT = borderline tuberculoid; BL = borderline lepromatous; 0 = no evidence of disease 14 months after re-challenge at necropsy.

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Figure 6.8: CD4 immunohistochemistry from SIV+ and SIV- animals at 2 day post inoculation (DPI). A - Monkey N264 (SIV+) showing dense CD4 recruitment. Arrow: cutaneous nerve, devoid of infiltrate. B - Monkey N209 (SIV-) showing dense CD4 recruitment. Arrow: cutaneous nerve, devoid of infiltrate. ML: persistent M. leprae infection status. C - Monkey N356 (SIV+) showing sparse CD4+ cell recruitment. Discrete non-specific staining is present (arrowhead). D - Monkey M726 (SIV-) showing weak, minimal CD4+ cell recruitment (center). Non specific staining is present at the edge of the section (upper left and lower right corners). Bar= 20 μm. Harris' hematoxylin counterstain.
Figure 6.9: Histopathology of skin inoculation sites at day 62 post-inoculation in SIV-negative animals (a) without evidence of persistent *M. leprae* infection (N209) and (b) with evidence of persistent *M. leprae* infection (M726). Bar = 300 µm.
See also Appendix 5 page 208.
Figure 6.10: Histopathology of skin inoculation sites at 62 DPI in SIV positive animals. (a) without evidence of persistent *M. leprae* infection – monkey N264 (a) evidence of persistent *M. leprae* infection – Monkey N356 (SIV+). Bar = 300 μm. See also Appendix 6 page 210.
6.3.6 **Flow Cytometry Evaluated of Cells Staining with γδ, CD14 and CD16 Markers**

The proportion of total γδ T cells in the blood in this study were similar to the ones described in rhesus monkeys (Average ± SD is 4.8% ± 4.4% versus 5.7% reported) (Wallace, Gan et al. 1994). The gamma-delta (γδ) T cells were not prominent in any of the sample analyzed or in any group of monkey studied. They were higher in the skin and LN as compared with blood (one tail heteroscedastic Student's t test p<0.001) (Table 6.4). More importantly, there was no difference in the proportion of γδ T lymphocytes over time, even in the skin, or between SIV+ and SIV- groups. Likewise, no suggestion of higher or lower proportions of γδ T cells in animals with or without persistent *M. leprae* infection was noted (data not shown).

**Table 6.4: Percentages of double positive, CD8+γδ+ and CD4+γδ+ T lymphocytes in the skin, lymph nodes (LN) and blood.**

<table>
<thead>
<tr>
<th></th>
<th>SKIN</th>
<th>LN</th>
<th>BLOOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8+γδ+</td>
<td>CD4+γδ+</td>
<td>CD8+γδ+</td>
</tr>
<tr>
<td>Number (%) analyzed</td>
<td>44 (81.5)</td>
<td>45 (83.3)</td>
<td>20 (62.5)</td>
</tr>
<tr>
<td>Mean</td>
<td>3.08</td>
<td>6.14</td>
<td>3.52</td>
</tr>
<tr>
<td>SD</td>
<td>2.92</td>
<td>12.01</td>
<td>3.23</td>
</tr>
</tbody>
</table>

SD= sample standard deviation. #= total number of tubes. Double positive fractions are presented. Not shown are the CD4- and CD8- γδ and double negative γδ T cells.

The proportions of CD14 cells in the lymphocyte gate remained low, even in the skin at day 26 and 62, in the range of 5 to 10%. In ungated samples, more CD14+ cells were observed, up to 25%. At day 62, the proportion of CD4+ CD14+ cells was very low, both gated and ungated. CD16+ cells were not observed at any time point in any system, possibly due to difficulties with the antibody conjugate used.

6.4 **DISCUSSION**

Results showed that, at 2 months (62 days post inoculation) after inoculation, SIV + animals had similar proportion of CD4+ T cells in the skin with higher percentage of CD4+CD45RO+ positive cells compared to SIV-negative monkeys, suggestive of compensatory mechanism and a more sustained
reaction in SIV+ rhesus monkeys. In addition and very importantly, disease development did not correlate with SIV positivity but rather with an early, vigorous CD4+ and CD3+ recruitment in skin inoculation sites.

Skin cell suspensions, without prior collagenase treatment, were analyzed by flow cytometry (FC) because histologic examination reveals that, at early time points, there is no significant granuloma formation and an absence of significant reticulin fiber formation. Gradient centrifugation was not feasible (Ficoll-Hypaque) due to low cell yields. As a result, small collagen debris, that non specifically adsorbed the monoclonal antibodies, consistently gave a diagonal pattern within the lymphocyte gate after staining with isotype controls. The CD4+ or CD8+ T lymphocytes identified by flow cytometry were most likely recruited cells instead of contamination with peripheral lymphocytes for two reasons. First, there was a strong correlation between the percentage of CD4 T cells obtained with FACS and IHC technology at early time points (2 and 5 DPI), times where it was most suspected that the analyzed cells by flow cytometry would be contaminated by peripheral blood lymphocytes. Second, analysis of variance (ANOVA) analysis of concatenated FACS data in the blood and skin revealed the %CD4+ from the skin to be statistically different from %CD4+ from peripheral blood at day 26 (p<0.02).

By flow cytometry, the median proportion of CD4+ T lymphocytes present at sites of *M. leprae* inoculation was significantly lower in SIV+ rhesus monkeys as compared with SIV negative animals, at all time points except at day 62 post inoculation. By immunohistochemistry and FC, a trend towards higher proportions of CD4+ cells were observed at day 12 in SIV+ animals. This trend is interestingly very similar to a previous study investigating the primary response to *M. leprae* in a naïve population of rhesus monkeys (Lathrop, Scollard et al. 1996). These combined results suggest delayed recruitment of CD4+ T lymphocytes at sites of skin inoculation after recall challenge of *M. leprae*. This suggests that, even though the SIV positive animals seem to have a decreased capacity to recruit CD4+ lymphocytes soon after bacterial inoculation, presumably due to their ongoing chronic retroviral infection, they still have the capacity to respond through the recruitment of these cells, resulting in a median maximum at day 12 post inoculation.

The proportion of activated/memory CD4+ T lymphocytes, as assessed by monoclonal antibody specifically binding to the short CD45R isoform of the common leukocyte antigen (CLA)
family of transmembrane protein, otherwise called CD45RO marker, is consistently higher in SIV+ animals in the skin and lymph nodes, in a significative manner day 62 in the skin. The same trend (not statistically significant) was observed in the LN at all time points. This result suggests that SIV infected animals can recruit a higher proportion of CD4 T cells with up-regulated CD45RO isoforms. Since these latter cells are believed to belong to the group of activated/memory lymphocyte subset (Merkenschlager, Terry et al. 1988), this may be due to a compensatory mechanism. Alternatively, this phenomenon may be non-specific, with higher activation levels of CD4 T cells related to persistent SIV infection, similar to what is observed in HIV infection (Copeland and Heeney 1996). The replication of HIV occurs preferentially in activated CD4+ lymphocytes (Copeland and Heeney 1996). In children infected in utero with HIV, higher numbers of CD4+CD45RO+ are infected with HIV provirus than naive CD4+CD45RA+ cells (Sleasman, Aleixo et al. 1996). In this latter study in infants and children, however, the normal age-related predominance of CD4+CD45RA+ T cells in the blood was maintained.

At day 62, no morphological difference in the nature and distribution of lymphocytes and granuloma was observed between the SIV+ and the SIV- animals, and the proportions of recruited CD4+ T cells or CD4:CD8 ratio was very similar. This is consistent with the published data concerning humans infected by both leprosy and HIV, where no difference in the nature of the established skin lesions was observed between the HIV+ and HIV- patients (studied and reviewed by (Faye, Mahé et al. 1996))(Machado, David et al. 1998). In these human studies, although no time frame data can be obtained, it is very likely that the lesions studied are older than 2 months, and in any case, events within the first month could not be assessed in human studies.

Examination of IHC and HE slides revealed the cellular response to be quite heterogenous in these animals, regardless of the SIV infection status of rhesus monkeys. Forty four percent (4/9) were early strong responders, including 2 SIV+ monkeys. This exactly correlated with absence of persistent infection with M. leprae in these latter animals.

Delayed type hypersensitivity (DTH) is characterized by strong cell mediated immunity (CMI) after injection of antigens. There is early (48-36 hours) dense perivascular accumulation of CD3+CD4+ T lymphocytes, fibrin and edema histo-pathologically. Grossly, the injection site is red and thickened. Since this study deals with inoculation with live Mycobacterium leprae instead of antigens, a
morphological picture at day 2 similar to a DTH reaction will be called a DTH-like reaction. Notably in this study of rhesus monkeys, the development of persistent *M. leprae* infection was inversely associated with a DTH-like reaction in the skin, independently of whether the subjects were chronically infected with SIV. In humans, a strong DTH to tuberculin associated with an absence of pulmonary lesions suggestive of tuberculosis is an indicator of resistance to disease among highly exposed contacts of tuberculosis patients (i.e. nurses) (Stead, Lofgren et al. 1985). Loss of the DTH response to tuberculin and other antigens in HIV+ patients has a high predictive value for subsequent development of AIDS, correlating with tuberculosis (Markowitz, Hansen et al. 1993) (reviewed by (Smith and Moss 1994)) and *Mycobacteria*-induced secondary opportunistic infection and disease (Birx, Brundage et al. 1993; Blatt, Hendrix et al. 1993). Moreover, and very importantly, there is a decrease of DTH and peripheral lymphocyte stimulation to tuberculin antigens in patients progressing to clinical *Mycobacterium tuberculosis* infection, such as pneumonia (Sanchez, Rodriguez et al. 1994; Surcel, Troye-Blomberg et al. 1994), reviewed in (Cooper and Flynn 1995).

In tuberculosis, most researchers believe that, whereas genetic factors of "innate immunity" are most important to determine whether an individual will develop tuberculosis after primary exposure to *M. tuberculosis*, acquired cell mediated immunity is thought to be crucial for the outcome upon re-exposure to tubercle bacilli and/or reactivation of tuberculosis disease (Bloom 1994). Our data suggest that the response to *M. leprae* infection and disease (i.e localization to nerves), it might be similar. The study of the cytokines expressed locally and regionally at sites of inoculations by these monkeys indicates that an early, vigorous Th1/type 1 response correlates with an absence of persistence and/or dissemination of the leprosy bacillus in rhesus monkeys.

Lastly, there was no preferential recruitment of γδ T cells in the skin or augmentation of γδ T cells in the LN, as evaluated by flow cytometry. The proportion of total γδ T cells in the blood were similar to the ones described in rhesus monkeys (Wallace, Gan et al. 1994). More importantly, there was no difference in the proportion of γδ T lymphocytes at early time points or over time in the lymph node or in the skin, where significant numbers of γδ T cells have been previously described early after inoculation with mycobacteria in rodents (Inoué, Yoshikai et al. 1991). In another study in mice, the γδ T cell recruitment paralleled but did not outnumber the accumulation of αβT cells in response to live
M. bovis strain BCG or *M. tuberculosis*-inoculated mice (Griffin, Harshan et al. 1991). In addition, the latter study did not find any prominent accumulation of \( \gamma \delta \) T cells in memory immune mice upon re-challenge as in this study.

Therefore, the recruitment however paralleled but did not outnumber the accumulation of \( \alpha \beta \) T cells in mycobacteria-inoculated mice, and no accumulation of \( \gamma \delta \) T cells in memory immune mice upon re-challenge was observed (Griffin, Harshan et al. 1991). This is similar to what was noticed in this study dealing with rhesus macaque re-challenged with *M. leprae*.

CD16+ cells, indicative of the natural killer cell subset, were not observed at any time point in this study, questioning the validity of this marker in rhesus monkeys, despite reports showing that it stained cells in this species (Reimann, Waite et al. 1994). This is consistent with previous studies revealing that, although the clone Leu11a, unlabeled or linked with FITC, stains lymphocytes in rhesus monkeys (Polacino-Firpo, Axberg et al. 1992; Sopper, Stahl-Hennig et al. 1997), the clone Leu11c linked with PE does not label rhesus macaque cells although both clones have been designed to label the same human marker, CD16 (Polacino-Firpo, Axberg et al. 1992).

In conclusion, this analysis showed that SIV+ animals are able to recruit CD4+ T cells in a manner similar to SIV- animal at early time points after a recall inoculation of *M. leprae* in the skin, with a delay of approximately one week. Protection against persistent *M. leprae* infection correlated with an early and vigorous recruitment of T lymphocytes primarily, but not exclusively, CD4 T cells, thereby mimicking the cell recruitment observed in a DTH reaction (Buchanan and Murphy 1997). In short, protection correlated with a DTH-like phenomenon, but not with the SIV status.

