DISSERTATION

ASSOCIATION OF THE PATHOPHYSIOLOGY AND THE IMMUNITY OF TUBERCULOSIS WITH THE *Mycobacterium tuberculosis* EXTRACELLULAR PROTEOME

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2005

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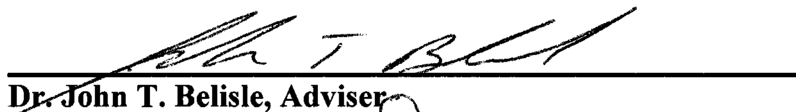

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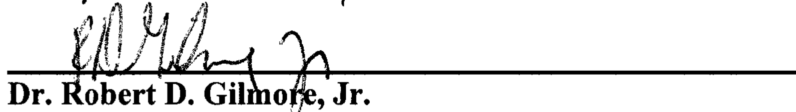

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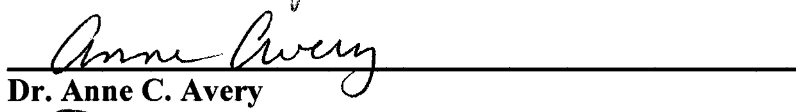

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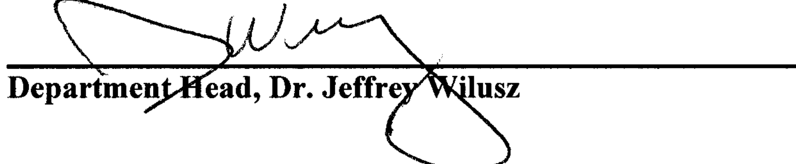

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ABSTRACT OF DISSERTATION

ASSOCIATION OF THE PATHOPHYSIOLOGY AND THE IMMUNITY OF TUBERCULOSIS WITH THE *M. tuberculosis* EXTRACELLULAR PROTEOME

Because of the devastating social and economic impact of tuberculosis on the countries of the world the need for an improved understanding of this disease is greater than ever. Chapter two of this research identified proteins with altered translocation patterns in a SecA2-/- mutant compared to wild-type *M. tuberculosis*. Superoxide dismutase (SodA), catalase-peroxidase (KatG), and Rv0393 contain no amino acid signal sequence, however, decreased levels of these proteins in the filtrate of SecA2-/- mutant cultures compared to the wild-type indicate a role for SecA2 in their export. Since the SecA2-/- mutant manifests truncated virulence in the mouse, and SodA and KatG are implicated in the detoxification of oxidative compounds produced by the macrophage, understanding the mechanisms by which this novel protein secretion pathway functions is of central importance.

Based upon the hypothesis that the changing environment encountered by *M. tuberculosis* during the course of infection will translate to altered protein production by the bacilli, chapter three of this work identifies proteins with increased production under stressed conditions believed to more closely reflect conditions encountered by the bacilli during infection. *M. tuberculosis* was grown under standard, microaerophilic, anaerobic, and alternate carbon source conditions. 2DE and MS/MS analysis of the filtrates of these cultures revealed four proteins with increased abundance under stressed conditions: Acr,

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BfrB, Ppa, and Ssb. These proteins and others known to be induced under stressed conditions were produced and purified for use in immunological studies.

In chapter four, the kinetics of the T cell response to these individual proteins during infection is determined overlaying leukocytes onto antigen-pulsed bone marrow derived dendritic cells. The response by T cells derived from the lungs and spleens of infected mice throughout 195 days of infection was specific to each protein and varied over the course of infection. The cytokines produced during the overlays were also examined and shown to be specific to each antigen and the length of infection. The protective potential of these proteins was determined in a vaccine study demonstrating that these proteins alone did not confer protection against subsequent challenge, but boosting BCG vaccination with these proteins augmented the protective efficacy of BCG alone significantly.

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LIST OF ABBREVIATIONS

2DE	two-dimensional gel electrophoresis
AG	arabinogalactan
AIDS	acquired immune deficiency syndrome
Ala	alanine
APC	allophycocyanin-labeled clone
APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	Bacillus Calmett-Guerin
CBA	cytometric bead array
CD	cluster of differentiation
CFP	culture filtrate proteins
CFU	colony forming units
CR	complement receptor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DDA	dimethyldioctadecyl ammonium bromide
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
DTT	dithiolthrietol
EDL	electron dense layer
ESI	electrospray ionization
ET	endotoxin
ETZ	electron transparent zone
FITC	fluorescein isothiocyanate-labeled clone
GAS	glycerol alanine salts
GDH	glycine dehydrogenase
GFP	green fluorescence protein
GKO	IFN-γ knockout
GlcNAc	*N*-acetylglucosamine
GM-CSF	granulocyte/macrophage colony stimulating factor
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HPP	hypoxia-induced protein pool
IFN-γ	interferon *gamma*
IL	interleukin
INH	isoniazid
iNOS	inducible nitric oxide synthase
IPG	immobilized pH gradient
KO	knockout
LAL	limulus amoebocyte lysate
LAM	lipoarabinomannan
LDA	low dose aerosol
LPS	lipopolysaccharide
MHC	multiple histocompatibility complex
MMR	macrophage mannose receptor
MPL-SE	monophosphoryl lipid A
MRI	magnetic resonance imagery

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MS	mass spectometry
MS/MS	tandem mass spectometry
MurNAc	*N*-acetylmuramic acid
NK	natural killer
NO	nitric oxide
NOS2	nitric oxide synthase 2
NRP	non-replicating persistence
OADC	oleic acid albumin dextrose catalase
OL	outer layer
ORF	open reading frame
OT	old tuberculin
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PerCP	peridinin chlorophyll protein-labeled clone
PM	plasma membrane
PPD	purified protein derivative
PZA	pyrazinamide
RD	region of difference
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RP	reverse phase
S_2DE	SecA2-dependent exported
SC	sub-cutaneous
SCID	severe combined immunodeficiency disorder
SDS	sodium dodecylsulfate
SP-A	surfactant protein - A
SP-D	surfactant protein - D
TACO	tryptophan asparagine-rich coat protein
TAP	transporter associated with antigen presentation
TCA	tricarboxylic acid
TCR	T cell receptor
TFA	trifluoroacetic acid
Th	T helper
TLR	Toll-like receptor
TNF-α	tumor necrosis factor *alpha*
WCL	whole cell lysate
WHO	World Health Organization

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CHAPTER 1: Literature Review

> The Captain of all these men of death that came against him to take him away, was the Consumption, for it was that that brought him down to the grave. [1]
>
> John Bunyan 1680

1.1 *Mycobacterium tuberculosis in historical perspective*

For thousands of years, humans have suffered from disease caused by *Mycobacterium tuberculosis.* Until recently, it was believed that this infectious agent was first introduced to man nearly 20,000 years ago during the domestication of cattle which were infected with *M. bovis*, a closely related species to *M. tuberculosis*. It was then believed that this bacterium evolved to more successfully fill the niche of a human pathogen. Recently, however, a whole genome approach to phylogeny has revealed that both *M. tuberculosis* and *M. bovis*, along with other pathogenic subspecies of the *M. tuberculosis* complex such as *M. africanum,* and *M. microti*, most likely co-evolved from a single unknown ancestor [2]. In fact, it appears more likely that *M. bovis* appeared on the evolutionary stage later than did *M. tuberculosis.* Whatever the evolutionary path taken by this organism, it is clear that disease conditions caused by *M. tuberculosis* have afflicted man from the beginning of recorded history. Evidence indicates that 1500 years ago Peruvians were infected by this bacterium [3], and more anciently, mummified bodies in Egyptian tombs show manifestations of granulomatous disease closely

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resembling tuberculosis pathology [4, 5]. Despite having been plagued by this agent throughout recorded history, tuberculosis remains to this day one of mankind's most devastating afflictions. As such, the World Health Organization (WHO), reports that nearly two-thirds of the world's population today is infected with *M. tuberculosis* [6]. Approximately 9 million new cases of tuberculosis are predicted to occur annually and this disease results in almost 2 million deaths per year [6].

Historically, diseases such as Scrofula, phthisis (from the Greek meaning "to waste away") and Pott's disease were characterized by cervical adenopathy, granulomatous lesions and skeletal deformities. Previous to the 19th century, it was believed that consumption (the most common form of disease caused by this agent) was not an infectious disease at all, but was rather brought on by a variety of "irritations". A weak moral character, indulging in unhealthy eating habits, a "bookish" and sedentary lifestyle, a family predisposition to weakness, and residing in a cold, wet climate were all believed to be antecedent to this affliction [7]. Based on this assumption and fueled by a general lack of medical knowledge about infectious disease as a whole, treatment was founded on restoring unbalanced humors in the body by changing the lifestyle of the patient and physicians commonly prescribed regimens based on this "constitutional treatment" [8]. Activities such as deep breathing plenty of fresh air, eating the most rich and "healthy" foods possible, exercise and exposure to the sun (heliotherapy) were thought to be particularly therapeutic [7]. Where necessary, the patient was encouraged to relocate from cold, wet environments to more sunny and dry locations. In spite of these health measures, consumption spread rapidly through the populations of Europe and America, especially in industrialized cities among the poor and working classes.

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Consumption became so prevalent and feared among the population that enormous public health campaigns were started in order to blunt its social and economical effects. Based on the assumption that this was primarily an affliction of the poorer classes - people who lived in dark rooms of broken down buildings and those who too frequently visited saloons and other morally decadent places of congregation - the government and medical communities sought reforms to construct playgrounds where people and families could assemble out-of-doors; tenement housing was restructured to allow for more fresh air and sunlight, water was purified and milk sterilized to allow even the poorest element of society to have clean food [9].

1.2 *Robert Koch: the father of bacteriology*

By the late 1800s the germ theory proposed by Louis Pasteur was gaining increasing acceptance in the scientific community. In 1865, Jean Antoine Villemin demonstrated that tuberculosis was transmissible from one animal to another: evidence that tuberculosis was a contagious disease [10]. It was believed by many, however, that the different manifestations of disease, whether pulmonary or miliary, were each caused by separate entities, and a causal organism had yet to be identified or observed. In August of 1881, Robert Koch, a German country doctor-turned-scientist known for the identification of the anthrax bacillus life cycle, using techniques of photomicroscopy and bacterial plating that he developed, began work to prove that the cause of tuberculosis was a parasite. A mere seven months later, on 24 March 1882, at a meeting of the Berlin Physiological Society, Koch announced his discovery of the tubercle bacillus. Three weeks later his research was published and disseminated throughout the world [11]. So

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thorough, complete, and convincing were his experiments that his findings were almost immediately universally accepted [12, 13]. By identifying a bacterial agent as the cause of nearly 1/7 of all deaths in the developed world, Robert Koch elevated bacteriology as one of the most important disciplines in medical science. Even today his experimental approach to infectious microbiology, called Koch's Postulates, is the foundation of research into the etiology of disease.