6.5 LITERATURE CITED


7 THE STRENGTH AND TIMING OF THE CYTOKINE RESPONSES TO RECALL M. LEPRAE INOCULATIONS IN THE SKIN OF RHESUS MONKEYS CORRELATES WITH OUTCOMES OF M. LEPRAE INFECTION

7.1 INTRODUCTION

T lymphocyte mediated immunity is now understood to develop along two pathways defined by the pattern of cytokines secreted in the local environments by at least CD4- and CD8-positive T cells, after exposure and stimulation with foreign antigens and pathogens. The T helper 1 (Th1) and the T helper 2 (Th2), each of which identifies a major immune response, are defined by precise arrays of cytokines with antagonistic consequences (Romagnani 1996). Both represent adaptive cellular immunity to distinct class of pathogens and both may have beneficial and detrimental effects. These arrays of cytokines are thought to reciprocally cross-regulate each other, in a complex manner (Muraille and Leo 1998). In leprosy, where a spectrum of immuno-pathologic manifestations characterizes the disease, both types of T cell help are present in fully developed disease (Modlin 1994). Among the exposed individuals that have developed active Hansen’s disease, type 2 T cell help (Th2) is present in lepromatous skin lesions and type 1 T cell help (Th1) is present in tuberculoid skin lesions (Yamamura, Uyemura et al. 1991). In T cell clones derived from these lesions and blood, two studies essentially confirmed the ex vivo findings (Salgame, Abrams et al. 1991; Misra, Murtaza et al. 1995), whereas another study using skin and blood derived T cell clones showed functional heterogeneity, that did not strongly correlate with disease classification, and phenotype evolution over time of cultured skin T cell clones (Howe, Wondimu et al. 1995). In this context, nothing is presently known about the evolution over time and critical aspects of the cytokine profile upon first exposure and re-exposure to Mycobacterium leprae in humans. Similarly, the manner in which these responses to M. leprae may be modified by HIV infection are unclear (Pönninghaus, Mwanjasi et al. 1991).

Most epidemiological (Pönninghaus, Mwanjasi et al. 1991; Frommel, Tekle-Haimanot et al. 1994; Munyao, Bwayo et al. 1994; Lienhardt, Kamate et al. 1996; Machado, David et al. 1998), immuno-pathological (Sampaio, Caneshi et al. 1995) and histo-pathological (reviewed in (Faye, Mahé 1995)).
et al. 1996) studies have failed to demonstrate any increase of the incidence of leprosy or downgrading of leprosy from tuberculoid to lepromatous disease in humans infected with HIV. However, since no reliable test can be performed to assess the ratio of the number of individuals suffering from leprosy to the number of individuals exposed to the leprosy bacillus without disease (leprosy disease rate: leprosy infection rate) in HIV-infected versus HIV-negative populations, the question of whether HIV infection and/or disease favors the development of leprosy after exposure to the pathogen remains unanswered and difficult to address. Likewise, there is an absence of data concerning the effect of leprosy and its different clinico-pathologic forms on the evolution of HIV infection/disease, in contrast with tuberculosis (Goletti, Weissman et al. 1996).

Experimentally, using large intra-venous and intra-dermal doses, inoculations of rhesus macaques (Macaca mulatta) have resulted in the development of persistent and, more rarely, disseminated *M. leprae* infection, resembling the development of human leprosy, in 26.5 to 45.7 % of them (Gormus, Xu et al. 1998). This has suggested that rhesus macaques, like humans and unlike nine-banded armadillo and sooty mangabey monkeys, have a heterogeneous response to the leprosy bacillus. Comparison of SIV+ and SIV- rhesus macaques has revealed higher percentages of SIV+ animals that eventually developed a leprosy-like disease, when inoculated with *M. leprae* at the time of SIV infection (Gormus, Murphey-Corb et al. 1989; Baskin 1990; Gormus, Murphey-Corb et al. 1998) or 8-10 months later, when these rhesus usually express advanced SAIDS (Gormus, Murphey-Corb et al. 1998). Non human primates may have an unexpected value and important applications in leprosy research and to study the interactions between leprosy and immunosuppressive lentiviruses (Meyers 1992; Meyers, Gormus et al. 1994). A previous pilot study, dealing with the cellular and the cytokine secretion pattern following a primary exposure to *M. leprae* in rhesus monkeys, had indicated a delayed recruitment and a delayed cytokine response in SIV positive animals as compared with SIV negative ones (Scollard, Gillis et al. 1997).

This study examined the consequences of a re-exposure with *M. leprae* and utilized 9 rhesus monkeys, 3 of which were chronically infected with simian immunodeficiency virus (SIV), addressing the question of the type of cytokine response in the skin and regional draining lymph node (LN) of rhesus macaques shortly after re-exposure to a live inoculum of *M. leprae*, taking advantage of the
recent optimization of primers for cytokine cDNA amplification in this species by RT-PCR (Villinger, Hunt et al. 1993; Villinger, Brar et al. 1995). The hypothesis of this study was that the SIV+ slow progressors would not mount an adequate Th1 but instead a Th0/Th2 response upon re-challenge with *M. leprae*. A related hypothesis was that differences in cytokine responses exist between the groups that do or do not develop persistent *M. leprae* infection.

7.2 MATERIALS AND METHODS

7.2.1 RNA ISOLATION

Ribonucleic acid (RNA) isolation was based on the property that RNA stays in solution when 8 M of guanidinium isothiocyanate is used to disrupt the nucleo-protein complexes. This property allows a rapid one step isolation procedure, which can be successfully applied to series of samples (Chomczynski and Sacchi 1987). This method was applied for all specimens of this study. However, for skin biopsies, where often low numbers of cells infiltrate the inoculation site, a longer procedure, including 2 lysing steps and 2 rounds of isopropanol precipitation overnight at -20°C, was used in an attempt to improve the yield and purity of nucleic acid isolates. The comparison of performance between the two methods was carried out using a skin sample and confirmed the better performance with better yields and slightly higher OD260:OD280 ratio using 2 lysing steps.

7.2.1.1 RNA Isolation from Lymph Node Cell Suspensions

Five millions (5 10⁶) cells from lymph node cell suspensions (LNCS) were lysed for 2 minutes using RNA-STAT60™ (Tel-test"B", inc., solution (Tel-test"B", Friendswood, TX) and immediately frozen at -70°C the day of sampling, during and after cell sorting. The isolation was later carried out in less than 90 minutes using 100 µl of lysing solution /10⁶ cells in 1.5-ml microfuge tubes with tipped ends (Sarstedt, Newton, NC), according to the manufacturer’s instructions, with slight modifications: the second ultra-centrifugation, after isopropanol precipitation, was carried out for 25 minutes instead of 15 minutes, due to the high content of nucleic acid noted by OD₂₅₀ reading in the supernatant. When pipetting out the aqueous phase, care was taken to leave 2-3 mm above the meniscus to prevent contamination by the lysing solution and unwanted DNA and proteins. After drying 20-30 minutes in a vacuum chamber, the isolates usually stayed in an aqueous phase and were dissolved in di-ethyl-phenyl-carbazine (DEPC)-treated distilled water up to a total volume of 100 µl. Four µl of the isolate was

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diluted 1:20 in 76 µL of DEPC water to determine the amount of nucleic acid by spectrophotometry (GenQuant™, Pharmacia Biotech Ltd, Cambridge, England, UK).

7.2.1.2 RNA Isolation from Frozen Skin Biopsies

Depending upon the abundance of the inflammatory infiltrate present, thirty to fifty 10 µm-thick sections from each skin biopsy were cut using a freezing microtome (Jung frigocut 2800E, Leica, Nußloch, Germany). These frozen sections were collected in 1.5 ml microfuge tubes (Sarstedt, Newton, NC) and kept at -27 °C until guanidinium isothiocyanate lysing solution was added while thawing. Isolation of the total RNA was performed using the RNA isolation kit (RNAgent™, Promega, Madison, WI), according to the manufacturer’s instructions for low to very low amounts of nucleic acid. The RNA isolate did not dry completely after 20 minutes in a vacuum chamber and was diluted to ~40 µL by adding 30 µL of DEPC water. When necessary due to high nucleic acid yield, the samples were put in a water bath for 3 min at 70 °C to melt the isolate. An aliquot of 4 µl diluted 1:10 and/or 1:20 was used to determine the quantity of nucleic acid by spectrophotometry, using the same instrument as for the lymph nodes.

Optical densities (OD) read at 260 and 280 nm were recorded to determine approximately the quantities and purity of nucleic acid isolates. Repeatability testing from blanks and specimen samples showed typically a precision of approximately +/- 0.010, which is about the reproducibility indicated by the manufacturer. In several samples where the reading had a high variability, serial dilutions at 1:40 and 1:80 were undertaken with more repeatable readings. This approach was also an opportunity to check for linearity of the corresponding OD values. Since we did not use any other method to determine nucleic acid concentration, the precision (accuracy) of the used spectrophotometer for these studies could not be determined. Comparison with other spectrophotometers indicated higher OD260:OD280 ratios, but very similar OD260 values.

7.2.2 TOTAL NUCLEIC ACID YIELDS AND PURITY

7.2.2.1 Skin

Absorbency of emitted UV light at 260nm from the ‘total RNA’ isolates from skin revealed typically heterogeneous and low total nucleic acid concentrations, especially at early time points. The average (AVG) ± standard deviation (SD) of calculated RNA concentrations was 10.1 µg ± 17.6 µg (n=42 isolates, including 6 repeats). After removing two extremes of >50 µg, the AVG ± SD was 6.5 ±
6.0 μg. The lowest yield was from day 2, with an AVG ± SD of 1.3 ± 1.3 μg. The average purity ± SD of the isolates, as assessed by the OD260:OD280 ratio, was 1.31 ± 0.13. The RNA isolates consistently had genomic DNA contamination, similar to the ones seen in chapter 4 (Fig. 4.3b page 99).

7.2.2.2 Lymph Node

There was no notable difference between the total RNA values from LN cell suspensions obtained at day 0, 5, 26 and 62. For example, at day 5, the AVG ± SD was 57.9 ± 28.5 μm (n=35). The average purity ± SD of the isolates, as assessed by the OD260:OD280 ratio at day 5, was 1.340 ± 0.15. This purity measure was low, but was still higher than on the other days. To ascertain that this was not merely an instrument-related phenomenon, 12 samples from day 5 were assayed with another instrument (Beckman DU 600, Beckman Coulter inc., Fullerton, CA). The OD260 values were similar. Table 7.1 summarizes the OD ratio findings. For purity assessment, relative performances have been taken into account, not absolute values.

Table 7.1: Instrument comparison in OD determination and ratio in LN nucleic acid isolates at day 5.

<table>
<thead>
<tr>
<th>number tested</th>
<th>OD ratio</th>
<th>Tot. Nucl. Acid</th>
<th>OD ratio</th>
<th>Total Nucl. Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVG</td>
<td>1.340</td>
<td>57.9</td>
<td>1.350</td>
<td>70.6</td>
</tr>
<tr>
<td>STD</td>
<td>0.151</td>
<td>27.6</td>
<td>0.130</td>
<td>34.4</td>
</tr>
</tbody>
</table>

7.2.3 Deoxyribonuclease I (DNase I) Treatment of Total Nucleic Acid Isolates

Amplification by polymerae chain reaction (PCR) without reverse transcription (RT) revealed that both lymph node cell suspension isolates and skin isolates had genomic DNA contamination, similar to what was noticed when RNA was isolated from Incomplete Freund's Adjuvant skin injection sites (Figure 4.3b page 99). Therefore, DNase I (GIBCO BRL – Life technologies, Gaithersburg, MD) treatments were performed prior to the reverse transcription, according to manufacturer's recommendations, on a total of 1 μg of nucleic acid isolate from both skin and LN. To minimize loss of messenger RNA during the treatment, the reaction was carried out at room temperature (~ 20-22 °C) for precisely 15 minutes.
7.2.4 Reverse Transcription and Polymerase Chain Reaction of the Ex Vivo Total RNA Isolates

7.2.4.1 Reverse Transcription

Half (0.5 μg of nucleic acid) of the Dnase I treated samples were reverse transcribed for 50 minutes at 42 °C using superscript II® (GIBCO BRL – Life technologies, Gaithersburg, MD) with slight modification of the manufacturer's recommendations. The total reaction volume was 40 μl instead of 20 μl and 0.5 unit of Rnase inhibitor (Rnasin® - Promega, Madison, WI) was added. The ratio of RNA isolate in μg to reverse transcriptase in units of specific activity was 1:1.

7.2.4.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out using a thermal cycler 480 (Perkin-Elmer Cetus Applied Biosystems, Norwalk, CT) using the following basic amplification program: melting 2 min. at 95 °C for 1 cycle, 1 min. at 95 °C, 1 min. at the optimal annealing temperature, then extension for 1 min. at 72 °C for n cycles and finally extension for 7 min. at 72 °C. Annealing temperature, cycle number and other parameters specific for each amplified cDNA are presented in Table 7.2. The primers and oligoprobe sequences for each rhesus macaque cytokine were published earlier (Villinger, Hunt et al. 1993; Villinger, Brar et al. 1995), except for IFNγ, for which primers designed for human IFNγ cDNA were used (Yamamura, Uyemura et al. 1991). All sequences are presented in Table 7.3.

Table 7.2: RT-PCR standardized conditions for the ex vivo cytokine study.

<table>
<thead>
<tr>
<th></th>
<th>β-actin</th>
<th>IFNγ</th>
<th>IL-4</th>
<th>IL-2</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>[primer] (μM)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Annealing T (°C)</td>
<td>63</td>
<td>55</td>
<td>62</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>[MgCl2] (mM)</td>
<td>2</td>
<td>2</td>
<td>2.5</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td>dNTP mixture (mM)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Cycles number</td>
<td>30</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Taq (Units)</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
</tbody>
</table>

M=moles.l
T= temperature
[MgCl2]= concentration in magnesium chloride
dNTP= deoxynucleotide tri-phosphate
Taq= *Thermus aquaticus* thermostable DNA polymerase
Table 7.3: Sequences of forward and reverse primers and biotin-labeled oligoprobes (PB) used to amplify rhesus macaques cytokines

<table>
<thead>
<tr>
<th>Cytokine Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>hIFNg - 5' AGT TAT ATC TTG GCT TTT CA</td>
</tr>
<tr>
<td></td>
<td>hIFNg - 3' ACC GAA TAA TTA GTC AGC TT</td>
</tr>
<tr>
<td></td>
<td>hIFNg - PB ATT TGG CTC TGC ATT TTT TGT CTG T</td>
</tr>
<tr>
<td>IL-2</td>
<td>Rh IL-2 - 5' ATG TAC AGG ATG CAA CTC CTG</td>
</tr>
<tr>
<td></td>
<td>Rh IL-2 - 3' CAC ATG AAT GTT GTT TCA GAT CCA</td>
</tr>
<tr>
<td></td>
<td>Rh IL-2 PB ACC CAG GGA CTT AAT CAG CAA CTAAA</td>
</tr>
<tr>
<td>IL-10</td>
<td>Rh IL-10 - 7x TGC TCT AGA AGG CAT GCA CTC AGC</td>
</tr>
<tr>
<td></td>
<td>Rh IL-10 - 6 TCT CAA GGG GCT GGG TCA GCT ATC CCA</td>
</tr>
<tr>
<td></td>
<td>Rh IL-10 PB GGC CGT GGA GCA GGT GAA GAA TGC CTT TAA</td>
</tr>
<tr>
<td>IL-4</td>
<td>Rh IL-4 - 5' ATG GGT CTC ACC TCC CAA CTG</td>
</tr>
<tr>
<td></td>
<td>Rh IL-4 - 3' TCA GCT CGA ACA CTT TGA ATA TTT CTC TCT</td>
</tr>
<tr>
<td></td>
<td>Rh IL-4 - PB CAG CAG TTC CAC AGG CAC AAG CAC C</td>
</tr>
</tbody>
</table>

The strategy for relative RT-PCR was as follows: from the RT product, 2 μl (5%) was sampled for cDNA standardization using the housekeeping gene, β-actin, amplified for 30 cycles, based on the saturation curve from half of a μg of DNase I treated nucleic acid (‘total RNA’) isolate (Fig. 7.1). Even though 40 cycles may have been at first analysis more appropriate to obtain signals visible after gel electrophoresis of PCR products, 37 cycles instead of 40 cycles were chosen after IFNγ amplification optimization from DNase I-treated samples from day 2 and 26 in the skin to avoid risks of product saturation. The number of cycles was the same for all the cytokines, for isolates from skin and LN (Table 7.2). Negative controls of PCR were water to check for contaminations before amplification. In a few PCR procedures, reverse transcribed RNA from normal rhesus skin were used as negative controls. Positive control cDNA mixtures for PCR cytokine detection were RNAs isolated from alloreactive mixed lymphoid cultures from rhesus monkey blood with 5 μg/ml of concanavalin A or 2 μg/ml of phyto-hemagglutinin-A. In addition, IL-4, which was only marginally detected or detected only after hybridization with biotin-labeled oligoprobes in alloreactive mixed lymphocyte cultures, and IFNγ benefitted of serial dilution of cloned pGEM plasmids containing IL-4 and IFNγ cDNA inserts as positive controls.
Figure 7.1: PCR saturation curves of (a) human β-actin cDNA and (b) rhesus lymph node cell suspensions (LNCS) cDNA. (a) 50 attomoles of a commercially available human β-actin cDNA positive control (Clontech, Palo Alto, CA) and (b) 0.5 μm of total RNA isolate from 3 representative rhesus macaque LNCS were used. Linear amplification was observed (a) between 17 and 25 cycles in the former and (b) between 25 and 35 cycle in the latter. The IOD values are based on the density of bands on agarose gels.

Twenty-five (25) percent of the PCR product was separated by electrophoresis, using a 2% agarose gel stained with 55 ppm ethidium bromide at 80 volts for 2 hours. The bands were photographed with constant standard exposure time and aperture (1 second, aperture 8) and scanned at 300 dpi per cm using an XRSTM 3cx scanner (X-Ray Scanner Corp., Torrance, CA) and PerfectScan™ software (Perfect Byte, Inc., Omaha, NE). An integrated optical density (IOD) value was obtained using Sunview™ software (Millipore Corp., Bedford, MA) with standardized parameters. This software
calculated the OD and surface of each scanned band and gave the integrated optical density (IOD) as a measure of the relative quantities of β-actin PCR products. Relative quantities of β-actin were standardized using the weakest signal of a given batch as an undiluted or minimally diluted reference. For each of the four cytokines, 23.75% (0.95/4) of the diluted standardized RT product was used: the remaining 5% was used for amplifying the adjusted sample for β-actin. After PCR amplification, ~25% of the product of each cytokine and the standardized housekeeping gene was separated by electrophoresis using the exact same protocol, and the bands obtained were scanned and an IOD value was obtained. Relative expression of each cytokine mRNA for each animal was obtained by calculating the IOD\textsubscript{cytokine} : IOD\textsubscript{β-actin} ratio (IOD \textsc{ratio}). Since the primary goal was to compare SIV+ versus SIV- groups, PCR runs and gels were usually grouped by sampling day and by sorted fractions. The consequence is that, unless of sufficient magnitude, day to day variation should be interpreted with more caution than intra-day variations observed between groups of animals.

7.2.5 SOUTHERN BLOTTING AND CHEMILUMINESCENT DETECTION OF PCR PRODUCTS NON VISIBLE ON THE GELS

When all the bands could not be directly detected after PCR amplification on ethidium bromide stained gels, the DNA from the gels were transferred overnight to a positively charged nylon membrane (Tropilon-plus\textsuperscript{TM}, Tropix, Bedford, MA) using an upward capillary transfer, i.e. a strong ionic gradient from a high salt, 10 x salt-sodium citrate (SSC) buffer to a low salt, 2X SSC buffer (Southern blot procedure) (Southern 1975) and subsequently fixed with 0.4M NaOH for 3 minutes (Brown 1993). Hybridization for each cytokine was performed using commercially available reagents (Southern-light\textsuperscript{TM}, Tropix) and oligoprobes specific for monkey IL-2, IL-4 and IL-10 cDNA (Villinger, Hunt et al. 1993) and human IFN\textgamma specific oligoprobe that specifically bound the rhesus IFN\textgamma cDNA sequence amplified from rhesus monkey RNA (sequence kindly provided by Sonia Montenegro-James, Tulane University, New Orleans, LA). Relative sensitivity of IL4 and IFN\textgamma detection was assessed by serial 10-fold dilutions of pGEM plasmids containing IL-4 and IFN\textgamma cDNA sequences from rhesus macaques (kind gift from Dr. Villinger, Emory University, Atlanta, GA). Results indicated similar detection performances, within 2 log\textsubscript{10} units, between the two probes. Oligoprobe affinity performance for IL-2
and IL-10 cDNA was not done, since we did not use cDNA inserted in plasmids of known quantity for these latter 2 cytokines.

Oligoprobe concentration in the hybridization buffer, hybridization temperature and film exposure times were standardized for all 4 cytokines, skin and LN and all time points at 2.5 pmoles/ml, 50°C and 1 hour, respectively. The chemiluminescent detection was done according to the manufacturer's instructions. The films were exposed to the nylon membrane for 1 hour immediately and one hour after the beginning of the luminescent chemical reaction (the reaction intensity peaks approximately 2-5 hours after its start).

To integrate results from strongly expressed cytokine(s) readily visible by ethidium bromide-stained gels, such as IFNγ, and data obtained from weakly expressed cytokine detected only by Southern blotting and chemi-luminescent detection, such as IL-4, the ranking system presented in Table 7.3 was used. This ranking system utilizes and combines qualitative criteria from the Southern blotting and ranks from intervals of the integrated optical density (IOD) ratio. It avoids the overestimation of weak signals when taking IOD ratio data alone. It does not, however, discriminate between levels of high expression. IOD ratio values are used to compare relative high expression of cytokine mRNA between groups. Because baseline expression in the lymph nodes was not homogenous between samples for a given cytokine, up-regulation or down-regulation of expression was assessed using IOD ratio adjusted on baseline, defined as follows:

\[ \text{Corr IOD ratio} = \text{IOD ratio}_{\text{day n}} - \text{IOD ratio}_{\text{baseline}}. \quad n = \{5, 26, 62\}. \]

The adjusted rank of IL-2, IL-10 and IFNγ was then determined according to the corrected IOD ratio adjusted on baseline defined as follows [rank (corr IOD ratio)]. IL-4 had no detectable baseline mRNA expression in the lymph nodes.

Table 7.3: Ranking system to compare weak and strong cytokine expression.

| Grade 0: no detectable band by southern blot hybridization (exposure time > 1 hour). |
| Grade 1: band visible only by southern blot hybridization |
| Grade 2: detection limit by gel - IOD ratio < 0.5 - usually strong hybridization signal. |
| Grade 3: detected by gel - 0.5 < IOD ratio < 1; cases of gel detection limit when β-actin bands are weak. |
| Grade 4: detected by gel - IOD ratio > 1 |

159
The acquired ranked data were used to generate Figure 7.3 through 7.6, where the data grouped by SIV status and disease outcome is presented as median ranks. Figure 7.2 is an illustration showing the flow of data acquisition to obtain IOD ratios of mRNA expression in the skin and lymph nodes. The example given is IL-2 from day 2 in the skin (Figure 7.2). A summary of the methods used are also mentioned in the legend.

7.2.6 Lymphocyte Stimulation Tests with *M. leprae* Sonicate

Three months (96 DPI) after re-challenge with *M. leprae*, 10-14 ml of heparinized peripheral blood was drawn from each monkey. Mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque™, Pharmacia, Uppsala, Sweden) washed and re-suspended in RPMI-10 at a concentration of 2 \(10^6\) cells per ml. Approximately 200,000 (1.94± 0.31 \(10^5\) ) mononuclear cells were plated in triplicate in round bottomed 96 well plates (Millipore, Bedford, MA ) in the presence of phytohemagglutinin (PHA) at a concentration of 4 \(10^{-6}\) G/mL, or *M. leprae* sonicate (ML-S) at 10 \(10^{-6}\) G/mL (1:10 dilution) and 5 \(10^{-6}\) G/mL (1:20 dilution). Negative control cultures received medium alone. Cells were cultured with PHA for 3 days, and with *M. leprae* and medium alone for 6 days. The cultures were pulsed for the last 18 hours with tritiated thymidine. Incorporated \(^3\)H was collected on filter paper, immersed in a scintillation fluid measured using a scintillation counter (Beckman LS 6000 IC, Beckman coulter corp., Fullerton, CA). Results were expressed in counts per minutes (CPM) (Fig. 7.9). Statistical analysis of the results was performed on count per minutes (cpm) and the natural logarithm of the cpm (lncpm).

7.2.7 Statistical Analyses

Tukey's studentized range test was performed on IOD ratio and ranked data, when comparing the four cytokines. When normally distributed as tested with an univariate procedure using SAS software version 6.12 (Cary, NC), further statistical analyses were performed using an analysis of variance (ANOVA) on ranked data (Krusval-Wallis) through the general linear model (GLM) procedure of the SAS software version 6.12 (Cary, NC). When the model was not valid, as for IOD ratio by SIV status in skin and LN due to a high variances, Wilkoxon's ranked sum tests were used.
Figure 7.2: Illustration of data acquisition and ranking of cytokine mRNA expression. This was performed by integrating gel electrophoresis band quantification (a, c) and semi-chemiluminescent detection of weak signals after Southern blotting (b).