1.3 *Physiology of the M. tuberculosis bacilli*

The mycobacteria are non-motile, rod-shaped bacteria belonging to the order *Actinomycetales* in the family *Mycobacteriacea*e, which consists of only one genus: *Mycobacterium*. These bacteria are typified by a high DNA GC content (66-71%), a lack of spore formation, cell wall mycolic acids made up of long acyl chains (C60-90), and the rare ability to retain carbol fushin dye in the cell wall even when washed with strong acids: hence their designation as "acid fast". Mycobacterial species can be divided into slow- and fast-growing bacteria. Slow growers, such as *M. tuberculosis*, *M. leprae, M. avium, M. bovis,* and *M. africanum,* are often associated with human and animal disease, while the fast growers, such as *M. smegmatis* and *M. phlei,* are classified as nonpathogenic.

The mycobacterial envelope is a highly complex physiological structure consisting of a plasma membrane (PM), a cell wall and an outer capsule layer. These three structures are typically divided into four sections based on their appearance by electron microscopy: 1) the plasma membrane, 2) the electron-dense layer (EDL), 3) the electron-transparent zone (ETZ), and 4) the outer layer (OL) [14].

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As in all prokaryotes, the PM of mycobacteria is a phospholipid bilayer that is 4-4.5 nm thick. This layer contains integral and associated proteins necessary for electron transport, ATP synthesis, solute and protein transport, cell signaling, and a host of other biosynthetic processes [15]. External to this PM is the cell wall core that spans the EDL and the ETZ, and is comprised of peptidoglycan (PG) covalently linked to arabinogalactan (AG), which in turn is covalently linked to mycolic acids [16]. More specifically, PG is a glycopeptide consisting of alternating residues of N-glycoylmuramic acid (MurNGly) and N-acetylglucosamine (GlcNAc) connected by β-1,4 linkages. Tetrapeptide chains on each MurNAc residue are cross-linked to each other via peptide bonds. Next, the tripartite AG consists of the proximal linker, medial galactofuran, and distal arabinofuran regions. The linker region of this molecule (rhamnose 1-3 GlcNAc) is connected by phosphodiester linkage to the MurNAc residues of the PG [17]. In turn the arabinosyl portion of the AG is covalently bound to α-alkyl, β-hydroxy fatty acids known as mycolic acids that contain C60-C90 alkyl chains radiating outward from the cell wall [16]. By weight, the mycolic acids make up the majority of the cell wall structure and are thought to act as a physical barrier protecting the bacillus from many forms of attack, including reactive oxygen intermediate (ROI) production by the infected macrophage [18] Finally, the outer capsule layer of the cell envelope is composed of noncovalently associated lipids, proteins, arabinomannan (AM), lipoarabinomannan (LAM), glucans, and xylan [19].

Associated with the mycolic acids of the cell wall core are various lipids, trehalose-6,6'-dimycolate, sulfolipids, and diacyl trehalose. Many of these molecules were discovered very early on to be virulence factors and antigens of *M. tuberculosis*.

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Trehalose-6,6'-dimycolate or "cord factor" was first described in 1952 by Sorkin as a lipid material responsible for the characteristic cord formation of *M. tuberculosis* observed under the microscope [20]. The antigenic properties [21, 22] and structure of this molecule [23] were characterized soon thereafter. The sulfolipids of mycobacteria are acylated trehalose 2-sulfates which are associated with the mycolic acid monolayer [24-26]. These sulfolipids are characterized as virulence factors because of their disruption of the normal phagocytic functions and activation of macrophages [27, 28]. Sufolipids are also immunogenic as they are recognized by sera from infected patients [29, 30]. Interestingly, only virulent species of mycobacteria, such as *M. tuberculosis, M. leprae* and *M. africanum,* possess sulfolipids in their cell wall [29]. Another molecule associated with the cell envelope that has received considerable attention is lipoarabinomannan (LAM). It is believed that LAM is associated with the PM as well as the capsule layer. The structure of LAM in *M. tuberculosis* is complex but can be divided into several regions: 1) a phosphatidylinositol mannoside bound to palmitic and tuberculostearic acid residues which insert themselves into the PM and mycolic acid portion of the envelope; 2) a mannan core; and 3) branched arabinofuran with mannose caps [31-33]. LAM interacts with numerous facets of the immune response of the host and contributes to protection of the bacillus by inhibiting IFN-γ activation of macrophages [34, 35], the binding of nonopsonized *M. tuberculosis* by macrophages [36], phagosome maturation [37], and protein kinase C [38]. LAM is also implicated in the scavenging of ROI within the infected macrophage [38]. Alternatively, other researchers have demonstrated that LAM is capable of binding the mannose receptors on macrophages thus inducing greater uptake into macrophages [39]. The ability of LAM to

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both inhibit and induce bacterial uptake into macrophages may seem to be contradictory, but it is clear that macrophages are capable of engulfing pathogens in a variety of different ways. Therefore, *M. tuberculosis,* being capable of preferentially directing the manner in which it is engulfed, could ensure its survival by circumventing antimicrobial mechanisms within the host.

In summary, this uncommonly complex cell wall is comprised of elements that are immunostimulatory while simultaneously retaining moieties that protect the pathogen from many conditions considered lethal to other bacterial species, including oxidative stress, desiccation and broad pH ranges. Furthermore, many components of this structure modulate normal immune responses of the host to infection, thus enabling the bacillus to persist and grow remarkably well in what is typically a very hostile environment as it faces the concerted antimicrobial forces of the host.

1.3.1 *The genome and proteome of M. tuberculosis*

The physiology of *M. tuberculosis* is one of the most unique and complex among the prokaryotes. Furthermore, the success of this organism in causing infection in a wide variety of animal species, overcoming innate and acquired immune mechanisms of the host, and being able to persist indefinitely within the host alludes to an extremely adept and responsive pathogen. As such, it was believed that the genome of this organism would reflect this complexity in the number of genes encoded, the variety of biosynthetic pathways represented, and the multitude of virulence factors and immune evasion mechanisms available. In 1998, Cole *et al.* published the complete genome sequence of *M. tuberculosis* and demonstrated that this was in fact true. The genome of *M.*

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tuberculosis comprises 4.4 million base pairs with a 65.6% G + C content, which code for 3,924 open reading frames (ORF) [40], a number that was subsequently increased to 3995 ORF [41]. It is clear from the genome that *M. tuberculosis* is primarily an aerobic organism producing ATP from oxidative phosphorylation and the electron transport chain. Its ability to survive under anaerobic conditions, however, is attested to by the presence of a number of genes involved in nitrate (*nar* family) and nitrite (*nir* family) reduction, taking the place of O_2 reduction to CO_2 when O_2 is not available. Another one of the many interesting findings was that when compared with other prokaryotes, an unusually high number of genes code for lipid synthesis and degradation. In fact, mycobacteria contain genes for many known polyketide and lipid biosynthetic pathways, including enzymes typically found only in plant and mammalian species. This fact helps to explain the vast and complex complement of lipid products found in the mycobacterial cell envelope.

In respect to transcriptional control, the genome of *M. tuberculosis* contains 13 sigma (σ) factors, 190 transcription regulators, 11 complete two-component signaling systems, and 11 protein kinases [40]. In prokaryotes the pentameric RNA polymerase is comprised of β, β', ω and α dimer subunits, and requires the association of a σ factor for efficient promoter recognition and optimal gene transcription. Several of the thirteen σ factors encoded in the *M. tuberculosis* genome, denoted σ^A – σ^M [40], possess specificity to a gene set via recognition of a consensus sequence in the promoter of the gene. While it is generally accepted that each σ factor possesses its own specificity, only six have been characterized thus far in *M. tuberculosis.* These include the primary, essential σ factor, *sigA* responsible for the transcription of all housekeeping genes as well as the non-

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essential *sigA*-like *sigB*, believed to act as a backup mechanism to ensure the transcription of housekeeping genes in the event of stress or inactivation of *sigA*. Another important σ factor is *sigF* ,induced in response to environmental stresses and entry into stationary phase in *M. bovis* and *M. smegmatis* [42] and is essential for full virulence of *M. tuberculosis* in mice [43]. *SigC* is involved in the transcription of at least 38 genes, which encode proteins involved in general metabolic processes as well as response to stress [44]. Another σ factor with a known consensus sequence is *sigE* which is induced during *M. tuberculosis* growth in human macrophages [45]. Finally, the last characterized σ factor to date is *sigH,* which is responsible for the transcription of 39 genes and is induced in response to oxidative stress, heat shock, and macrophage infection [45, 46]. While much work has yet to be done to fully characterize all 13 σ factors of *M. tuberculosis* it is clear that the regulated expression of these σ factors is a major strategy by which mycobacteria can ensure its survival by providing for the global regulation of gene transcription in response to various stimuli and physiological needs.

An additional gene regulation mechanism found in both prokaryotes and eukaryotes, the two-component signaling system, is comprised of a transmembrane histidine kinase protein and a cytoplasmic response regulator. While two-component systems vary, generally the histidine kinase receives an environmental signal that is transmitted via a phosphorelay to the partner response regulator protein located in the cytosol. The phosphorylated response regulator, in turn, directs the transcriptional activation or repression of specific genes. The two-component signal transduction couplets that have been identified in *M. tuberculosis* are DevR/DevS (Rv3133c/Rv3132c), KdpD/KdpE (Rv1028c/Rv1028c), MprA/MprB (Rv0981/Rv0982),

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MtrA/MtrB (Rv3246c/Rv3245c), PhoP/PhoR (Rv0757/Rv0758), PrrA/PrrB (Rv0903c/Rv0902c), SenX3/RegX3 (Rv0490/Rv0491), TrcR/TrcS (Rv1033c/Rv1032c), TcrX/TcrY (Rv3765c/Rv3764c), NarL/NarX (Rv0844c/Rv1736c), and Rv0600c/Rv0601c/TcrA with other sensor histidine kinases (Rv0845, Rv1626, Rv2027c, and Rv3220c) and response regulators (Rv0260c, Rv2884) yet to be paired [40]. Two-component systems play a vital role in the adaptation of bacteria to changing environmental conditions and are pivotal to the ability of a pathogen to infect and persist within host tissues. Several of these couplets have recently been shown to be essential for virulence and persistence of infection in mycobacteria. DevR/DevS is responsible for the hypoxic response of the organism and transcriptional control of α-crystallin [47, 48]. MprA/MprB [49] deletion results in attenuated virulence of BCG *in vivo* [50]. MtrA/MtrB,is induced in BCG upon entry into phagocytes [51]. PhoP/PhoR is essential for intracellular growth in mouse-derived phagocytes [52]. PrrA/PrrB is expressed during growth *in vivo* but not in culture media [45]. SenX3/RegX3 deletion results in slowed growth of the bacilli in both immunocompetent and immunodeficient mice [53]. In summary, a careful analysis of the *M. tuberculosis* genome reveals an extremely complex and highly responsive organism that is superbly equipped to modulate its gene expression profile and provide all the essential components of biosynthesis, degradation, and post-translational or post-synthetic modifications necessary to survive in a multiplicity of environments, including those conditions encountered during intracellular invasion and persistence.