Frozen skin biopsies, from which the RNA isolates were obtained, are arranged in the same ascending order in a, b, c, and d. The same method was applied to the lymph node cell suspensions.

a: relative RT-PCR: 0.5 µg of DnaseI treated RNA isolate was reverse transcribed using Superscript II® (Gibco BRL) and standardized samples were amplified using PCR. Upper panel of the gel: β-actin cDNA band at 947 base pairs (bp). Lower panel: IL-2 cDNA at 346bp. L: 100 bp ladder; +C = positive control; C = negative control

b: Southern blot: The gel was blotted overnight on a positively charged nylon membrane (Tropilon-plus®, Tropix), fixed with 0.4M NaOH for 3 minutes and hybridized with rhesus macaque cytokine cDNA-specific biotinylated oligonucleotides at 50°C overnight. Hybridization was detected using CSPD® based chemiluminescent kit (Southern Light®, Tropix). Exposure time was 1 hour.

c: IOD ratio: IOD data were acquired by scanning the photo shown in a and using Sunview® software (Millipore, corp.). Histograms of the integrated optical density ratio IODIL-2:IODβ-actin (IOD ratio); lines at 0.5 and 1 delimit ranks 2, 3 and 4 (see table 7.3) in bands visible with gel electrophoresis.

d: Data integration: IOD data, IOD ratio, chemiluminescent hybridization signals and ranks obtained by combining the IOD ratio data from the histogram in c and the Hybr. Signals in blot in b. Ranks were obtained using the criteria used in the table 7.3 shown above. Southern blots primed above IOD ratio data, i.e. no band detected is ranked 0, whatever the IOD ratio value.

Also shown are the SIV status of the animals and whether they developed persistent *M. leprae* infection (DZ outcome +) or not (DZ outcome -) [see also chapter 5 pages 112-113].

The acquired ranked data were used to generate Figure 7.3, 7.4 and 7.7 through 7.10, where the data grouped by SIV status and disease outcome is presented as median ranks.
7.2a

Standardized b-actin
30 cycles

7.2b

IL-2 cDNA
37 cycles

7.2c

Relative RT PCR - skin - IL-2 Day 2 PI

IOD b-actin 0.51 0.29 0.63 0.61 1.17 1.35 0.54 1.64 0.44
IOD IL-2 0.08 0.08 0.03 0.11 0.72 0.07 0.69 1.66 0.06
IOD RATIO 0.15 0.28 0.05 0.17 0.62 0.05 1.28 1.61 0.14
Hybr. signal + + + + + ++ + +++ -

7.2d

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RANK
7.3 RESULTS

7.3.1 GAMMA INTERFERON mRNA EXPRESSION IS ELEVATED IN ALL ANIMALS STUDIED

Gamma interferon was the dominant cytokine mRNA expressed in the skin (Fig. 7.3d) and post-panned unfractionated cells from lymph node cell suspensions (LNCS) (fig. 7.4d), including the CD4-enriched and CD8 fractions of the LNCS (7.5c, 7.5d, 7.6c, 7.6d), in all the 9 animals studied.

7.3.1.1 Skin:

At the inoculation sites in the skin, when all the monkeys were considered (n=9), the maximum expression was at day 26, with a mean rank of 3.55 and a mean IOD ratio of 1.88 ± 1.15 (mean ± SD, n=9) and was significantly higher than day 2, 5 and 12, but not day 62 (p<0.05) (Fig. 7.3d).

Similar levels of IFNy mRNA expression were observed up to day 26 in SIV- and SIV+ monkeys (Fig. 7.5a and fig. 7.7d). At day 62, SIV+ monkeys had significantly higher IFNy mRNA expression than did SIV- animals (p<0.05), (fig. 7.5a). Significantly higher IFN-γ levels were present at day 5 and at day 26 in animals that did not have any evidence of persistent/disseminated *M. leprae* infection at the termination of the study compared with those which did develop persistent lesions (Fig. 7.6a and 7.8d; p<0.05).

An unexpected result was the low expression of IFNy observed at day 12 in the skin, where only 2 bands were visible on the gel and where hybridization was required to detect most products, despite β-actin levels similar to the other days, such as day 5 and day 26 (Fig. 7.3d). Even though the possibility of day-to-day variation must be mentioned and considered, this may suggest a real down regulation of inflammation and pro-inflammatory cytokines such as IFN-γ at day 12, that also correlates partially with observations at gross inoculation sites where fewer inoculation sites were visible (Fig. 5.6 page 116).

7.3.1.2 Lymph Nodes

In the unfractionated LNCS, IFNy expression overall was only moderately and not significantly increased at day 5 and 26 compared to baseline expression, as assessed by both the mean rank (Fig. 7.4d) and the median IOD ratios.
Figure 7.3: Average local (skin) cytokine response observed in the entire group of 9 monkeys analyzed over time. Results are expressed as mean rank (bars) ± standard deviation (error bars). Ranked are defined in Table 7.3. Integrated optical density (IOD) ratios, from which the ranks were partly determined, were normally distributed, when tested with a univariate procedure using SAS software version 6.12 (Cary, NC). (a) IL-4 mRNA expression in the skin (b) IL-2 mRNA expression in the skin (c) IL-10 mRNA expression in the skin (d) IFNγ mRNA expression in the skin.
Figure 7.4: Average regional (lymph node) cytokine response observed in the entire group of 9 monkeys analyzed over time. Results are expressed as mean rank (bars) ± standard deviation (error bars). Ranked are defined in Table 7.3. Integrated optical density (IOD) ratios, from which the ranks were partly determined, were normally distributed, when tested with a univariate procedure using SAS software version 6.12 (Cary, NC). (a) IL-4 mRNA expression in the skin (b) IL-2 mRNA expression in the skin (c) IL-10 mRNA expression in the skin (d) IFNγ mRNA expression in the skin.
Figure 7.5: Gamma-interferon mRNA expression found in the skin and the unsorted and sorted lymph node cell suspensions, by SIV status. Comparison of median integrated optical density (IOD) ratio of IFNγ mRNA expression, standardized on β-actin mRNA, by SIV ratio in (a) the skin, (b) unfractionated LN cell suspensions, (c) CD4 T lymphocyte enriched fraction and (d) CD8 fraction sorted immuno-magnetically. In the skin, day 12 is not shown.

Significant differences are indicated by (*) (p<0.05).
Figure 7.6: Gamma-interferon mRNA expression found in the skin and the unsorted and sorted lymph node cell suspensions, analyzed by persistence of *M. leprae* infection. Comparison of the median IOD ratio of IFNγ mRNA expression, standardized on β-actin mRNA, between animals that did not show any evidence of persistent *M. leprae* infection (ML−; animal number n=4) and animals that show pathologic evidence of persistent *M. leprae* infection (ML+; n=5). In the skin, day 12 is not shown.

Significant differences are indicated by (*) (p<0.05).
In the CD4 and CD8 fractions, however, a sharp increase of IFNγ mRNA expression was noted at day 5-post inoculation (Fig. 7.5c, d and fig. 7.6c, d). In the unfractonated LNCS, the expression of gamma-interferon returned to or below baseline levels after the slight increase at day 5 and day 26 (p<0.05). Due to possible day-to-day variations, it is difficult to know whether there was, on average, a true down regulation of IFNγ mRNA expression at day 62 in unfractonated LNCS. Interestingly, this sharp decline in IFNγ expression was also observed on sorted fractions of LNSC, but earlier compared with unfractonated LNCS, at day 26, both in the CD4-enriched fraction and the CD8 fraction, suggesting a peak of IFN-γ at day 5 with no sustained expression at day 26 in lymphocytes (Fig. 8.2c, fig. 8.2g, fig. 8.2d and fig. 8.2h). A statistically significant higher expression compared to baseline is noted at day 5 both in CD4 enriched (IOD ratio ± SD was 3.64 ± 1.91) and CD8 fractions (IOD ratio ± SD was 6.61 ± 3.39) of lymph nodes (p<0.001).

In the lymph nodes, and in contrast to the skin, higher expression of IFNγ mRNA was consistently observed in SIV+ animals at day 5 and 26 as compared with SIV- animals in unfractonated LNCS (p<0.05 - Fig. 7.5b), whereas in sorted CD8 fractions, SIV+ animals had a trend of lower IFNγ expression levels at day 5 (Fig. 7.5d). Notably, no difference of IFNγ expression was observed at any time point between SIV+ and SIV- animals in the CD4-enriched fraction (Fig. 7.5c).

**7.3.2 IL4 mRNA Expression Is Not A Feature of the Secondary Response to *M. leprae* in the Skin But in Lymph Nodes Is Predictive of Persistent Infection**

Overall and in contrast to IFN-γ, IL-4 mRNA expression was weak and detected mainly after southern blotting, hybridization and chemiluminescent detection, and hence assumed rank values of 0 or 1, giving mean rank values between 0 and 1 (Fig. 7.3a and fig. 7.4a).

**7.3.2.1 Skin**

In the skin, IL-4 mRNA expression was observed in a minority of animals, only after Southern blotting, hybridization and chemiluminescent detection (Fig. 7.3a). Since this highly sensitive technique was necessary to detect IL-4 mRNA message, it can be stated that upon re-inoculation of *M. leprae* in the skin, only low/minimal expression of IL-4 is noted in rhesus macaques. At day 26 post-inoculation, the highest frequency of animals (4/9) with a detectable IL-4 mRNA was observed (Table 7.4). This detection corresponded to the same animals which had early high mRNA expression of IL-2 and IFNγ.
at day 2 and 5. Interestingly, these same 4 monkeys with detectable IL-4 mRNA at day 26 also had
time-wise the highest expression of IL-2 locally at day 2 and IFNγ locally. In the skin at day 26, there
was no statistically significant difference of IL-4 mRNA expression between the SIV+ and SIV- (table
7.4, $\chi^2$-square test $p>0.40$). However, the presence of a weak IL-4 signal in the skin at day 26, but not at
day 12 or day 2, was associated with animals which had the highest IFN-γ and IL-2 mRNA expression
and presented no development of persistent *M. leprae* infection and lesions (Table 7.4 $\chi^2$-square test $p<$
0.05 and fig. 7.8a,7.8b and fig 7.8d).

### Table 7.4: IL-4 mRNA expression in the skin – frequency counts and
statistical analysis

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<th>IL-4 RANK results</th>
<th>day 2 observed</th>
<th>day 12 observed</th>
<th>day 26 observed</th>
<th>day 62 observed</th>
<th>expected (if no effect)</th>
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Notes: No IL-4 was detected at day 5 post inoculation and was not shown.
SIV+: Rhesus monkeys with *M. leprae* inoculation and persistent, chronic J976 simian immuno-deficiency infection.
SIV-: Rhesus monkeys inoculated with *M. leprae*.
ML+: Rhesus monkeys with persistent *M. leprae* infection at the completion of the study, regardless of the SIV status.
ML-: Rhesus monkeys without signs of any persistent *M. leprae* infection at the completion of the study, regardless of the SIV status.

#### 7.3.2.2 Lymph Nodes

Baseline IL-4 was absent in all animals in all LN cell fractions. Messanger RNA for IL-4 was
mainly detected in the unfractionated cells of lymph nodes (Fig. 7.4a). Rare positive bands were also
detected in the sorted CD4-enriched fractions at day 26 and 62 (Table 7.5). Hybridization was required
to detect the PCR product in all but one animal (M736), which had a visible cDNA band in agarose gel
at day 5 (Table 7.5). No expression of IL-4 was observed in sorted CD8 cell fraction.

At day 5 post-inoculation, the highest frequency of animals (4/9) with a detectable IL-4 mRNA
was observed (Table 7.5). SIV status was not predictive of the IL-4 mRNA expression in lymph nodes
($\chi^2$ test – $p=0.48$). However, IL-4 mRNA expression was predictive of disease outcome: persistent *M.
leprae* infection and lesions developed in 4/5 (80%) animals with a detected IL-4 mRNA signal in the

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LN at day 5 (χ² test - p<0.05), and corresponded to lower levels of IFNγ in the unfractionated LN cells among this latter group (fig. 7.6b).

### Table 7.5: a IL-4 mRNA expressions in the post-panning, unfractionated and the sorted CD4-enriched lymph node cell fractions, with b the sum of ranks and statistical analysis on unfractionated LNCS.

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<th>SIV</th>
<th>ML*</th>
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<td>1</td>
<td>ND</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ML: 0 = no pathological evidence of persistent *M. leprae* infection. 1 = persistent *M. leprae* infection. Animal sorted according to this criteria.
SIV: 0 = SIV-negative; 1 = SIV-positive.
ND: not done.

<table>
<thead>
<tr>
<th>b</th>
<th>IL-4, unfractionated</th>
<th>Sum of ranks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>day 5 observed</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>ML- (n=4)</td>
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<td>0</td>
</tr>
<tr>
<td>chi-square</td>
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<td>0.24</td>
</tr>
</tbody>
</table>

Notes: see figure 7.9 and 7.10 pages 170 and 171.
Figure 7.7: Median ranked relative mRNA expression in the skin inoculation sites grouped by SIV status of (a) IL-4, (b) IL-2, (c) IL-10 and (d) IFN-γ. The ranks are defined in Table 7.3.
Figure 7.8: Median ranked relative mRNA expression in the skin inoculation sites grouped by disease outcome of (a) IL-4, (b) IL-2, (c) IL-10 and (d) IFNγ. Disease outcome is defined as the positive persistence (ML+) or negative persistence (ML-) of *M. leprae* infection at the termination of the study. The ranks are defined in Table 7.3. Statistically significant differences (p<0.05) are marked by (*).
Figure 7.9: Median ranked relative mRNA expression in unfractionated lymph node cell suspensions grouped by SIV status of (a) IL-4, (b) IL-2, (c) IL-10 and (d) IFNγ. The ranks are defined in Table 7.3.
Figure 7.10: Median ranked relative mRNA expression in the post-panning unfractionated lymph node cell suspensions grouped by disease outcome of (a) IL-4, (b) IL-2, (c) IL-10 and (d) IFNγ. Disease outcome is defined as the positive (ML+) or negative persistence (ML-) of *M. leprae* infection at the termination of the study. The ranks are defined in Table 7.3. Statistically significant differences (p<0.05) are marked by (*).
7.3.3 **Early Vigorous IL2 mRNA Expression Correlates with Failure to Develop Persistent M. leprae Infection**

Overall, IL-2 mRNA expression in the skin and lymph node was intermediate between that of IFN-γ and IL-4 (Fig. 7.3b and fig 7.4b).

### 7.3.3.1 Skin

The highest levels of expression of interleukin 2 were observed at day 2 and then progressively went down to lower levels over time, the lowest mRNA expression levels being seen at day 62 (Fig. 7.3b). IL-2 mRNA expression in the skin was detectable in agarose gels in most animals until day 12 post-inoculation, after which it came back to levels detectable most often only with Southern blot hybridization. The average IL-2 IOD ratios at day 2 for the 9 monkeys studied was not statistically higher than those at day 5 or 12 (p>0.07). No significant difference of expression of IL-2 mRNA in the skin was observed between SIV+ and SIV- animals (Fig. 7.7b).

However, when disease outcome was used to group IL-2 IOD values (data not shown) and the corresponding ranks in the skin (Fig 7.8b), the difference is striking: the animals that did not develop persistent *M. leprae* infection had a much higher level of IL-2 at day 2 in the skin than the animals that did develop the persistent/disseminated *M. leprae* infection and lesions, which had no and barely detectable levels (p<0.001). A higher IL-2 mRNA expression was noted at day 12 in animals that did develop persistent infection than the (Fig. 7.8b), but this result was not statistically significant when compared to the animals that resisted persistent *M. leprae* infection, comparing both the IOD ratio (p=0.07) or median ranks (p>0.05). Subsequent days did not have any significant difference between animals that did and did not develop the persistent infection.

### 7.3.3.2 Lymph Nodes

Significantly lower mean rank of IL-2 mRNA expression was observed at day 5 in the unfractionated LN cells when all monkeys were considered, compared to IL-2 mRNA mean ranks obtained at baseline and day 26 (Fig. 7.4b). Baseline levels of IL-2 mRNA expression were similar between SIV+ and SIV- rhesus monkeys, as those at the other sampling time points (Fig. 7.9b). Significantly higher median ranked IL-2 mRNA expression was observed in animals that resisted persistent *M. leprae* infection at baseline, a few days before re-inoculation. Other time points did not have any significant difference between animals with and without persistent *M. leprae* infection (Fig. 175).
7.10b). Higher levels of IL-2 mRNA expression at day 5, as compared with baseline levels, were observed in only 2 SIV- animals that developed persistent M. leprae infection.

7.3.4 IL10 mRNA EXPRESSION

The level of expression of IL-10 mRNA was very similar to IL-2 in magnitude. However, IL-2 mRNA was mainly expressed at early time points, generally before IFN-γ, whereas IL-10 was expressed later than IFN-γ (Fig. 7.3c and 7.4c).

7.3.4.1 Skin

In the skin, IL-10 expression was not detectable in ethidium bromide stained gels at day 2, 5 and 12 in the majority of animals. When all animals were considered, the highest mean rank was observed at day 26 post-inoculation and then waned at day 62 (Fig. 7.3c). At no time was any significant difference observed IL-10 mRNA expression in the skin between SIV+ and SIV- animals (Fig. 7.7c) or between animals that did not or did that developed persistent M. leprae infection (Fig. 7.8c).

7.3.4.2 Lymph Nodes

In the lymph nodes, there was no significant difference of IL-10 mRNA expression between SIV+ and SIV- animals or between monkeys that did and did not that develop persistent M. leprae infection (fig. 7.9c and 7.10c). Slight, not significant up-regulation of IL-10 expression was observed at day 5 in rhesus monkeys that developed persistent M. leprae infection, when each animal was analyzed according to ranked data from IOD ratio adjusted on baseline IOD ratio (data not shown). However, ranking of data without baseline adjustment did not reveal a higher median level of IL-10 mRNA in this group (Fig. 7.10c). Moreover, baseline and day 62 levels of IL-10 were consistently but not significantly higher in animals that did not develop the disease (Fig 7.10c).

7.3.5 PBMC STIMULATION TEST CONFIRMS LOWER PROLIFERATION CAPABILITIES TO M. leprae IN SIV+ ANIMALS

Lymphocyte stimulation assays run 96 days post re-inoculation in the 9 animals revealed a significantly higher proliferative response to M. leprae sonicate (ML-S) at both concentrations tested, as compared to unstimulated cells (p<0.05). Cell proliferations from SIV+ rhesus monkeys consistently had lower cpm than those recorded from SIV- animals. These results were significant when stimulated with PHA and the higher concentration of ML-S (10) (p<0.05), but not the lower concentration of ML-S (5 µg/ml) (p=0.755) (Fig. 7.11). The proliferation of PBMC stimulated by both concentrations of ML-
S, as assessed by cpm, from SIV+ animals was not significantly higher than unstimulated cells from the SIV+ group (p>0.20), whereas it was significantly higher in the SIV-negative group at both concentrations of ML-S than unstimulated cells from the SIV-negative group (p<0.01).

Figure 7.11: Lymphocyte stimulation tests from PBMC 96 days post-inoculation. Mls = M. leprae sonicate. Mls-1 = 1:10 dilution corresponding to 10μg/ml of Mls; Mls-2 = 1:20 dilution corresponding to 5 μg/ml. PHA = phytohemagglutinin. None = culture medium only. Bars and error bars represent the group mean ± standard deviation (M±SD).

Lower counts per minutes (cpm) were consistently found in SIV+ animals as compared with SIV- animals, except for baseline (the 'none'treatment group). Comparisons between SIV+ and SIV- monkeys by treatment group that are statistically significant are marked by (*) (p<0.05) All comparisons achieve significance at p<0.001 except for baseline when the natural logarithm (ln(cpm)) of the count per minutes is used as responder variable.

When the natural logarithm transformation of the cpm variable (ln(cpm)) was analyzed statistically, it upgraded the significance levels from less than 5% to less than 1% in most comparisons.

Of note is the significantly lower ln(cpm) at the 5 μg/ml concentration of ML-S in SIV+ animals than

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Incpm from SIV- monkeys (p<0.001). However, the cells from SIV+ monkeys stimulated by 10 μg/ml of ML-S, but not 5 μg/ml of ML-S, did have significantly higher Incpm than Incpm from unstimulated cells from the same group (SIV+) of animals (p<0.02 and p=0.261, respectively). Combined with the cpm data, this statistical analysis suggests that SIV+ monkeys are still capable to respond, albeit to a lesser magnitude, to *M. leprae* sonicate at both concentrations, like the non-SIV-infected animals.

We obtained unexpectedly low counts per minutes, even though the triplicates were all consistent both per animal and with regard to treatments, in this experiment: the mean±standard deviation (M±SD) of the maximum triplicate cpm was 964.8±73.2 (phytohemagglutinin treatment) and the M±SD of the minimum triplicate cpm was 82.6±4.3 (no treatment). This was interpreted retrospectively as a technical error since an inappropriate scintillation fluid for LST was used.

Figure 7.12: CD4:CD8 ratios of simian immunodeficiency (SIV)-positive and SIV-negative animals. The ratios of SIV-positive monkeys are consistently and significantly lower than the SIV-negative animals (p<0.01). This graph was generated from flow cytometry data and analysis (see chapter 6).
Besides the consistently and significantly lower proliferation potential of SIV+ animals to PHA and *M. leprae* sonicates, these animals have consistently and significantly lower CD4:CD8 ratio throughout the experiment than the SIV-negative animals (Figure 7.12).

7.4 DISCUSSION

7.4.1 THE STUDY HYPOTHESIS

This study indicates that, in the skin, upon re-challenge with *M. leprae* 26 months after the initial *M. leprae* infection, the SIV+ survivors that had been challenged with SIV J943 2 weeks prior to the first *M. leprae* infection, have very similar levels of expression to that of the SIV-negative for all the cytokines studied, especially at early time points.

Results indicate that all the animals mounted an initial type 1 cytokine profile in the skin, suggesting that the immunologically crippled SIV+ monkeys are still capable of eliciting locally an adequate cytokine response that is similar to the SIV- counterparts, despite systemic signs of diminished cell-mediated immunity. However, the animals that eventually developed persistent/disseminated disease had a delayed and weaker up-regulation of type 1 cytokines in the skin and an inadequate Th0/Th2 cytokine up-regulation in the lymph nodes draining the skin inoculation sites.

The level of expression of IL-2 at day 2 in the skin is not statistically different between the SIV+ and the SIV-negative groups. At later time points, the SIV+ monkeys tend to have a more sustained mRNA expression, such as, for example, in the case of IFNγ at 62 DPI, where significantly higher levels are observed in SIV+ monkeys, suggestive of compensatory mechanisms in the skin and/or a more persistent local inflammatory response in SIV+ animals. Likewise, in the LN, there were similar levels of secreted cytokines between the SIV+ and the SIV- animals overall.

This result is quite surprising and is different from the cytokine mRNA expressed in broncho-alveolar lavage (BAL) of HIV+ and HIV- patient with pulmonary tuberculosis: in BAL of tuberculous patients, steady state (*ex vivo*) IFN-γ mRNA was decreased in 4 HIV+ patient compared with 4 HIV-patients (Law, Jagirdar et al. 1996). Surprisingly, very few studies focused on cytokine expression at sites of inflammation in other mycobacterial infections in HIV+ patients, such as for example in *M. avium* infection where, to our knowledge, only macrophage cytokine expression are published (Denis and Ghadirian 1994).
However, these results are consistent with evidence concerning the interactions of leprosy and HIV infection at other levels of investigation, such as epidemiology (Meeran 1989; Leonard, Sangare et al. 1990; Frommel, Tekle-Haimanot et al. 1994), (Faye, Mahé et al. 1996). At the level of cytokine production in leprosy lesions in HIV+ patients, there is so far no report concerning cytokine expression between HIV+ and HIV- leprosy patients. One study did examine cytokine mRNA expression evaluation from PBMCs and noted no differences (Sampaio, Caneshi et al. 1995). The major studies of *ex vivo* cytokine expression in the skin lesion of leprosy patients did not investigate whether HIV+ patients would have altered cytokine expression (Yamamura, Uyemura et al. 1991). This is therefore the first study 1) documenting cytokine expression at inoculation sites of *M. leprae* and 2) addressing the question of a difference of cytokine expression between lentivirus-infected and lentivirus non-infected animal models.

7.4.2 Kinetics and Cell Fraction Findings

Kinetics of mRNA expressions over time must be interpreted with caution due to possible day-to-day variations related to experimental design: samples were tested per day to primarily compare groups of animals. However, the results confirm some previous findings and provide some interesting insights on the dynamics of cytokine secretion in response to *M. leprae* inoculations in the skin.

In the skin inoculation sites, when the mean per sampling time point of the ranks of each cytokine obtained from each monkey was determined, it was clearly found that IL-4 and IL-10, classified as type 2 cytokines secreted by type 2 T cells (Th2), have a maximum of mRNA expression both at day 26 whereas the maximum of mRNA expression of IL-2, classified as type 1 cytokines secreted by type 1 T cells (Th1), is at day 2. The mean rank of interferon-γ mRNA expression in the skin is characterized by a peak at day 5 and a maximum at day 26. It can therefore be concluded that, overall, the local cytokine response to *M. leprae* is initially a type 1 response and then evolves towards a type 0 response at around 1 month post inoculation. Interestingly, this very trend has also been observed in a murine model of pulmonary tuberculosis, where the initial cytokines secreted *in situ* were IL-2, IFNγ and IL-12 followed at ~ 28 days by IL-4 mRNA expression up-regulation (Hernández-Pando, Orozco et al. 1996).
In the regional draining lymph nodes, and in contrast with the inoculation sites, no peaks or maxima of mean rank of mRNA cytokine expression over time was noticed. There is a trend towards a down regulation of IL-2 and an up-regulation of IL-4 at day 5 post-inoculation. Slight up-regulation of IL-10 was present at day 26. No conclusion could be drawn for IFNγ mRNA expression using the mean rank since the IOD ratios in most cases were well above 1. The initial ex vivo cytokine milieu in the LN was therefore a type 0, without early predominence of the set of cytokines defining the type 1 cytokine response, like what was observed in the skin.