Characterization of the genome of an organism is a tremendous asset toward understanding the life of that organism with all of its physiological aspects, capabilities of

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adaptation and potential responses to environmental pressures, but it is no more nor less than that: a description of its potential. The genome is simply a set of theoretical possibilities, but cannot pinpoint actual states. In an effort to form a more accurate picture of actual events, scientists have long looked at the products of gene transcription including the proteome to understand how an organism interacts with its environment and reacts to nutritional needs and environmental pressures.

Research into the physiology of *M. tuberculosis* is no different. The history of tuberculosis research began with Koch's initial observations of the bacillus [11] and subsequent work to characterize preparations of the organism [54], but biochemical evaluation of specific components of the pathogen were decades away. Nearly a half century after Koch's work, Florence Seibert succeeded in isolating a purified fraction from *M. tuberculosis* cultures which she termed "tuberculin purified protein derivative" (PPD) [55]. Later, building upon her previous labors, she was able to isolate individual proteins (A, B, C, and D) and polysaccharides (I and II) from non-denatured preparations of *in vitro* cultures and perform biochemical evaluation of these substances [Seibert 1949]. Since that time many researchers have worked to characterize the protein and non-protein components of *M. tuberculosis* using a variety of different fractionation, extraction and separation techniques. Of particular and obvious focus in this area has been the identification and characterization of the immuno-relevant mycobacterial components [56-59]. Table 1.1 enumerates the protein antigens of *M. tuberculosis* discovered to date.

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Table 1.1 Protein antigens of *M. tuberculosis*

Gene	Protein Name(s)	Location	Function	Immune Reactivity	Reference(s)
Rv0400c	Mtc28, 28-kDa	CFP	unknown	T cell	[60, 61]
Rv0054	Ssb	cytosol, CFP	DNA binding	T cell	[62]
Rv0125	PepA, MTB32a	CFP	serine protease	T cell	[63]
Rv0129c	Ag85C, FbpC	cell wall, CFP	mycolyl transferase	T cell and sera	[62, 64-68]
Rv0288	CFP-7, 10-kDa	CFP	unknown	T cell	[69, 70]
Rv0350	DnaK, Hsp70?	CFP	heat shock protein, chaperone	T cell and sera	[62, 71]
Rv0440	GroEL2, Hsp65	cytosol, cell wall, CFP	heat shock protein, chaperone	T cell	[62, 72]
Rv0538		cell wall, CFP	PTRP (Pro-Thr Repetative Protein)	sera	[73]
Rv0577		cytosol, CFP	conserved hypothetical	T cell	[62]
Rv0652	RplL	cytosol	50s ribosomal protein	T cell	[62]
Rv0733	Adk	cytosol	adenylate kinase	T cell	[62]
Rv0798c	CFP-29, 29-kDa	cell wall, CFP	unknown	T cell	[74]
Rv0915c	Mtb41, PPE14		unknown	T cell	[75]
Rv0916c	Mtb10	CFP	serine protease	T cell and sera	[75]
Rv0934	PstS1, PhoS1	CFP	phosphate binding protein	T cell and sera	[62, 71, 76-78]
Rv0951	SucC	cytosol	succinyl-CoA synthetase	T cell	[62]
Rv1037c	EsxI, Mtb9.9D	CFP	ESAT-6 like protein	T cell	[79]
Rv1174c	Mtb8.4	CFP	ESAT-6 like protein	T cell and sera	[80-82]

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Rv1196	Mtb39A, PPE18		PPE family protein	T cell	[83]
Rv1198	EsxL, Mtb9.9C	CFP	ESAT-6 like protein	T cell	[79]
Rv1211		cytosol	conserved hypothetical	T cell	[62]
Rv1240	Mdh	cytosol	malate dehydrogenase	T cell	[62]
Rv1287	CFP-17	CFP	conserved hypothetical	T cell	[84]
Rv1361c	Mtb39B, PPE19		PPE family protein	T cell	[83]
Rv1352		CFP	conserved hypothetical	T cell	[62]
Rv1626		cytosol	2-component response regulator	T cell	[62]
Rv1793	Mtb9.9A, EsxN	CFP	ESAT-6 like protein	T cell	[79]
Rv1810		CFP	conserved hypothetical	T cell	[62]
Rv1818c			PE-PGRS family protein	T cell and sera	[85]
Rv1827		cytosol, CFP	conserved hypothetical	T cell	[62]
Rv1837c	GlcB	CFP	malate synthase	sera	[86]
Rv1860	ModD, MPT32, Apa	CFP	putative molybdenum uptake protein	T cell	[62, 87-89]
Rv1886c	Ag85B, FbpB	cell wall, CFP	mycolyl transferase	T cell and sera	[62, 64-68]
Rv1908c	KatG	cytosol, CFP	catalase/peroxidase	sera	
Rv1926c	MPT63, MPB63	CFP	unknown	T cell	[61, 66, 67]
Rv1932	Tpx	cytosol, CFP	thiol peroxidase	T cell	[62, 90]
Rv1980c	MPT64, MPB64	CFP	unknown	T cell	[62, 66, 91]
Rv1984c	CFP-21	CFP	cutinase	sera	[84]
Rv2031c	Acr, HspX, α-crystallin	cytosol, CFP	heat shock protein	T cell and sera	[62, 92, 93]
Rv2346c	EsxO, Mtb9.9E	CFP	ESAT-6 family of proteins	T cell	[79]
Rv2376c	Mtb12, CFP-2	CFP	unknown	T cell	[94]
Rv2428	AhpC2	cytosol	alkylhydrogenase reductase	T cell	[62]
Rv2461c	ClpP	cytosol	protease subunit	T cell	[62]

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Rv2626c		cytosol	conserved hypothetical	T cell	[62]
Rv2780	Ald	cytosol	alanine dehydrogenase		[95, 96]
Rv2827c	DsbE, MPT53	CFP	unknown	sera	[97]
Rv3004	CFP-6	CFP	unknown	T cell	[98]
Rv3017c	EsxQ	CFP	ESAT-6 family of proteins	T cell	[69]
Rv3019c	EsxR	CFP	ESAT-6 family of proteins	T cell	[69]
Rv3028c	FixB	cytosol	electron transfer flavoprotein α-subunit	T cell	[62]
Rv3029c	FixA	cytosol	electron transfer flavoprotein β-subunit	T cell	[62]
Rv3044	FecB	cell wall, CFP	$Fe3^{+}$ dicitrate transport	T cell	[62]
Rv3246c	MtrA	cytosol	2-component response regulator	sera	[73]
Rv3367		cell wall, CFP	PE-PGRS family protein	sera	[73]
Rv3417c	GroEL, Hsp60	cytosol	heat shock protein	T cell and sera	[71, 99]
Rv3418c	GroES, MPT57	cytosol	heat shock protein	T cell and sera	[62, 71, 90, 100, 101]
Rv3478	Mtb39c, PPE60		PE family protein	T cell	[83]
Rv3596c	ClpC	cytosol	protease subunit	sera	[102]
Rv3597c	Lsr2		unknown – Fe regulated	T cell and sera	[103-105]
Rv3619c	EsxV, Mtb9.9D	CFP	ESAT-6 family protein	T cell	[79]
Rv3763	LpqH, 19-kDa lipoprotein	cell wall	lipoprotein	sera, neutrophil activation, IFN-γ inhibition	[102, 106-108]
Rv3803c	MPT51, FbpC1	cell wall, CFP	mycolyl transferase precursor		[66, 109, 110]

Rv3804c	Ag85A, FbpA	cell wall, CFP	mycolyl transferase	T cell and sera	[62, 64-68]
Rv3810	Erp, PirG	cell wall, CFP	unknown	sera	[73, 111]
Rv3841	BfrB	CFP	bacterioferritin	T cell	[62]
Rv3846	SodA	cytosol, CFP	superoxide dismutase	sera	[67, 102, 112, 113]
Rv3874	CFP-10	CFP	conserved hypothetical	T cell	[60, 114, 115]
Rv3875	ESAT-6	CFP	early secreted antigen target	T cell	[62, 114, 116]

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1.3.2 *In vivo veritas*

While evaluation of individual mycobacterial components is a necessary step to full understanding of the pathogenesis and immunity of disease, physiological processes must be evaluated in light of the host environment during infection. It was demonstrated early that metabolic, pathogenic and immunogenic differences exist between bacilli grown *in vitro* (H37Rv) and bacilli passaged through a host (LRv) [117-119]. One potential explanation is the observation that *in vivo* and *in vitro* bacilli exhibit a different lipid profile, specifically the lack of tuberculostearic acid, phthioic acid and phthiocol and decreased sulfolipids in the LRv organisms [120-122]. The complexity of this issue is highlighted in the paradoxical observation that while H37Rv confers greater protection than LRv when used as a vaccine, LRv-infected mice succumb to infection twice as quickly as H37Rv-infected mice [118]. This may be due to the time requirement for an infection to produce a strong memory response, with LRv infection progressing too quickly and precluding effective generation of a memory population. Recognizing the significance of this observed dichotomy, researchers have proceeded to characterize protein expression and gene transcription patterns of bacilli during intracellular infection and *in vivo*-mimicking conditions compared to flask-grown cultures. Early Western blot experiments used hemagglutination by rabbit anti-sera as a method to evaluate different fractionations of mycobacteria cultures that are recognized during infection [123, 124]. Gradually, individual protein and non-protein antigens were purified and evaluated in much the same manner. Through these efforts a number of potent immune modulators

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and antigens were discovered and shown to be present during actual infection, including "cord factor" [20], LAM [34, 38, 125, 126], α-crystallin [127-129], and the Antigen 85 complex of proteins [73, 130, 131]. More recently, broad-spectrum analysis has been performed on whole cell fractions and complex mixtures of mycobacterial products to assay pathogenic relevance. These analyses provided many advances, including the discovery of 19 novel T cell antigens in mouse and human infection, the identification of over 30 sera-reactive proteins, and the development of rapid and simple diagnostic and analytical methods for determining biologically important proteins [62, 88, 132, 133].

1.4 *Pressures of in vivo survival*

Differences between *in vivo* and *in vitro* physiology are surely the result of numerous pressures and influences on the organism. Upon invasion into the host, the bacilli is confronted with numerous stress conditions and environmental changes to which it must adapt. First, as the bacillus is inhaled into the lungs of the host, it comes in contact with the alveolar surfactant lipoprotein complex comprised mostly of phosphatidylcholines and multifunctional proteins [134]. In addition to the detergent activity of these phospholipids that could potentially strip the bacillus of protective envelope lipids, surfactant proteins A and D (SP-A, SP-D) possess antimicrobial characteristics. SP-A and SP-D possess high affinity for lipids, in which the mycobacterial cell wall is rich, but exhibit opposite functional consequences on the phagocytosis of *M. tuberculosis* by alveolar macrophages. SP-D has been specifically shown to bind the mannosyl residues of LAM with a resulting increase in bacterial agglutination and decrease in macrophage phagocytosis [135, 136], while SP-A mediates

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the increased uptake of mycobacteria through up-regulated mannose receptor activity on the macrophage surface [137-139]. The fact that SP-A is 10 times more abundant than SP-D in the lungs and has greater phospholipid affinity alludes to an immuno-stimulatory effect of surfactant during *M. tuberculosis* infection rather than any significant SP-D immunosuppressive contribution [140].

The next stress encountered by the organism is that of toxic compounds and proteins, ROI, and acidification of the phagosome within the macrophage. Several molecules shown to have anti-mycobacterial activity are free fatty acids, such as oleic, linoleic, and arachidonic acid, produced by activated macrophages [141-143]. In addition, several proteins present within the phagosome of the macrophage have demonstrable antimicrobial traits [144]. Of these, a cytolytic protein present in CTLs and NK cells, granulysin, is implicated in mycobacterial killing in humans, though is not existent in mice [145, 146]. Granulysin was first shown to exhibit anti-microbial effects against bacteria, fungi and parasites by Stenger in 1998 [147]. With specific relevance to mycobacterial infection, granulysin was shown to kill tubercle bacilli directly, and in concert with perforin, demonstrated the ability to kill intracellular mycobacteria [147]. Other powerful bactericidal mechanisms employed by macrophages are the production of ROI and reactive nitrogen intermediates (RNI). ROI activity includes the production of peroxide (H_2O_2) and other oxygen radicals. While having been shown to be lethal to *M. microti* [148] the effective role of ROI in the killing of *M. tuberculosis* and control of infection is a matter of debate [149, 150]. Mycobacteria are well equipped to counter ROI compounds by the production of superoxide dismutase (SodA), which converts O_3^- to H_2O_2, and catalase-peroxidase (KatG), converting H_2O_2 to H_2O and O_2 [151].

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Consequently, it is not surprising that ROI production is insufficient to control the growth of mycobacteria within infected macrophages. On the other hand, RNI with the subsequent production of nitric oxide, has been demonstrated to play a significant role in the activity of macrophages against mycobacterial infection in both mice [150, 152-154] and humans [155, 156], though the latter is a matter of some debate [157]. While it has been demonstrated that high levels of iNOS are induced in patients with progressive tuberculosis [155, 156], the protective effects of this induction have not been proven.

Yet another weapon of the macrophage against infection is the acidification of the phagosome by lysosomal fusion and the incorporation of proton ATPases in the membrane of the endosome [158]. While *M. tuberculosis* has developed ways of arresting the maturation of the endosome, the inhibition of this mechanism is incomplete, as the endosomal pH does drop to 6.3-6.5 [158, 159]. Nevertheless, it is clear that upon engulfment of the bacilli by the macrophage and subsequent initiation of acquired immunity with the induction of antimicrobial measures, *M. tuberculosis* is bombarded with a host of compounds and mechanisms meant to destroy the organism. As such, the successful survival of the pathogen to this attack demands the ability to adapt to and withstand an extremely hostile environment.

If the *Mycobacterium* survives this multifaceted attack, the pathogen then receives the "silent treatment". The characteristic granulomatous formation at the nucleus of infectious foci is an attempt to sequester the pathogen, limit damage, and ensure against invasion into surrounding vessels and tissues. Starving a pathogen of vital nutrients such as iron and essential salts is the body's answer to a variety of infections. In fact, the anemia often observed in people during illness is the result of a spike in the production of

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ferritin and other chelators as an attempt by the host to bind up and sequenster soluble iron concentrations [160]. The host response to *M. tuberculosis* infection follows this pattern, as well [161]. Even before it became known that "consumption" was caused by a microbe, the importance of iron starvation to the control of tuberculosis was recognized. During a medical symposium in 1872, one physician emphasized that iron should be restricted in the diets of tuberculosis patients because it was observed that the common practice of prescribing whole animal blood tonics was detrimental to the outcome of disease because of the large concentration of readily available iron [162]. The prognosis for TB patients immediately improved once this recommendation was followed.

Perhaps the most important nutrient restricted from the pathogen within the granuloma is oxygen. It has been hypothesized that the dense structural architecture of the granuloma serves to limit oxygen diffusion into its core where *M. tuberculosis*-infected macrophages reside [163]. While the evidence is largely circumstantial in this regard, both the observed physiological shutdown of the bacilli and the compact construction of the granuloma support this idea. On the cellular level, however, empirical evidence does exist; using electron paramagnetic resonance, an oxygen-sensitive probe delivered to phagocytes demonstrates that the phagosomes of activated macrophages have a measurably lower oxygen concentration than those of resting macrophages [164]. Furthermore, strong evidence for the existence of hypoxic conditions within the host come from the multitude of genes that are transcribed in response to hypoxia *in vitro* that are essential for growth *in vivo*, especially during persistent infection [48, 49, 128, 165-172], not the least of which are genes necessary for the glyoxylate shunt pathway [167, 168]. In summary, from the first interaction between the mycobacterial bacillus and the

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host at the alveolar surfactant layer to the final state of hypoxia and nutrient starvation, the pathogen is exposed to sub optimal environmental conditions and aggressive antimicrobial attack, requiring a legion of adaptive mechanisms and alternative survival factors in order for mycobacteria to survive and persist.

1.5 *Protein secretion in prokaryotes*

The ability of an organism to interact with its environment is crucial for the survival of the organism. Moreover, direct interaction between a pathogen or its constituents with the host determines the character of the immune response against that pathogen, as well as the ultimate outcome of disease. While all components of a bacterial cell are potential immune determinates, those factors that are exported or present on the surface of the pathogen are uniquely positioned to direct pathogenesis and immunity. One of the most simple and direct ways the pathogen can actively influence its environment is for the bacterium to insert specific molecules into its cell wall or secrete them into its immediate surroundings. The remarkable aspect of this process is that many of the molecules exported are largely hydrophilic in nature and must overcome the hydrophobic barrier in the cell wall and membrane in order for translocation to occur. Therefore, bacterial species have developed various mechanisms of exporting gene products into the environment. These mechanisms are best described in the Gram negative prokaryotes and are termed Type I-IV protein secretion [173]. Type I protein secretion is characterized by a trans-envelope structure that spans from the cytoplasm of the cell to the exterior environment allowing the secretion of the protein directly to the outside of the cell [173]. Type II protein secretion is characterized by the shuttling of a

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signal sequence-containing pre-protein to a plasma membrane embedded complex that exports the protein, cleaves the signal sequence, and delivers the protein into the periplasm of the cell envelope [174]. Type III secretion, by contrast, delivers proteins from the bacterial cytoplasm directly into the cytoplasm of host cells, and employs numerous accessory proteins to form this intercellular bridge through which these proteins are delivered [175]. Finally, type IV secretion is known as "self-directed" secretion since the C-terminal region of the nascent protein itself forms a pore through the outer membrane through which the protein is exported [176]. As these methods of secretion are represented in Gram negative prokaryotes, there is no evidence for these in mycobacteria. However, other secretion mechanisms have been discovered in Gram positive bacteria [177, 178]. It would be expected that in mycobacteria, where the envelope of the bacillus presents a very unique structural hurdle for protein secretion, multiple mechanisms would be utilized to accomplish this essential function. Furthermore, as an intracellular bacterium, *M. tuberculosis* is uniquely dependent upon the ability to affect its environment and to influence the response of the host it infects in order to survive. Protein secretion mechanisms, therefore, are key to the progression of tuberculosis as well as the host immune response to infection.

1.5.1 *Sec-dependant protein translocation*

In 1975, Gunter Blobel proposed that various proteins slated for transport out of eukaryotic cells are endowed with an amino acid sequence that is hydrophobic in nature allowing for anchoring in the cell membrane [179, 180]. This cleavable signal sequence is synthesized at the amino terminus of the pre-protein and is the portion of the gene

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product that is recognized by the translocation components of the cell and allows for the eventual transport of the protein across the hydrophobic membrane. The chaperone SecB, has been described in many bacterial systems and serves as the first contact of the pre-protein with the secretion apparatus [181]. This chaperone binds the pre-protein as it is being translated at the ribosome where it prevents translocation-inhibiting folding of the pre-protein and protein aggregation. In addition, SecB serves to convey the nascent protein to the SecA homodimer bound in the plasma membrane [181].

SecA is an ATPase that can reside as either a cytosolic or membrane-bound protein which, along with binding to the pre-protein and SecB, also binds to the phospholipid bilayer of the cell membrane as well as the translocase molecules, SecG, SecY and SecE. After the dissociation of the pre-protein with SecB, translocation occurs in an ATP-dependant manner as SecA co-inserts with the pre-protein through the translocation channel, SecYEG. Upon insertion into the membrane, SecA dissociates from the polypeptide and the translocase, but leaves the protein chain in the membrane [174]. After withdrawal from the membrane, SecA once again binds the protein chain and through multiple rounds of ATP hydrolysis, translocation of the polypeptide is accomplished. During this action, the signal sequence is undergoing cleavage by one of two signal peptidases on the periplasmic side of the cell membrane, depending on the ultimate fate of the protein. LepB is the signal peptidase that has been characterized for cleavage of signal sequences associated with proteins bound for the extracellular milieu or to be inserted into the cell wall of the bacteria [182], while LspA cleaves signal sequences linked to future lipoproteins that will be inserted into the periplasmic side of the inner and outer cell membranes [183, 184].

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The characteristics of the signal sequence itself are essential for this interaction to occur. The standard signal sequence is composed of a positively charged N-terminus of roughly 10 amino acids with a hydrophobic stretch of 15-20 amino acids followed by a cleavage site characterized typically by an Ala-X-Ala motif. The lipoprotein signal sequence expectedly differs only in the cleavage site, which carries a large, hydrophobic amino acid at site –3 and a cysteine residue in the +1 position, which cysteine becomes acylated prior to the cleavage of the signal sequence [182, 185].