In all animals studied, the maximum mRNA expression of IFNγ as assessed by IOD ratio was noted at day 5 in all fractions of lymph nodes and at day 26 in the skin inoculation sites. Sorted fractions in the lymph node indicated that the main source of IFNγ was the lymphocytes, since the highest mRNA expression and up-regulation at day 5 were in CD4-enriched and CD8 fractions, without apparent preferential expression between these fractions. This finding must be interpreted with caution, however, since CD8 fractions were near pure (>95% purity) whereas the CD4 fraction attained on an average only (± SD) 74.2 ± 17.7% purity (see chapter 5) with 9.5 ± 16.0% of remaining CD8+ cells. Several lines of evidence suggest that CD8+ T lymphocytes can be high producers of IFN-γ and have very similar capabilities as compared to CD4+ T cells in terms of the array and preferential expression of type 1 and type 2 cytokines (Croft, Carter et al. 1994; Sad, Marcotte et al. 1995). In addition, both type 1 and type 2 cytotoxic CD8+ T cells (Tc1 and Tc2, respectively) may be able to induce DTH reactions in mice (Li, Sad et al. 1997).

At day 5, slightly higher levels of IFN-γ were noted in the non-fractionated LNCS and similar to slightly lower, but non statistically significant, levels in the CD4-enriched and CD8 fractions of SIV+ animals as compared with SIV- animals. This finding, which would need confirmation with larger numbers of animals, raises the possibility that other sources of IFN-γ production cells such as, for example, macrophages and NK cells as early high producers of IFN-γ (Billiau, Hermans et al. 1998; Gessani and Belardelli 1998; Kos 1998). Such compensatory mechanisms of IFNγ secretion by non-CD4 non-CD8 T cells may be important in SIV+ animals responding to a moderately virulent infectious challenge such as M. leprae. For example, a murine mice malaria model, has recently shown that NK cells producing high levels of IFNγ and IL-12 are important, in addition to CD8+ T cells, for the
adaptive immunity against malaria (Doolan and Hoffman 1999). This underscores the complexity of the immune response against intracellular pathogens and addresses the currently poor knowledge about the relative importance of adaptive CMI mediated by non-CD4 and non-CD8 T cells in leprosy.

In summary, LN CD4 and CD8 fractions, with the highest expression at day 5, were the main source of IFNγ in regional lymph nodes in response to \textit{M. leprae} challenge.

7.4.3 Correlation of Cytokine mRNA Expression with \textit{M. leprae} Persistence Outcome

The outcome of \textit{M. leprae} persistent infection was assessed in \textit{post-mortem} samples of nerves, skin and nasal mucosa. Persistent infection is compatible with mild leprosy disease or, at least, persistent \textit{M. leprae} carriers with multiple site lesions, and mainly neural involvement (see chapter 5; table 5.2).

Significantly higher IL-2 and IFN-γ expression at day 2 and day 5 locally in the skin, respectively, but not in the LN, were observed in the group of rhesus monkeys which resisted to persistent \textit{M. leprae} infection compared to the group which developed persistent \textit{M. leprae} infection. Higher median level of IL-2, but not IFNγ, were observed in the CD4-enriched fraction of lymph node cells in animals which resisted to persistent \textit{M. leprae} infection. The highest relative IL-2 mRNA expression was observed at day 2 in the skin of monkeys resistant to \textit{M. leprae} persistent infection.

These findings strongly support the concept that IL-2 and IFN-γ are critical cytokines secreted early after re-exposure to a CMI-eliciting agent, in particular after a second challenge with mycobacteria. Interferon-γ is known to be very important for the successful management of invading mycobacteria (Barnes, Fong et al. 1990; Barnes, Lu et al. 1993; Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993). Our study also re-emphasizes the importance of early vigorous IL-2 secretion in a recall immune response against inoculated mycobacteria, although recent emphasis have been on the secretion of IL-12 in the primary response (Zhang, Gately et al. 1994; Cooper, Magram et al. 1997). A recent study, studying the immune response and disease outcome in a murine model of \textit{Mycobacterium tuberculosis} primary infection in CD4 and MHC class II deficient mice, reported a 2-week delay or an absence of a peak of IFN-γ mRNA, respectively, at the site of infection (lung); both of these correlated with development of disease and death, whereas the wild-type controls successfully managed the challenge (Myers Caruso, Serbina et al. 1999). Surprisingly, IL-2 was not detected in this study,
probably because the first sampling time was at day 7. Our kinetic results are consistent with this finding, showing similar delays; a 10 day-delayed secretion of IL-2 and a 24 day-delayed secretion of IFN-γ in animals that developed persistent or progressive *M. leprae* infection. Timing of protective cytokine secretion is therefore crucial both for *M. tuberculosis* and *M. leprae* infection in rodent and primate model of infection, respectively.

There was no significant difference in the magnitude and the timing of IL-2 and IFNγ mRNA expression between the SIV+ and the SIV- groups at day 2 and day 5, suggesting that the SIV+ slow progressor animals were able to respond adequately to restrict persistent *M. leprae* infection. A similar proportion of SIV+ animals compared to SIV-negative monkeys were indeed able to restrict persistent *M. leprae* infection. The low number of SIV+ animals in this study, the fact that the available SIV+ animals for re-challenge were slow progressors for SAIDS and that they were leprosy-free up to 26 months after the initial *M. leprae* inoculation, may explain the difference in the disease outcome results compared to previous reports about the interaction of *M. leprae* and SIV in rhesus macaques (Gormus, Murphey-Corb et al. 1989; Gormus, Murphey-Corb et al. 1998). Flow cytometry and immunohistochemistry data both indicated that the 2 SIV+ animals that did not develop persistent *M. leprae* infection were different in terms of relative proportions of CD4 and CD8 T cells. Whereas one animal had CD4 recruitment at day 2 essentially similar to SIV- animals, the other had weak CD4 and strong CD8 cell influx in the skin. These data suggest that the source of type 1 cytokines may be CD8 T cells, which compensate for the defective recruitment of CD4 cells in this animal. This hypothesis has been very elegantly demonstrated in a model of tuberculosis in CD4 knock out mice, where the main source of IFN-γ was shown to be CD8 T cells and was a compensatory mechanism, albeit delayed and partly inefficient (Myers Caruso, Serbina et al. 1999).

In regional LN, there was little or no cytokine mRNA up-regulation the in SIV-negative, *M. leprae*-resistant animals at day 5, and very little in SIV-positive that had subsequently developed persistent *M. leprae* infection, suggesting that the cytokine component of the immune response against *M. leprae*, whether protective or not, is essentially local in SIV — animals. In contrast, both resistant and susceptible SIV+ animals up regulated IFN-γ expression at day 5 in the LN. This produced a Th1 profile at day 5 in the two *M. leprae*-resistant SIV+ monkeys and a Th0 response in the only *M. leprae*-
susceptible SIV+ animal. The higher IFN-γ mRNA expression in the unfractionated LN of the SIV+ group as compared with the SIV- group, may reflect a compensatory mechanism or it may be a consequence of chronic SIV infection in the LN. An earlier up regulation of the cytokine response in SIV+ M. leprae-resistant animals compared to SIV- animals (e.g. IFNγ at day 5 versus day 26), supports the compensatory hypothesis. Also, this regional response could be associated with an intermediate level of resistance locally in the SIV+ animals. Persistent chronic SIV infection may also induce inflammation in the LN, however, and play a direct role in the regional IFN-γ up-regulation in the response to M. leprae challenge, regardless of the outcome in terms of persistent progressive M. leprae infection. In HIV patients, higher expression of IFN-γ in lymph nodes have been described compared to controls (Graziosi, Pantaleo et al. 1994). In SIV+ rhesus monkeys, similar results were found in the lymph node (Khatissian 1996).

Very importantly, the results of this study suggest for the first time that in leprosy, successful containment and destruction of the bacilli resulting in resistance to persistent M. leprae infection (i.e. the absence of disease development), is strongly associated with a local, early and strong type 1 cell mediated immune response at site of invasion upon re-challenge, and not with early cytokine mRNA expression in the LN.

In the lymph nodes, expression of IL-4 and up-regulation of IL10 was present at day 5 in all the animals but one that developed persistent M. leprae infection, suggesting that an early type 2/type 0-cytokine response in the LN is associated with progression to persistent M. leprae infection. This may be interpreted in 2 ways: with the sensitivity of RT-PCR, cytokine up-regulation may be detected in the regional LN of monkeys that have dissemination of M. leprae there, as an anticipation of future persistent disease and inflammation. Alternatively, antigen presentation and an appropriate immune response may take place mainly in the skin early (at day 5) in M. leprae-resistant SIV- animals, with minimal response in the LN, whereas in susceptible animals, significant antigen presentation and inappropriate cytokine secretion may take place in the LN early (~ 5 DPI), regardless of bacillus dissemination.

In SIV+ resistant animals, and in contrast to SIV- resistant animals which had slight up-regulation of any cytokine, type 1 cytokines are up-regulated in the LN whereas all 4 cytokines are
expressed in the animals which developed disseminated \textit{M. leprae} infection. Even though these findings must be interpreted with caution due to day-to-day variation, the consistency of up-regulation of all 4 cytokines in the LN of the all 5 animals that developed persistent \textit{M. leprae} infection, but in none of the SIV-, resistant animals is a positive argument to validate biological relevance of these results.

Re-inoculation was not randomly administered, but involved animals that had not developed any evidence of persistent infection with \textit{M. leprosy} despite inoculation 26 months earlier with large doses of armadillo derived \textit{M. leprae} (Gormus, Xu et al. 1998). It is therefore not very surprising that these animals had rather high IFN\(\gamma\) expression levels in the skin and in CD4 and CD8 lymphocyte fractions in LN. Likewise, the SIV positive group, although showing systemic evidence of impaired CMI responses 96 days as assessed by LST and peripheral CD4/CD8 ratios, was composed of slow progressor animals. It is therefore somewhat surprising that a high proportion of SIV- animals (4/6) developed the disease whereas only 1/3 SIV+ did. Several factors could potentially be responsible, the first one being higher selective pressure in SIV+ rhesus macaques challenged by large doses of \textit{M. leprae} compared with control, SIV- animals.

Multiple exposures, occurring in the context of a susceptible genetic background, have never been experimentally tested in any animal model of leprosy. It would be the predisposing factor for disease development most readily tested in rhesus macaques, in conjunction with rhesus MHC typing that is already used to test for SIV disease susceptibility in rhesus monkeys (Baskin, Bontrop et al. 1997).

\subsection*{7.4.4 Type1 Versus Type2 Response}

Interferon-\(\gamma\) was the predominant cytokine expressed locally and regionally in the skin and lymph nodes of all the animals studied. IL-2 was the earliest cytokine reaching a maximum in the skin. IL-4 and IL-10 expression either were either absent or minimal and followed later at lower levels the expression of IFN-\(\gamma\) in all animals at all sampling time points in the skin.

Low expression of IL-4 was noted in a few animals studied, both in the skin and lymph nodes. Interestingly, up to 26 DPI in the skin, weak IL-4 expression was associated with the animals that developed the strongest CMI as assessed by histology and immunohistochemistry and the most vigorous type 1 cytokine mRNA expression. This suggests that, up to 1 month, IL-4 may act as a physiologic

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down-regulator of a vigorous initial type 1 response in the skin rather than driving an inadequate, strong type 2 reaction or a type 0 response. In fact, vigorous early IL-4 secretion and other type 2 cytokine production remain to be shown in any mycobacterial infection at the site of disease (Hernández-Pando, Orozco et al. 1996). IL-4 and IL-10 expression were not, at any time point, strongly up-regulated either in SIV+ animals or among the group that developed persistent *M. leprae* infection in the skin. At the last sampling time point (62 DPI), type 1 cytokines, represented by IFN-γ expression, still dominated the response in the skin. In an attempt to extrapolate those results in humans, it may be proposed that the type 2 cytokines observed in established lepromatous leprosy lesions in humans (Yamamura, Uyemura et al. 1991) would appear after at least several months after exposure. This could also be tested in sooty mangabey monkeys that are highly susceptible to lepromatous leprosy (Meyers, Gormus et al. 1994).

In the lymph nodes, there was minimal differences between different LN cell fractions with respect to expression of IL-10 and IL-2 expression. The response was therefore a type 1 or a type 0 response in all animals at all time points. In the lymph nodes, it is only after subtraction of the baseline levels of IL-10, IL-2 and IFNγ expression for each monkey and mRNA up-regulation/down regulation analysis that type 2 responses were evident in some animals. The predominant baseline IFN-γ expression and type 1/type 0 profile may be associated with the species studied. Analysis of T cell clones in rhesus macaques have shown that a large majority of Th1 and Th0 clones predominated compared to Th2 clones in SIV- rhesus monkeys (47%, 24% and 4%, respectively). In SIV+ animals, this trend is even more pronounced, with relatively more Th1 clones (21%, 60% and 9%, respectively) (Ansari, Mayne et al. 1994; Rosenberg, Lewis et al. 1994). Whether this is due to genetic or environmental factors or a lesser capability of rhesus monkey Th2 clones to survive is not known. It is not an artifact, however, since predominant Th2 clones were found in other monkey species, such as the sooty mangabey (Ansari, Mayne et al. 1994; Rosenberg, Lewis et al. 1994). Also cytokines do not all have the same levels of mRNA expression and protein secretion in an inflammatory focus: IFN-γ and IL-10 have usually higher mRNA production and quantities of proteins secreted, whereas IL-2 and IL-4 have lower, more discrete and brief mRNA up regulation and protein secretion.
In the lymph nodes, when analysis was based on up or down-regulation after adjustment for baseline expression, IL-4 and IL-10 signals were detected only in animals that developed persistent \textit{M. leprae} infection, at day 5. IFN-\(\gamma\) expression levels were either unchanged or down-regulated in these animals at the same time (day 5).