The above pathway is the most well understood method of protein export in mycobacterial species to date. A few differences have been observed, however, between the general motif of sec-dependent protein secretion and that found in mycobacteria. First, characteristic of other gram-positive bacterial species, a *secB* homologue is absent from the genome of *M. tuberculosis* [40], which, in itself, is not of great concern since the genome of *M. tuberculosis* encodes another chaperone, Rv2916c (*ffh,* fifty-four homolgue signal recognition particle), which binds nascent proteins as they emerge from the ribosome and acts in concert with Rv2921c (*ftsY,* signal recognition particle receptor) to target proteins to the membrane. Additionally, SecA can function as a soluble, cytosolic chaperone itself [186]. The second, more interesting distinction from the model was in the discovery of a second homologue to SecA [40], termed called SecA2, that has been observed in multiple mycobacterial species and subsequently identified in other Gram positive bacterial species, as well.

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1.5.2 *Sec-independent protein translocation*

Another mechanism of protein secretion described for bacterial systems is known as the twin arginine translocase, or Tat. In *E. coli,* Tat has recently been shown to translocate specialized proteins involved in periplasmic biosynthesis and cell division across the cell membrane [187-190]. Although this pathway does not utilize the Sec machinery, it does require an N-terminal signal sequence motif of S/T-R-R-X-F-L-K containing the R-R amino acid cleavage site in the pre-protein [191]. This precursor protein, in the majority of cases, is seen to bind cofactors and fold or oligomerize before translocation occurs [191]. This differs from Sec-dependent translocation where only non-aggregated, unfolded proteins can be exported as they are threaded through the SecYEG translocase one section of the amino acid sequence at a time. It has been suggested that Tat translocation might occur by the formation of a large pore in the cytoplasmic membrane being formed by members of the Tat pathway. Pore formation in *M. tuberculosis* has recently been observed and research is ongoing to determine the association of this phenomenon with the observation that many extracellular proteins of *M. tuberculosis* are extremely large and multidimeric [192, 193]. While no Tat-dependent substrates have been observed in mycobacteria as yet, the discovery of genes coding for fundamental Tat components in *M. tuberculosis* [40] corroborates the existence of this translocation system in mycobacteria and ensures future identification of Tat-translocated proteins.

One area of particular focus in the field of mycobacterial pathogenesis is the significance of genes located within a chromosomal region deleted in every strain of BCG yet present in all virulent strains of *M. tuberculosis* [194, 195]. This 9.5 kb region

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is known as region of difference 1 (RD1) and contains nine open reading frames, Rv3871-Rv3879c. The role that these genes play in virulence is confirmed by the fact that deletion of this region alone from *M. tuberculosis* results in an attenuated phenotype identical to BCG. Two of the gene products within this region, Rv3875 (ESAT-6) and Rv3874 (CFP-10), are powerful T cell antigens and are recognized by patient sera [196-198]. Of particular interest regarding protein secretion is the fact that these two proteins are observed in culture supernatant in the absence of cell lysis [199], yet contain no known amino acid signal sequence by which it would be recognized by classical secretion mechanisms for translocation [40]. Recent work using transposon mutagenesis has demonstrated that other proteins located in the RD1 region (Rv3871, Rv3876, Rv3878, and Rv3879c) are essential for the secretion of these antigens [199-202]. Rv3870, a likely ATP-dependent chaperon located just upstream of the 5' end of the RD1 region, is also necessary for secretion. Based upon biochemical motifs Rv3866 and Rv3871 are putative integral membrane proteins. Another neighboring gene outside of the RD1 region (corresponding to Rv3881c in *M. tuberculosis*) also plays an essential role in secretion of ESAT-6 and CFP-10 from *M. marinum* [199]. Rv3877 is another putative integral membrane protein within the RD1 region but its role in protein secretion has not been evaluated. Taken together, the gene products of RD1 likely form a multimeric secretion system to tranlocate the antigenic ESAT-6 and CFP-10, although the precise mechanisms by which this translocation occurs in the absence of a signal sequence motif present on the secreted proteins has yet to be elucidated.

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1.6 *Immunology of tuberculosis*

1.6.1 *The macrophage*

The macrophage is a critical determinant of the host response to infection by *M. tuberculosis* and the success of this pathogen to cause disease is characterized by its ability to survive and multiply within the phagosome of resting alveolar macrophages. Activated macrophages, however, present a far more hostile environment and are capable of killing the organism and controlling infection [149, 203, 204]. The characteristics, therefore, of resting versus activated macrophages and the mechanisms by which each state is defined and influenced by infection is central to the progression of disease and the prognosis to the host.

Tuberculosis is typically transmitted by the expulsion of an aerosol droplet containing one or two bacilli from an infected host and inhaled by a new host [205]. Once the bacilli reach the alveolar space phagocytosis by resident macrophages may occur through a number of different pathways, including complement receptor binding, mannose receptor binding, Fcγ receptor binding by opsonized bacteria, surfactant protein A receptor binding [139], and class A scavenger receptor binding by mycobacterial sulfolipids [206].

Typically, upon ingestion of a bacterium, the pathogen-containing vacuole undergoes acidification by acquiring proton ATPase complexes in the membrane of the phagosome [158]. This is followed by fusion of the phagosome with lysosomes. The lysosome vesicles contain high concentrations of hydrolytic enzymes, ROI and H_2O_2, in an acidic environment, which are extremely toxic to the pathogen. This process is

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precedent to antigen processing and presentation of bacterial peptides in the context of MHC class I and II molecules. These bacterial antigen:MHC complexes are recognized by antigen-specific $CD8^+$ and $CD4^+$ T cells resulting in the stimulation of effector functions within the T cells as well as the presenting macrophage. Arguably the most important effector function of $CD4^+$ T cells is the production of IFN-γ [207]. The secretion of this molecule by $CD8^+$ T cells also contributes to the antimicrobial functions of macrophages [208-211]. One of the powerful effects of this molecule is the induction of the macrophage to produce RNI, such as nitric oxide (NO), and higher concentrations of ROI [150, 212, 213].

When pathogenic mycobacteria are inhaled, however, an alternate series of events transpire. After activation of the alternative complement pathway, *M. tuberculosis* can be opsonized by C3b and iC3b followed by phagocytosis via interaction with complement receptors CR1, CR3, and CR4 with 80% of complement-mediated engulfment occurring through CR3 [39]. Usually, when CR3-mediated phagocytosis of an organism occurs, signaling pathways are activated that result in phagosome-lysosome fusion and enhanced killing of the engulfed bacterium. Phagocytosis of live mycobacteria, however, results in the inhibition of cytosolic [Ca^{2+}] increase, which is essential for calmodulin activation and calmodulin-dependent protein kinase II [214, 215]. Since phagosome-lysosome fusion is dependent upon the binding of endosome-associated calmodulin with cytosolic Ca^{2+} [215] the ability of live mycobacteria to inhibit the increase of cytosolic [Ca^{2+}] is an effective defense against a powerful antimicrobial mechanism of the macrophage.

In addition to opsonic-mediated phagocytosis through CR3, mycobacteria can also bind to an alternate domain on CR3 leading to uptake of non-opsonized bacilli into

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the macrophage [216]. Interestingly, internalization of non-opsonized mycobacteria is observed only in pathogenic species, such as *M. tuberculosis, M. avium*, and *M. kansasii* while the non-pathogenic *M. smegmatis* does not exhibit this characteristic [216, 217]. It has been hypothesized that this clandestine mode of entry allows the bacillus to circumvent normal trigger mechanisms in the macrophage that would lead to superoxide production and bacterial lysis [218].

Another mode of bacterial recognition utilized by the macrophage is the expression of mannose receptors (MMR) on its surface. Mannose is a six-carbon sugar that, while present in mammalian species [219], is far more plentiful in the envelope many prokaryotes. *M. tuberculosis,* as well as other pathogenic mycobacteria, are covered with mannose-capped LAM which bind the MMR leading to engulfment of the bacterium [220]. In fact, this phagocytic pathway participates in the engulfment of a significant proportion of invading mycobacteria as demonstrated by a nearly 50% decrease in engulfment when *M. tuberculosis* is incubated with anti-ManLAM antibody [221].

Once within the macrophage, however, not all of the bactericidal effects of RNIs are neutralized by the multitude of *M. tuberculosis* defense mechanisms, as evidenced by the fact that mice lacking NOS2 activity exhibit increased susceptibility to acute and chronic mycobacterial infection [154], but the bacilli has demonstrated effective mechanisms to overcome, at least to an extent, their lethal effects. This is confirmed by transposon mutagenesis research identifying multiple genes and pathways essential for *M. tuberculosis* to overcome the effects of NO [222]. In other words, both the NOS2 activity of the host and the protective antioxidative effects of mycobacterial gene

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products are required for the observed standoff between host and pathogen during the infection process. If either side is inhibited in its abilities in these regards the balance immediately shifts and overwhelming infection on one hand or clearance of the bacilli on the other is the result.

In summary, invasion of the macrophage is a critical process in the establishment of infection by *M. tuberculosis*, and the multiple methods by which bacilli uptake occurs and is encouraged attests to the pathogen's prurient intracellular habitation. As such, once within the macrophage, the bacillus has a large array of weapons to subvert normal operations of the phagocyte and control its environment, thus ensuring its survival in an extremely privileged site.

1.6.2 *$CD4^+$ T cells*

The central dogma of immunity to *M. tuberculosis* infection is that $CD4^+$ T cells recognize MHC class II-presented bacterial antigen, which secrete IFN-γ to activate infected macrophages. In turn, these activated macrophages become increasingly hostile to phagocytosed bacteria because of the release of ROI and RNI into the phagosomes. Additionally, activated macrophages secrete chemokines and cytokines that recruit and direct immune cells in the formation of the classical granuloma observed in tuberculosis patients [223, 224]. While research into tuberculosis immunity commenced immediately upon discovery of the bacillus the road to this understanding has taken the better part of a century.

Shortly following Koch's discovery of the *M. tuberculosis* bacillus, it was demonstrated that resistance to infection by this pathogen could not be conferred through

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sera from infected individuals [225]. Over the past century, the precise protective contribution of various components of the immune system has been a major focus of research around the world. During the middle of the 20th century, work by Suter and Mackaness demonstrated that, although antibodies could not confer resistance to disease, the acquired immune response is essential for protection [226-228]. A short time later, concurrent research in two labs demonstrated that thymus-derived lymphocytes (T cells) mediated protection to *M. tuberculosis* infection [229, 230]. In support of these findings it was subsequently revealed that the protection observed from the BCG vaccine was mediated by T lymphocytes [231]. These findings were confirmed by Orme and Collins when they demonstrated that protective immunity to subsequent infection could be conferred by adoptive transfer of T cells into sub-lethally irradiated mice [232].

While it was known that resistance was mediated by lymphocytes, the precise population responsible for protection was not known until 1987 when Orme demonstrated by adoptive transfer of purified T cell subsets from infected mice that $CD4^+$ ($L3T4^+$) cells were able to confer protection early in infection. This protection was greatest from mice infected for 20-30 days and dropped off significantly after 60 days. Alternatively, $CD8^+$ (Lyt-2+) cells appeared to offer significantly less protection throughout when compared to $CD4^+$ T cells, and occurred later in the infection [224]. Recent work implicates the preeminent role of $CD4^+$ T cells in protection by demonstrating that CD4 knockout (KO) mice succumb to infection at a time corresponding to the initiation of acquired immunity [209].

The activation and expansion of T lymphocytes requires that mycobacterial antigen be presented to the T cell receptor (TCR) of naïve T cells in the context of MHC

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class II:peptide complexes on professional antigen presenting cells (APCs). Whether the interaction between APC and T cell occurs at the site of initial bacterial engulfment or at other sites in the body is a matter of current research and speculation. On this topic, it has been demonstrated that dendritic cells isolated from the lungs of mice were capable of bacterial phagocytosis and secretion of IL-12, resulting in the activation of $CD4^+$ T cells to secrete IFN-γ [233]. Furthermore, since a favorable outcome to infection requires the fast turnover of dendritic cells at the site of infection and rapid migration to the draining lymph nodes of the lung where antigen presentation may occur [234], it becomes clear that dendritic cells within the lung engulf invading *M. tuberculosis*, migrate to the lymph nodes and prime naïve T cells resulting in a circulating population of activated, antigen-specific lymphocytes, which then migrate back to the site of initial infection as effector cells.

1.6.3 *$CD8^+$ T cells*

$CD8^+$ T cells possess a number of mechanisms by which they can contribute to a favorable immune response during infection. In addition to being major producers of the synergistic, inflammatory cytokines IFN-γ and TNF-α, $CD8^+$ T cells can mediate the killing of target cells through Fas/Fas ligand binding, perforin-mediated introduction of granzyme molecules, and direct microbicidal action of granulysin. However, the precise role of this subset in protection against *M. tuberculosis* infection is an area of intense research and debate. One reason for the disagreement among researchers is the method by which $CD8^+$ cells recognize antigen. $CD4^+$ T cells recognize antigen in the context of MHC class II molecules on the surface of APCs. This antigen becomes associated with

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the MHC class II molecule in a vacuole that is part of the endocytic pathway. Alternatively, $CD8^+$ T cells recognize antigen that is presented on MHC class I molecules [235]. These molecules are loaded with peptides from cytosolic proteins that have been processed by the proteasome and translocated into the endoplasmic reticulum by tranporters associated with antigen processing (TAP). Common class I-associated antigens include tumor or viral proteins produced within the nucleus or cytoplasm of the cell [235]. As *M. tuberculosis* is a phagosomal-dwelling bacterium it is expected that antigen presented to lymphocytes would be presented in the context of MHC class II molecules exclusively.

In opposition to this assumption, early research demonstrated that adoptive transfer of $CD8^+$ T cells into lymphocyte-depleted mice conferred protection against subsequent infection, but the level of protection was not as high as that observed from $CD4^+$ T cells [236]. In addition, antibody-mediated depletion of $CD8^+$ T cells illustrated that this population contributed to protection, as well [237]. The cytolytic function of $CD8^+$ T cells was also shown to be important in studies involving specific immunogenic antigens like the 38kDa glycoprotein [238], 19 kDa lipoprotein [239] and ESAT-6 [211]. Further studies showed that $CD8^+$ T cells can recognize mycobacterial antigens presented in class I MHC molecules [210]. While these experiments present evidence for the involvement of $CD8^+$ T cells in the immune response to tuberculosis, the manner in which these cells act is far less cut and dried.

A number of genetically modified strains of mice have recently been utilized to identify relative contributions of MHC class I-dependant cell populations and effector mechanisms. These strains include β_2-microglobulin deficient (β_2m-/-), TAP1-/-, CD8α-

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that the depletion of $CD8^+$ T cells during the chronic stage of infection resulted in reactivation of latent tuberculosis in mice, giving further evidence that this T cell subset is essential for control of latent or persistent infection [242]. While it is difficult to pinpoint the exact mechanism of defense utilized by the immune response in such a dynamic and complex system as *M. tuberculosis* infection, it is tempting to speculate that the contribution of perforin activity exclusively in late, but not early, stages of infection could account for their role in the immune response during chronic infection [243-245].

1.6.4 *Cytokines*

Of critical importance to the control of tuberculosis are the secretion of cytokines and chemokines by immune cells. The list of these effector molecules and their interconnected functions is lengthy and continues to grow, but there are three key cytokines that are critical to an effective host response to infection: IL-12, IFN-γ, and TNF-α.

IL-12 is a heterodimeric molecule, composed of two subunits (p35 and p40) and is produced primarily by macrophages and dendritic cells in response to infection [246]. In the context of *M. tuberculosis* infection, the primary activity of IL-12 is the stimulation of NK and T lymphocytes [247, 248]. In this manner, IL-12 functions to coordinate cytolytic and effector activities of both the innate and acquired immune response. In regard to the latter, IFN-γ production is induced in T cells in response to IL-12 alone in activated T cells or in concert with accessory cells in naïve T cells [249, 250]. The essential nature of this cytokine was demonstrated by the extreme susceptibility of IL-12 KO mice to intravenous infection with *M. tuberculosis*, in which uncontrolled bacterial

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growth and lack of IFN-γ production were hallmarks [251]. Interestingly, the deletion of p40 had a more detrimental effect upon the outcome of disease than did the deletion of p35, suggesting that the p40 subunit of IL-12 may function other than as part of IL-12. A newly discovered cytokine, IL-23, which is composed of p40 and a p19 subunit has been implicated in the protective response to tuberculosis [252], since the p19 subunit is induced in host tissues during tuberculosis infection. IL-12 functions by binding to IL-12R molecules on the surface of lymphocytes resulting in the production of IFN-γ [247] and it is presumably the interruption of this function that accounts for the high mortality observed in IL-12 KO mice. Interestingly, exogenous IL-12 given concurrent to intravenous mycobacterial infection results in the retarded progression of pathology and a lower bacterial burden in the host, even when the infection occurred in an animal strain that is typically extremely sensitive to infection [253]. These data underscore the pivotal nature of this cytokine during the course of infection with *M. tuberculosis.*

IFN-γ is a homodimeric protein produced by NK and T lymphocytes and is considered the most critical cytokine in the protective immune response to mycobacterial infection. This molecule has a multiplicity of biological activities critical to the effective functioning of an antimicrobial response. IFN-γ enhances presentation of peptide antigens on APC-bound MHC class I and class II molecules to antigen-specific $CD8^+$ and $CD4^+$ T lymphocytes, respectively [254]. In addition, IFN-γ induces the production of protective RNI by infected macrophages [203, 255]. The contribution of RNI to the control of mycobacterial infection is crucial to inhibiting mycobacterial growth [203]. The most convincing indication that this cytokine is indispensable is seen in the infection of IFN-γ-KO (GKO) mice with mycobacteria. These mice exhibit uncontrolled bacterial

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growth, increased pathology, and rapid mortality when infected by the aerosol or intravenous routes [153, 256]. Perturbation of other cytokines that affect the production of IFN-γ (i.e. IL-18 and IL-12) manifest similar phenotypes of disease progression as the GKO animals [257]. Taken together, this evidence supports the conclusion that IFN-γ is absolutely essential for a protective immune response to mycobacterial infection.

TNF-α is produced primarily by macrophages and acts in an autocrine fashion to stimulate the Th1 immune response and heighten bactericidal functions. This cytokine plays an important role in the trafficking and migration of cells, including neutrophils [258], lymphocytes, and monocytes [259]. Further, TNF-α is significant in respect to tuberculosis, because of its role in granuloma formation [260, 261]. The utilization of TNF-KO mice, TNF receptor deficient mice, and TNF neutralization immunotherapy techniques have established the importance of this cytokine. Upon infection with BCG [262] and *M. tuberculosis* [263], TNF-depleted and TNFRI mice, respectively, exhibited a marked inability to control bacterial growth, decreased granuloma development, and earlier mortality. The granuloma formation observed in TNF-KO mice challenged with *M. tuberculosis* exhibited necrotic central regions marked by early infiltration of neutrophils with few macrophages and no lymphocytes [264]. Interestingly, while it is clear that TNF operates in the early activation of the immune response and initial establishment of a functional granuloma, it is also required for control of a persistent infection. Research has shown that depletion of TNF during the chronic stage of infection results in the rapid onset of destructive pathology and breakdown of granulomatous integrity within the lung [265].

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IL-10 is produced by both macrophages and lymphocytes and is generally considered to have anti-inflammatory functions. During infection with *M. tuberculosis,* the production of this cytokine results in the down-regulation of IL-12 production by macrophages followed by a subsequent decrease in the levels of IFN-γ [266-269]. IL-10 directly inhibits $CD4^+$ lymphocyte functions as well as limiting APC activity of infected macrophages [270]. As further evidence to the role of this cytokine to inhibit antimycobacterial activities, mice that constitutively express IL-10 showed an inability to control BCG infection [271] and in another experiment were seen to reactivate latent infection accompanied by decreased IFN-γ production [272].

IFN-α is a mixture of subtype interferons that are produced by both lymphocytes and monocytes/macrophages in response to a variety of stimuli. IFN-α is a potent stimulator of the maturation of monocytes into DCs [273]. After infection with mycobacteria, both macrophages and DCs produce high levels of IFN-α [274, 275]. Interestingly, however, infection with *M. tuberculosis* inhibits the effects of this cytokine and skews the maturation of monocytes into the macrophage phenotype [276].

TGF-β is another cytokine considered to by immunosuppressive during infection with *M. tuberculosis.* This cytokine is produced by monocytes and inhibits IFN-γ–induced NOS2 induction [277]. In addition, TGF-β inhibits the T cell activation response to infection [266, 278]. In combination with IL-10, the effects of this cytokine have been linked to the ability of tuberculosis to reactive in infected hosts [266].

IL-6 is a pleiotropic molecule with both pro- and anti-inflammatory capabilities depending upon the immediate environment and type of cells producing the cytokine as well as those affected by it [279, 280]. Although IL-6 is produced mainly by stimulated

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monocytes and macrophages, other sources include endothelial cells [281, 282], T and B lymphocytes [283, 284], and mast cells [285]. IL-6 production by monocyte/macrophages is induced by IFN-γ [286], GM-CSF [286], LPS [287, 288], IL-1α [289, 290], and TNF-α [287, 290], and is inhibited by IL-4 [291] and IL-10 [267]. In all non-monocyte/macrophage derived IL-6, IL-1 and TNF-α appear to be the dominant stimuli. This cytokine has been implicated in the immune response to *M. tuberculosis* infection as well. T cell responses are suppressed by IL-6 produced by mycobacteria infected macrophages [292]. Upon low dose aerosol infection, increased bacterial count in the lungs, as well as decreased IFN-γ production, were observed in IL-6-/- mutant mice, compared to wild-type mice, suggesting that IL-6 is involved in the initial innate response to infection [293].