In conclusion, there is an interesting regional effect observed in IL-4 and IL-10 expression. Whereas in the skin, up to day 26, IL-4 and IL-10 seem to act physiologically to down-regulate the strongest a type 1 cytokine responses, early (5 DPI) up regulation of IL-4 and IL-10 in lymph nodes (i.e. a type 2 milieu) is predictive of development of persistent \textit{M. leprae} infection.

In the case of lymph nodes, it is important to mention that, from unfractionated LN cell suspensions, only two out of five animals that developed the persistent \textit{M. leprae} infection had moderate IL-2 and IFN\(\gamma\) mRNA expression up-regulation compared with baseline values. These findings indicate that those 2 animals most probably develop a type 0 response, whereas the last 3 developed a type 2 response in the regional LN draining the inoculation sites. Analysis of \textit{in vitro} stimulated PBMCs and LNCSs for cytokine expression and T cell clones at day 5 in lymph nodes draining an \textit{M. leprae} first and second challenge would be extremely useful to clarify this finding.

To summarize these data with regard of the Th1/Th2 model, in the LN, the detection of a signal and up-regulation for IL-4 together with IL-10 may be considered as a strong up-regulation of type 2 cytokines, because absent or low up-regulation of type 1 cytokines were seen regionally at the same time. In contrast, in the skin, type 2 cytokine expression consistently lagged in magnitude and time behind type 1 cytokines and this was interpreted as physiologic down-regulation locally in the skin inoculation sites. Taken together with the known properties of these cytokines, the results of this study confirm that a type 1 response occurs in the skin, and that progression to persistent \textit{M. leprae} infection is associated with delayed and weaker type 1 responses in the skin rather than a type 0/ type responses.

\textbf{7.4.5 LST Results: Systemic Versus Local Influence of SIV Infection}

Comparison of results of LST in PBMC and cytokine mRNA expression indicate that peripheral blood lymphocytes of the SIV+ rhesus monkeys have a diminished capability to proliferate against \textit{M.leprae} Ag. However, they retained the capability to induce a strong local CMI and strong type 1 cytokine mRNA expression in the skin. Even though there was no test performed to assess the
capability of recruited skin lymphocytes to proliferate against *M. leprae* antigens, this difference of response between the skin and peripheral blood is very similar to the results found in HIV infected persons with BT leprosy (Sampaio, Caneshi et al. 1995). We found a similar proportion of SIV+ animals able to contain *M. leprae* inoculation and avoid the development of progressive of *M. leprae* infection in nerves and nose as compared with controls. Due to the low number and the slow progressor nature of SIV+ animals, this result should not be generalized, but clearly indicates the possibility of adequate response against *M. leprae* inoculation in the skin among SIV+ rhesus monkeys. In previous experiments of inoculation of larger numbers of rhesus monkeys, a higher percentage of SIV+ animals developed persistent *M. leprae* infection (Gormus, Murphey-Corb et al. 1989; Gormus, Murphey-Corb et al. 1998). In addition to the compartmentalization observed between the blood and the skin, there is also an interesting regional effect observed between cytokine expression in the skin inoculation sites and cytokine up regulation in the draining LN in SIV+ monkeys. Whereas IFN-γ is present at similar levels in the skin up to day 26 between SIV+ and SIV- animals, early (5 DPI) strong up regulation of IFN-γ that is a type 1 milieu is observed in lymph nodes of all the SIV-infected animals, regardless of resistance or persistence of *M. leprae* infection. Even though, again, the finding does not address *M. leprae*-specific immune response capabilities between SIV+ and SIV- animals in the LN, this finding suggests a compartmentalization of the local and regional responses against *M. leprae* in SIV+ animals. This may be possibly due to a combination of SIV persistent infection in the LN that dysregulate innate and acquired immunity capabilities (Pantaleo and Fauci 1995) with recent *M. leprae* re-challenge. In contrast, it is improbable that innate defense mechanisms in the skin are impaired in this model. In fact, Langerhan’s cells from homozygous twins discordant for HIV-1 infection were shown to mediate similar immune responses in humans (Blauvelt, Clerici et al. 1995; Blauvelt, Chougnet et al. 1996).

In summary, our study suggests that, despite lower frequencies of circulating T cells capable to specifically respond to *M. leprae* antigens in SIV+ monkeys, there is a similar local response resulting into the adequate management of *M. leprae* infection in a similar proportion of SIV+ as compared with SIV- animals. This finding also suggests an appropriate or better local recruitment of the fewer responding T lymphocytes in SIV+ animals and probably adequate chemoattraction.
The apparent compartmentalization of the effect of SIV on CMI against *M. leprae* is intriguing and is consistent with observations in humans infected with HIV. There is compelling evidence that the effect of lentiviral persistent infection may differ in different tissues. The most striking example of this is the discovery that primary replication of SIV in rhesus monkeys shortly after infection is in the activated lymphocytes of the gastro-intestinal tract (Veazey, De Maria et al. 1998). In this system, CD4 depletion is profound and rapid in the intestinal lamina propria whereas the CD4:CD8 ratio and CD4 counts remains unremarkable in the peripheral blood. Another example is the persistent HIV and SIV infection in lymph nodes, in particular germinal centers (Fox, Tenner-Rácz et al. 1991; Staprans, Hamilton et al. 1995), during the asymptomatic stage of these lentiviral diseases (Pantaleo, Graziosi et al. 1991; Embretson, Zupancic et al. 1993; Pantaleo, Graziosi et al. 1993), (reviewed in (Pantaleo and Fauci 1995), (Khatissian 1996). The germinal centers are the privileged sites of immune response with a high concentration of activated lymphocytes, which are the main target of HIV and SIV for virus replication. It is now well established that concurrent infections and disease, whether successfully contained or not, may favor the progression to more advanced clinical features of AIDS by favoring virus replication, among others (Pantaleo, Graziosi et al. 1993)(reviewed by (Copeland and Heeney 1996). Immune activation elicited by vaccination and diseases like influenza and tuberculosis, also favor replication and higher HIV-1 mRNA copies in the blood, at least transiently (Ho 1992; O'Brien, Grovit-Ferbas et al. 1995; Staprans, Hamilton et al. 1995; Zhang, Nakata et al. 1995; Goletti, Weissman et al. 1996; Stanley, Ostrowski et al. 1996; Garrait, Cadranel et al. 1997; Nakata, Rom et al. 1997; Honda, Rogers et al. 1998).

The reaction of the lentivirus-infected host to *M. leprae* is unique when compared to other mycobacterial infections such as tuberculosis (reviewed in (Shafer and Edlin 1996) and (Bloom 1994) and opportunistic species of mycobacteria such as *Mycobacterium avium*-intracellulare complex infections (Wallace and Hannah 1988; Benson and Ellner 1993; Race, Adelson-Mitty et al. 1998), which rank among the main life-threatening secondary infection in HIV-infected and AIDS patients. This uniqueness may be related to unique pathogenic properties of *M. leprae*, characterized by intracellular location and slow growth, among other characteristics. It is as if the SIV-infected animals...
were able to focus the CMI defense apparatus in the very sites of inoculation, with apparent success, in
the half of the animals of the group.

In experiments performed in other rhesus monkeys in which SIV was inoculated 8-10 months
before, M. leprae primary inoculation with resulted in diminished proliferation to Concanavalin A lectin
stimulus in co-infected animals and an absence of proliferative response to ML-S (Gormus, Murphey-
Corb et al. 1998). A majority of these animals developed persistent M. leprae infection. This indicates
that the timing of the relative inoculations of SIV and M. leprae is important when studying the
interactions between the two. In our model, when M. leprae was inoculated at the time of the SIV
primary syndrome, we did not find any major difference between the two groups.

7.4.6 SUMMARY AND CONCLUSION

In summary, an important finding of this study is that slow progressors, SIV positive animals
are still capable of mounting an adequate type 1 immune response against M. leprae locally and
regionally, with similar or higher levels of IL-2 and IFN-γ compared with SIV- monkeys. This response
was present despite significantly lower levels of circulating, significantly lower stimulation indexes of
peripheral blood mononuclear cells to M. leprae antigens and slightly lower recruited CD4 T cells. The
patterns of response present in both SIV+ and the SIV- animals correlated quite well with the degree of
cell infiltration of the skin lesions and the disease outcome. The animals that did not develop signs
and/or lesions characteristic of a persistent M. leprae infection and an evolution towards leprosy had
consistently more rapid and more vigorous expression of IL-2 and IFNγ locally in the skin, indicating a
rapid and vigorous Th1 response. Th2 cytokines (IL-4, IL-10) do not appear to be important in the skin
at early time points, but are up-regulated in the lymph nodes in animals which developed persistent M.
leprae infection, with microscopic lesions as assessed by post-mortem findings. According to our results
on a limited number of animals, SIV infection appears to have little importance in the type and
magnitude of the cell mediated immune response to a Mycobacterium leprae re-challenge. The rhesus
monkey is a useful model in which to explore the causative factors of the defective CMI in leprosy, that
appears in only a proportion of animals and does not seem to be strongly affected by SIV infection,
corroborating results of epidemiological studies of leprosy in HIV+ individuals.
7.5 LITERATURE CITED


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8 SUMMARY AND CONCLUSION

There is a general consensus among leprosy researchers to consider that the disease following infection with *Mycobacterium leprae* is more a consequence of immunopathologic processes than a manifestation of the direct action of the agent. This mycobacterium is a well-adapted, stealthy, minimally toxic pathogen that hides in the host, with a predilection for peripheral nerves and the cooler parts of the body (reviewed in (Job 1994)). Another important factor is the apparent genomic monomorphism of *M. leprae* (Williams, Gillis et al. 1990), suggesting that most, if not all of the disease spectrum is a consequence of the host’s capabilities or inability to react against the parasite.

One central question of leprosy research concerns the pathogenesis of lepromatous leprosy. It is still not known, more than a century after the discovery of the etiological agent by Harmauer Hansen, whether the primary defect in lepromatous leprosy is an initial failure to recognize and/or process antigens from *M. leprae* or is a progressive down regulation of the cellular arm of adaptive immunity, a consequence of uncontrolled replication of the organism (Harboe 1994). Since this non-cultivable organism is a slow grower in animal tissues, one is tempted at first analysis to favor the first hypothesis. However, as molecular microbiologists are unraveling the complexity of strategies in mycobacteria-host relationship (reviewed in (Bloom 1994; Ernst 1998; Aderem and Underhill 1999), the situation might be more complicated. It could be a combination of the above, with partial failure to process antigens and therefore mount an efficient CMI, subsequent augmentation of the bacterial load and secondary down regulation of the CMI. According to the Th1/Th2 paradigm, this down regulation of CMI would then be characterized by a shift from a local type 1 (Th1) to a local type 2 (Th2) cytokine possibly mediated by type 2 cytokines, such as IL-4 and IL-10. This situation may also be valid for tuberculosis in humans. These kinetic aspects of cytokine responses in mycobacterial diseases, and in leprosy in particular, have seldom been tested in animal models.

Whereas individuals exposed to *Mycobacterium tuberculosis* develop an extremely strong CMI, illustrated by a positive tuberculin test that indicates a strong DTH, individuals suffering from active, progressive tuberculosis usually have a Th0 or Th2 cytokine profile, with occasional tuberculin test negativity (Sanchez, Rodriguez et al. 1994; Surcel, Troye-Blomberg et al. 1994) (reviewed in (Cooper and Flynn 1995; Lucey, Clerici et al. 1996)). Kinetic studies on a murine model of progressive...
pulmonary tuberculosis in Balb/c mice have clearly shown a progressive shift from a type 1 to a type 2 cytokine milieu, that correlates with progression to pneumonia and corroborates the hypothesis of CMI down regulation (Hernández-Pando, Orozcoe et al. 1996).

Another puzzling observation in leprosy research concerns the apparent lack of increased incidence of leprosy, severity of lesions and down-grading from tuberculoid to lepromatous in HIV-infected Africans and Asians. The reasons of this observation, if true, are not presently understood. The apparent ability of individuals immunologically crippled by chronic HIV infection to compensate and avoid a worse manifestations of leprosy after chronic *M. leprae* infection may be related to the apparent slow growth of the pathogen. In addition, the often limited, local infection elicited by *M. leprae* in tuberculoid leprosy may still be mastered by a partially impaired host immune response whereas it would not be if *M. leprae* infection was characterized by a rapid overwhelming of the immune system (Sampaio, Caneshi et al. 1995). Presently, the situation is as if the general loss of the immune capabilities observed in HIV-infected persons evolving towards AIDS had little consequence on how *M. leprae* will behave in the human host. This issue is still not settled and more epidemiological studies will be helpful to clarify the situation. This is in any case unique to *M. leprae* since tuberculosis is definitively worse in HIV+ than in HIV- patients and mycobacterial opportunistic infections are highly frequent in the former group.

Carefully controlled experimental situations such as rhesus macaques inoculated with *M. leprae* (ML), with or without SIV infection, reveals a higher incidence of persistent ML infection in ML-SIV co-infected animals compared with ML-inoculations alone (Gormus 1989) (Gormus, Murphey-Corb et al. 1998). This suggests that sooty-mangabey-derived SIV might have a detrimental affect on the outcome of large doses of *M. leprae* inoculated in rhesus macaques. This detrimental effect has been observed in two distinct situations: at the initial time of ML exposure, or later during progression towards SAIDS. However, the mechanism of the effect of SIV infection on *M. leprae* persistent infection and disease has not been determined in rhesus macaques. Similarly, the possible detrimental effect of SIV infection on *M. leprae*-associated persistent infection and disease has not been apparently tested when ML was inoculated several months before SIV challenge. Lastly, the effect of
M. leprae-associated persistent infection on the primary infection complex and the evolution of SIV infection and disease in rhesus macaques has not been explored.

Moreover, it is not known yet what type of cytokine milieu is present initially at site of inoculation in the skin of rhesus macaques whereas it is already known in humans with established leprosy lesions (Yamamura, Uyemura et al. 1991; Modlin 1994). Preliminary studies have been done to test the cell recruitment and the cytokine milieu in the primary immune response to M. leprae in rhesus macaques (Lathrop, Scollard et al. 1996; Scollard, Gillis et al. 1997). These studies revealed delayed recruitment of CD4+ lymphocytes and delayed mRNA expression of IL-2 in SIV+ macaques.

To investigate the memory immune response against a second challenge of M. leprae, we inoculated 9 rhesus macaques, 3 of which were SIV positive. Our study hypothesis was that a type 1 cytokine milieu initially exists in rhesus macaques and that an inadequate local and regional response against M. leprae is present in SIV+ animals. Results indicated that there was indeed, in the initial phase of host response after challenge, a type 1 cytokine response in the skin with a predominance of IFNγ and IL-2 mRNA expression locally in the skin, and surprisingly, that there was no significant difference between SIV+ and SIV- animals. In the lymph node, a strong Th1 response was present at day 5 when sorted lymphocytes were studied for mRNA expression, with notably high levels of IFNγ mRNA. In contrast, in the unfractonated cell suspension, a proportion of animal had a type 0 response at day 5 since IL-4 mRNA was detected. As in the skin, there was no significant difference between SIV+ and SIV- monkeys. However, when the rhesus macaques were grouped according to persistence of M. leprae infection, 4 of 5 animals with persistent infection had IL-4 mRNA in the lymph node at day 5, suggesting that the early up-regulation of type 2 cytokines in the LN rather that at the site of infection in the skin, was predictive of future development of persistent infection in this species.

The results presented in this work, despite low numbers of animals, are the first in any animal model of leprosy that clearly indicates that a locally delayed, weaker adaptive cytokine response is uniformly present in the skin of the group of monkeys that subsequently had persistent M. leprae infection (ML+). This weaker and delayed response is characterized by lower levels of Th1 cytokines, such as IL-2 and IFN-γ, rather than an initial Th2 response, and reduced recruitment of lymphocytes, especially of CD4+ T cells. This is in contrast with the regional response in the LN, where early up-
regulation of IL-4 is present at day 5 in animals that will subsequently develop persistent ML infection. Two notes of caution should be considered in the case of the animals that developed persistent M. leprae infection (ML+): (1) One cannot conclude that the development of persistent M. leprae infection (ML+) in rhesus macaques was entirely due to the re-inoculation, even though they all appeared healthy, since they had been infected previously with M. leprae. (2) Whether these results can be applied to humans exposed per-cutaneously to M. leprae remains to be proven, since rhesus macaques do not exhibit the same features of leprosy as humans (Gormus, Xu et al. 1998).

A rapid and vigorous secretion of IL-2, and of IFN-γ immediately thereafter, was invariably present in the 4 animals that failed to develop persistent lesions and M. leprae infection (ML-), regardless of their SIV status. These latter monkeys were able to recruit large numbers of CD3+ lymphocytes in the skin inoculation sites at 2 DPI, the majority of which were CD4+ T cells, except one SIV+ animal that had a large majority of CD8+ T cells. No statistically significant difference was observed between SIV+ and SIV- animals, both in cytokine responses and cell recruitment, reflecting similar proportions of ML- animals among the 2 groups.

The possibility that a type 2 cytokine milieu may act as down regulator of CMI in mycobacterial infection, rather than being the motor of early inadequate response, was supported by the results of IL-4 expression in the skin: IL-4 was expressed in a very limited, minimal manner, without any recognizable pattern, until day 26 in all animals. At day 26, IL-4 mRNA was detected in the skin in the ML-negative animals that had had the highest initial Th1 response.

The value of performing longitudinal studies stressing the kinetics of cell recruitment and cytokine mRNA expression or cytokine protein secretion will probably be the key for a better understanding of the innate and adaptive immune response to mycobacteria and other infectious agents. Moreover, they will help solve some of the apparent discrepancies observed in studies dealing with a single sampling time point from peripheral blood.

Several issues, however, need to be addressed. Our results suggest that there is a group of animals with an inadequate secondary anamnestic immune response, regardless of the SIV infection status in rhesus monkeys. This latter group of animals could be characterized with planned breeding and further study, and they could give important insights into possible genetic defects in the processing and
handling of \textit{M. leprae} antigens and possibly other mycobacteria. They would possibly complement quite interestingly the studies in humans and laboratory rodents, where several genes have been characterized (de Vries and Young 1994), because these animals are phylogenetically closer to human beings. Another avenue of research would be the testing, from this sub-group of rhesus macaques that develop persistent infection, of the influence of the lepromatous leprosy on the outcome of acute SIV infection, since it has already been proven that tuberculosis has a negative effect on HIV infection (Goletti, Weissman et al. 1996; Garrait, Cadranel et al. 1997; Nakata, Rom et al. 1997; Honda, Rogers et al. 1998).

In conclusion, this study enabled us to get important insights into the pathogenesis of leprosy by stressing the key role of a precocious and strong expression of an adequate type 1 cytokine milieu, characterized by early and strong IL-2 and IFN\textgamma{} expression, after re-challenge of \textit{M. leprae} in the skin of rhesus macaques that controlled \textit{M. leprae} and avoided persistent infection. Animals that developed persistent \textit{M. leprae} infection had delayed and weaker expression of IL-2 and IFN\textgamma{} mRNA, rather than an initial inadequate type 2 cytokine environment. There was no significant differences between SIV-infected and SIV-non-infected macaques, corroborating epidemiological studies in human leprosy and AIDS. Further studies exploring the basis of this difference of strength and precocity of type 1 cytokine expression between resistant and susceptible animals would be highly interesting and of uppermost importance.

8.1 LITERATURE CITED


APPENDICES

APPENDIX 1: *M. LEPRAE* PERSISTENCE OUTCOME COMPARISONS BETWEEN PRESENT AND PUBLISHED STUDIES
Appendix 1

Comparison of the results of disease outcome by SIV status between already published data and the present study.

<table>
<thead>
<tr>
<th></th>
<th>Previous study (Gormus, Xu et al. 1998) (n=38)</th>
<th>Present study (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distrib.</td>
<td>%</td>
</tr>
<tr>
<td>No visible disease</td>
<td>24</td>
<td>63</td>
</tr>
<tr>
<td>Disease development</td>
<td>14</td>
<td>37</td>
</tr>
<tr>
<td>TT^</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Neuritic TT^</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>TT/Ind^</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>BB^</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>BL^</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>LL^</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

§ Including SIV positive rhesus macaques
¶ Leprosy immunological classification according to (Ridley and Jopling 1966) modified in (Gormus, Xu et al. 1998).

References


APPENDIX 2: PHOTOMICROGRAPHS ILLUSTRATING THE HISTOPATHOLOGY AND CYTOLOGY OF INCOMPLETE FREUND'S ADJUVENT (IFA) INJECTION SITES

See also page 92 and page 98.
APPENDIX 3: PHOTOMICROGRAPHS ILLUSTRATING RNA ISOLATION FROM IFA INJECTION SITES

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APPENDIX 4: PHOTOMICROGRAPHS ILLUSTRATING *M. LEPRAE* SKIN INOCULATION SITES AND GROSS LESIONS 26 DAYS POST-INOCULATION (DPI)

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APPENDIX 5: PHOTOMICROGRAPHS ILLUSTRATING CD4 IMMUNO-HISTOCHEMISTRY IN *M. LEPRAE* SKIN INOCULATION SITES AT 2 DPI

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APPENDIX 6: PHOTOMICROGRAPHS ILLUSTRATING TOPOGRAPHICAL HISTOPATHOLOGY AT 62 DPI IN SIV NEGATIVE RHESUS MACAQUES

See also page 138.
Figure 6.9
APPENDIX 7: PHOTOMICROGRAPHS ILLUSTRATING
TOPOGRAPHICAL HISTOPATHOLOGY AT 62 DPI IN SIV
POSITIVE RHESUS MACAQUES

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Figure 6.10
VITA

Thomas Xavier D. Lemarchand was born in 1967 in Caen, Normandy, France. In 1974, his parents decided to move in Brittany and he had the luck to be raised in the countryside in Locmariaquer, a village along the Morbihan Gulf, a beautiful and touristic area in southern Brittany. His child wish were to become a farmer and biologist. He attended the “école Saint Gildas” catholic private school from the 4th grade to the 7th grade, then “Le Verger” public college (highschool) in the 8th and 9th grade in Auray. At that time, he realized that biology and geology was most interesting and importantly that it was easy to be bold in this discipline with moderate studying, a situation not present in other disciplines such as languages and mathematics! He then moved for three years to the “Lycée Benjamen Franklin”, Auray, France, a lycee named after the great American scientist and politician, who landed there after a long travel through the Atlantic Ocean in the late 18th century to discuss alliances with the young French Republic against the British Crown. There, he focused his efforts on Experimental Sciences and Mathematics and finished his highschool curriculum by taking and passing the “Baccalaureat série C” in the spring of 1985. Shifting from his initial wish of farming, he decided to become a veterinarian and enrolled in pre-veterinary preparation in Lycée Chateaubriand, Rennes, France and took the competition in the spring of 1986. He luckily passed the written and oral part of the competition and was accepted as a veterinary student in “The Ecole Nationale Vétérinaire de Nantes” (ENVN) in fall 1986. While focusing on the harsh theory courses of the first 2 years, he gradually got involved in a non-lucrative association founded by students known as the “Ferme Expérimentale des élèves de l’ENVN” (ENVN students’ Experimental Farm) dealing with many aspects of small ruminant and poultry medicine and zootechniques. Noticing the recent isolation of feline immunodeficiency virus (FIV) by Dr Pedersen in Davis, California, he got involved in 1989 on research investigating the lesions associated with the natural disease caused by this virus, and decided in 1990 to learn further veterinary pathology by enrolling in a 3 year residency in veterinary anatomic pathology. He defended his veterinary thesis entitled “Etude anatomo-pathologique de 25 chats naturellement infectés par le virus de l’immunodéficience feline” (Pathology of 25 cats naturally infected with FIV) in the fall of 1992 and focused, for his residency research project, on the neurologic changes associated with natural FIV.
infection. Meanwhile, in an attempt to learn more in large animal pathology and achieve excellence in pathology, he decided, for the third year of his residency, to follow a practical training after the kind invitation of Dr Michel Morin from the University of Montréal, Québec, Canada. He completed his training there in the early summer of 1993, and took and passed successfully the requirements for receiving the recently created “Diplôme d’études spécialisées en anatomie pathologique vétérinaire” from the French Ministry of Agriculture. He then moved to Albuquerque, New Mexico, United States of America, to fulfill a one year temporary position in the New Mexico Department of Agriculture’s Veterinary Diagnostic Services. Seeking for further knowledge and competency, he decided in 1994, with the warm support of his wife Christine, to diversify his previous training and devote himself to learning research philosophy and techniques in pathology and immunology, and to accept an assistantship in the Department of Veterinary Pathology, Louisiana State University. His career goals are to continue further developments in the understanding of the mechanisms of disease and practice the expertise acquired in the last 9 years in diagnosing animal diseases and in counseling his colleagues and veterinarians.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Thomas Xavier D. Lemarchand

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Early Local and Regional Immune Response to Recall Inoculations of Mycobacterium leprae in the Skin of Rhesus Macaques (Macaca mulatta)

Approved:

Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

19 October 1999