In summary, the coordinated movements and activities of multiple and divergent cell populations of the host immune system is absolutely required for the control of tuberculosis and the amelioration of the pathophysiological manifestations of disease. This coordination is orchestrated at every stage by the directed secretion and recognition of cytokine and chemokines concentrations, without which infection with *M. tuberculosis* would rapidly overwhelm the host.

1.7 ***Vaccine development***

Immediately following the discovery of the bacillus, Robert Koch began efforts to produce a protective vaccine for tuberculosis. Founded on work by Jenner and Pasteur, Koch prepared a sterile filtrate from cultured bacteria, which became known as "old tuberculin" (OT) which he erroneously claimed in 1890 was a cure for tuberculosis [294].

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/-, perforin-/-, and CD1-/- mice [240]. The mutant with the least impact on control of infection as measured in survival time and bacterial load in the lungs was the CD1d -/- mutant, demonstrating no difference when compared to the wild type. The other strains exhibited varying levels of resistance to infection from strongest to weakest, as follows: perforin -/-, CD8α-/-, TAP1-/-, and β_2m-/- [240]. Interestingly, because none of these mutants displayed the same level of susceptibility to infection, the actions of each component are independent from each other and cannot be explained by any one deficiency. For example, the lack of CD8$^+$ T cells could not fully account for the susceptibility seen in β_2m-/- mice, suggesting an alternative role associated with this molecule. In addition, it is clear that perforin-dependent CTL activity is not the only means whereby CD8$^+$ cells contribute to resistance. Another point of argument, whether or not antigens find their way into the cytoplasm of resident macrophages in order to be processed through the endogenous pathway, is addressed by the observation that TAP1-/- mice show significant loss of resistance to infection [240]. Additionally, it was recently demonstrated that *M. tuberculosis* resides within a permeable phagosomes within the macrophage [241], which could explain the presence of mycobacterial proteins within the cytoplasm.

While it has been demonstrated that the control of initial infection from *M. tuberculosis* depends on CD4$^+$ T cells, evidence is coming forth to demonstrate the need for CD8$^+$ T cells for control of later-stage infection. The first observance of the kinetics of CD8$^+$ T cell contribution during infection was during the afore mentioned research by Orme in 1984. In this experiment, the time at which these cells gave the most protection was 30-60 days after the peak of CD4$^+$ immunity [236]. More recent work has shown

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Unfortunately, after broad-range use of this treatment, it was soon determined that more infected patients died from therapy than were aided by it [295]. Through subsequent studies to unravel this problem, Koch observed that injecting OT into the skin of infected animals, a characteristic edema resulted at the site of injection within 1-2 days. This "Koch phenomenon" later came to be known as delayed-type hypersensitivity (DTH). While this was not a cure for tuberculosis as Koch had hoped, the true value of this work was the discovery of a tool still used today for the diagnosis of infection. In 1941, because of problems resulting from its heterogeneous nature, OT was replaced by an ammonium sulfate precipitation of culture media known as purified protein derivative (PPD) [55] and is currently the standard diagnostic tool for testing humans and animals for infection with *M. tuberculosis.*

Building on subsequent work by Trudeau in 1891 showing that attenuated strains of *M. tuberculosis* showed the most promise as vaccine candidates, scientists Albert Calmette and Camille Guerin began working on attenuating a strain of bovine tuberculosis (*Mycobacterium bovis*) in 1906. After 13 years and 231 passages on potato slices with ox bile, they isolated bacteria that did not cause disease when injected into guinea pigs, cattle, rabbits, or horses [296]. This organism was first tested as a prophylactic vaccine in 1921 on an infant whose mother had died of tuberculosis. The test was considered a significant success when the child did not subsequently develop disease even though he resided with an infected grandmother [297]. This vaccine, termed BCG for Bacillus Calmette-Guerin, became widely distributed and administered starting in 1924. Since that time it has been almost universally accepted throughout the world as a cheap, safe, stable, long-lasting vaccine with added advantage of being safe for neonatal

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administration. In addition, BCG is not only used as a vaccine against tuberculosis, but also leprosy, and, interestingly, utilized since 1976 in various treatments for bladder cancer [298-300]. While the exact antitumor modality of BCG is unknown its clinical efficacy may stem from synergistic effects of increased BCG invasion into dedifferentiated cells exclusively resulting in the subsequent production of cytotoxic concentrations of NO within the infected urothelial cells [301], and MHC class I, class II, and non-MHC, CD1-restricted antigen presentation resulting in a characteristic Th1 cytokine production enabling recruitment, proliferation and activation of cytotoxic NK and $CD8^+$ T cells [302-306]. Because of its ability to elicit this broad-ranged response from the host, BCG has been used with some measure of success throughout the world.

Ascertaining the precise cause of attenuation in Calmette and Guerin's original strain has been nearly impossible owing to the fact that the original strain no longer exists, save in the form of numerous genetically similar, but not identical daughter strains throughout the world. Based on the sequenced genome of *M. tuberculosis*, a whole genome microarray approach was employed to identify deleted regions in current BCG strains relative to the virulent *M. tuberculosis* H37Rv strain [194]. These deleted segments, 16 in all, were termed regions of differentiation, or RD1-RD16. From this, an evolutionary timeline was constructed detailing the specific branchpoints of the various extant BCG strains and when each RD region was likely to have been deleted. While six regions appear to have been deletions occurring sometime after the 1921 clinical debut of BCG, nine regions are present in *M. tuberculosis* missing from BCG and all virulent *M. bovis* strains tested, and thus are believed to be initial deletions occurring in the 1921 strain. Of particular interest is RD1 which codes for genes Rv3871-3879c, including the

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dominant antigens ESAT-6 (Rv3875) and CFP-10 (Rv3874) [194]. The fact that this region codes factors critical to *M. tuberculosis* virulence was suggested by complementation of BCG with the RD1 region, termed a "knock-in mutant" resulting in a restoration of virulence in the mouse model, while other RD regions, likewise restored to the BCG genome, had no such effect [307]. These findings, however, may be overstated slightly since the level of pathology and bacterial burden in the lungs of mice receiving the complimented strain of BCG do not demonstrate the same level observed in *M. tuberculosis* infected mice.

The protective efficacy of BCG has been called into question, however, as numerous studies report extreme variations ranging from 0-80% efficiency in protecting against disease [308]. Different hypotheses have arisen to explain why there is such a broad range of protection. One such interpretation is the presence of atypical mycobacteria in the environment where the vaccine is being administered [309]. In addition to developing host tolerance to avirulent mycobacteria resulting in the failure of the vaccine to produce a memory population of protective lymphocytes, the presence of environmental mycobacteria is thought to raise the baseline of natural resistance to infection caused by mycobacterial species, thus skewing results toward lower observed efficacy [310, 311]. Other sources of protective variation include the method of preparation or strain of BCG used in the study [312, 313], length of the studies, and the analytical readout of success or failure [314], and genetic factors of our diverse human population [315]. In addition to inconsistent performance, BCG presents several other drawbacks, including interference with surveillance programs that utilize the DTH reaction of intradermal PPD injection, the danger of administering BCG to

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immunocompromised individuals (a growing serious issue in light of the ballooning population of HIV-infected children) [316, 317], and apparent waning of protective efficacy after adolescence.

In response to the obvious and urgent need for a more effective preventative measures, scientists have worked for decades on the development of a vaccine with more consistent and positive results that protects throughout the life of the individual. Since $CD4^+$ T cell proliferation and IFN-γ production are critical for an anti-tuberculosis protective response, production of a Th1 response and a pursuant memory population resulting from vaccination is required. Candidates have included live attenuated, whole-cell killed, DNA, subunit, and prime-boost vaccines, as well as combinations of the above.

1.7.1 *Live attenuated vaccines*

Mycobacterial vaccine formulations from typically pathogenic species which are modified to be less virulent, such as BCG, and attenuated *M. tuberculosis,* as well as species of mycobacteria that are nonpathogenic in humans, such as *M. microti* have been assessed in animal and human trials for their protective and therapeutic efficacy. Modification of the existing BCG bacillus in an attempt to increase the immune response is a common approach. Such modifications include augmented expression of mycobacterial antigens like Ag85A and heat shock proteins and the addition of exogenous cytokine, and haemolysin genes [318-321]. On the other hand, because of potential dangers in administering BCG to immunocompromised neonates, auxotrophic mutants of BCG have been produced to render the bacillus less virulent [322-324]. The

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attenuation of *M. tuberculosis* itself has also been an attractive approach, especially in the realm of targeting biochemical processes that occur during the latent phase of infection, thus resulting in a clearable, yet longer lasting and more pathogenic infection than BCG which would, in theory, yield a more effective population of protective memory cells. This goal was also attempted by the use of other nonpathogenic mycobacteria as early as the 1950s. While most of these trials produced results similar to those seen from BCG alone, several promising candidates exhibited significant efficacy.

1.7.2 *Whole-cell killed vaccines*

Coupled with the belief that active secretion of mycobacterial cell products by live bacilli is required for effective priming of the memory response, and the knowledge that killed *M. tuberculosis* fail to generate an antigen-specific protective T cell response, the study of inactivated whole-cell vaccines against tuberculosis has received little attention. Relatively recently, however, it was demonstrated that a killed preparation of *M. vaccae* does in fact elicit a mycobacteria-specific cytotoxic T cell response and confer protection in animal models [325]. Indeed, further testing in humans demonstrated this organism's ability to produce IFN-γ and lymphoproliferative responses in healthy and HIV-infected individuals [326].

1.7.3 *Subunit vaccines*

Since Koch's work with Old Tuberculin in the late 19th century it has been recognized that individual components of *M. tuberculosis* can elicit a memory response and might thus be used as an effective vaccine. Recent subunit formulations utilizing

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specific gene products known to be immunogenic during natural infection have focused on the secreted and surface-bound proteins of *M. tuberculosis*. Preeminent of these has been the entire CFP, the Ag85 complex, and the low molecular weight ESAT family [67, 327-330]. In an effort to boost uninspiring results demonstrated in initial studies, subsequent trials have used combinations of various antigenic proteins, peptides, and adjuvant to boost protection to levels that rival BCG. A recent success has been the development of a fusion protein termed Mtb72F. This protein linearly combines the C terminus of Mtb-32 to Mtb-39 followed by the N terminus of Mtb-32. Utilization of this vaccine induced strong CD4 and CD8 responses in the mouse and afforded significant protection in both the mouse and guinea pig against subsequent challenge [331, 332].

1.7.4 *DNA vaccines*

Building on the work by Wolff in 1990 demonstrating that injection of plasmid DNA into the muscle of mice resulted in expression of the plasmid-encoded gene [333], multiple researchers began assessment of the efficacy of DNA vaccines targeting cancer and infectious disease [334-336]. The principle behind DNA vaccination is this: plasmid DNA is injected into the muscle and taken up by the cells, the gene encoding the antigen is transcribed and translated, and the protein is then processed and peptides are presented on MCH class I and II molecules where they provide strong stimulation for T-cell mediated immunity development. Research has demonstrated that both humoral and cell-mediated immune responses develop, and some measure of protection against subsequent challenge has been achieved with HIV and influenza vaccines [337, 338]. Similar immune activity is observed in vaccination with mycobacterial DNA yet results have

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been varied. Vaccination with plasmid DNA encoding the major secreted antigen Ag85A was observed to provide significant protection against subsequent challenge [339]. On the other hand, increased necrosis and lung pathology were observed in mice and guinea pigs given hsp60 or Ag85 DNA injections in both prophylactic and therapeutic vaccination schemes [340, 341].

1.8 *The phenomenon of tuberculosis latency*

The ability of the *M. tuberculosis* organism to persist within its host for extended periods of time, even in the face of aggressive innate and cell-mediated immune mechanisms to combat infection, is a hallmark of tuberculosis infection and helps to explain why nearly two-thirds of the world's population harbors this pathogen. The term "dormant" is often used to describe the physiological state of the bacillus as it persists in the host in a sub-clinical state, while "latency" refers to the remission of disease rather than specific reference to the bacterium. Clinical and laboratory evidence alludes to many mechanisms possessed by *M. tuberculosis* by which it can persist *in vivo* without causing outward disease – sometimes as long as decades after initial infection - only to become active when conditions within the host favor recrudescence. To explain the uncanny ability of this pathogen to persist, research has focused on several aspects, including genetic and physiological factors of the bacterium, host-pathogen interaction, and immunopathology of the host.

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1.8.1 *Models of persistence*

Before an attempt to describe the putative host and pathogen factors that could lead to persistence, it would be helpful to describe the *in vitro* and *in vivo* models that have led to our current understanding.

1.8.1.1 *In vitro models*

Early work to describe the environment of bacilli within the granulomatous lesion using human lung resections led to the idea that the pulmonary granuloma is hypoxic or microaerophilic in nature. Evidence to support this include 1) the lack of angioneogenesis within the granulomatous lesion to facilitate oxygenation would preclude efficient gas transfer in the more densely packed tissue 2) the ability of the organism to enter a stage of non-replicating persistence upon hypoxic exposure, and 3) the induction of genes upon hypoxic challenge and *in vivo* growth that code for nitrate reduction.

The most well known of these hypoxic models of dormancy was developed by Wayne wherein *M. tuberculosis* cultures were subjected to a gradual reduction of [pO_2] providing time for the bacilli to adapt to the changing environment: a feature Wayne claimed would exist within the host during granuloma formation [167, 342]. This research led to the description of a two-stage shiftdown into hypoxic persistence, designated NRP1 and NRP2 (for non-replicating persistence stages 1 and 2). NRP1 occurs when the pO_2 concentration reaches 1% saturation and is characterized by the continued utilization of ATP, indicative of ongoing cellular metabolism and processes, and a sharp increase in the production of glycine dehydrogenase (GDH). NRP2 occurs

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when the oxygen saturation level falls to 0.06%, considered truly hypoxic, and is characterized by decreased GDH production and acute susceptibility to metronidazole [342]. Several factors argue against the "Wayne Model" as a true representation of *M. tuberculosis* dormancy (as defined by metabolic quiescence). First, metronidazole is a nitroimidazole class antibiotic that is taken up by anaerobic bacteria and reduced by cellular mechanisms only present during anaerobiosis. It is this reduced form of the drug that is capable of inhibiting bacterial nucleic acid synthesis ultimately leading to death of the bacillus. This mode of action would argue that any bacilli susceptible to this drug are not completely dormant but continue cellular processes involving gene transcription at least at a basal level. As experimental testament, the only time during a murine infection model when metronidazole demonstrates clinical efficacy, albeit limited efficacy, is during the chronic phase, precisely the stage when hypoxic conditions are predicted to prevail [343]. In addition, Wayne also observed an increase in nitrate reduction during NRP2 and proposed this process as a marker for metabolic shiftdown [344]. Yet, once again, nitrate reduction is testimony to the occurrence of cellular activity. Finally, the bacilli in Wayne's NRP stage 2 were fully capable of immediately forming colonies on plates, differing from the observation of "stainable but not culturable" bacilli isolated *in vivo*. For these reasons it has been argued that the Wayne model is more accurately a representation of bacterial adaptation to conditions that could lead to the formation of dormant bacteria, but falls short of representing true dormancy itself. Indeed, by lengthening the duration of hypoxia in the Wayne model from 14 days to 4 months researchers recently showed that non-culturable bacilli (many of which were coccoid)

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were produced that could only be resuscitated by subsequent repeated passage, precisely what was observed in the animal and human specimens [345].

1.8.1.2 *In vivo models*

Beginning in the 1950's, McDermott and McCune developed what is known as the Cornell model of dormancy [346, 347]. In this mouse model, *M. tuberculosis* infected mice were treated with PZA and INH for 3 months resulting in the cessation of disease and the inability to culture bacilli from the spleens of these mice. Three months following chemotherapy, however, nearly one third of the mice experienced relapse of disease [346]. This number increased to 100% when corticosteroids were given to suppress the immune response of the animal [347]. It is argued that these results demonstrate the bacteria in a truly non-culturable, dormant state where they are not resistant to antibiotics, as subsequent sensitivity upon reactivation of disease would show, but merely refractive in their non-metabolic state. While the high number (33%) of spontaneously recrudescent "patients" is problematic and may actually represent a lag-phase phenomenon or slowed re-growth of a few metabolically active bacilli rather than a shift to and from the dormant phenotype, the cases of reactivation upon immunosuppression may more nearly reflect the phenomenon of human latency in that a vast majority of secondary tuberculosis cases are brought on by incapacitation of the host immune response, such as by HIV infection or old age. While it is well accepted that *M. tuberculosis* may indeed lie dormant within a host for decades and activate when conditions are favorable, the precise mechanisms and factors that initiate this state of quiescence or signal reactivation are not well understood.

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1.8.2 *Physiological aspects of persistence*

Wayne was the first to identify a bacterial factor induced in a dormancy model that was required for persistence of the bacilli *in vivo* [167]. This factor was isocitrate lyase (Icl), an enzyme of the glyoxylate shunt pathway. This enzyme is induced under hypoxic conditions and upon phagocytosis of the bacilli by macrophages [45]. Its expression is localized to the non-necrotic lesions *in vivo* as no mRNA for *icl* is detected by *in situ* hybridization in the central necrotic areas of human granulomas [166]. Interestingly, the *icl* gene is not required for the survival of the bacilli under aerated or hypoxic culture conditions *in vitro* nor for initial infection *in vivo,* but is essential for survival of the bacilli beyond the initiation of acquired immunity in the host [168].

Sigma factors are global regulators of an organism's transcriptome responding to the requirements of the organism and environmental pressures exerted upon it. As such, two of the thirteen sigma factors found in the *M. tuberculosis* genome, SigF and SigB, have been implicated in survival of the organism during persistent stages of animal infection. SigF is found only in the slow-growing, virulent mycobacteria and is induced by a variety of stress conditions, including hypoxia [348], oxidative stress, cold shock, nitrogen depletion, and entry into stationary phase [349]. *In vivo,* the contribution of SigF regulation is less clear since there is no difference in intracellular survival when the gene is deleted, as observed in macrophage culture. Decreased virulence of the mutant is observed, however, in murine infection [43]. This disparity could be explained by the fact that macrophage cell culture does not present the same environmental conditions as *in vivo* growth, namely lymphocytic activation of macrophages, physical architecture of

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the granuloma leading to possible hypoxia, depletion of essential compounds such as by iron chelation, and exposure to toxic antimicrobial factors produced by immune cells other than macrophages.

One of the most intensely studied mycobacterial products has been the 16 kDa heat-shock protein, α-crystallin homologue or Acr. This immunodominant protein was originally identified in 1985 and was later observed to be highly induced in the Wayne model of hypoxia [172, 350] as well as exposure to reactive nitrogen [351]. Further evidence for the protective contribution of this gene product during *in vivo* survival and persistence comes from the fact that Acr is induced during intracellular growth [127] and a mutant strain lacking the *acr* gene exhibits decreased survival in macrophages [128]. Recently, the two-component response regulator dormancy response regulator (DosR) was demonstrated to be the fundamental mediator of the hypoxic response in *M. tuberculosis* [170, 352]. When *M. tuberculosis* is exposed to nitric oxide, the bacilli exhibit characteristics of nonreplicating persistence and the regulon under the control of DosR is induced, implicating DosR in the shift to an NRP state. Furthermore, several genes under the control of this response regulator, including *acr,* are shown to be up-regulated within an animal infection after the initiation of Th1 immunity [353, 354].

The stringent response in bacteria was first studied in *E.coli* and is a transcriptional program involving almost 100 genes [355-357]. Induced by nutrient starvation, it is essential for survival of the organism under extreme conditions where full metabolic activity is not desirable or possible. In *M. tuberculosis* this stringent response is regulated by hyperphosphorylated guanine (ppGpp), which is synthesized by the protein ppGpp synthase I [358]. Deletion of this gene yielded deficiencies both *in vitro*

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and *in vivo*: *in vitro* and in macrophage cell culture, mutant mycobacteria were able to survive as well as the wild type initially, but exhibited attenuated survival during stationary phase and late-term infection, respectively [359]. Furthermore, mice infected with the RelA-/- mutant did not sustain bacterial numbers during chronic infection as typically observed. This attenuation late in disease was observed as a lack of classical granuloma formation seen in wild-type infections [360]. This stringent response regulator has been linked to the expression of numerous cell-wall biosynthesis enzymes, virulence factors, secreted antigens, and heat shock proteins, highlighting the critical role of this protein during persistent infection [360].

1.8.3 *Immunological aspects of persistence*

In the course of a typical bacterial infection the host responds in a characteristic, aggressive manner to destroy the invading organism. First, the phagocytic macrophages and dendritic cells at the site of infection engulf the organism, which is sequestered within an intracellular compartment known as the phagosome. Phagosomal maturation includes acidification and the production of highly destructive reactive oxygen and nitrogen intermediates. Subsequently, the phagosome is fused with lysosomes within the cell. These lysosomes contain toxic molecules such as superoxide radicals, lysozyme, defensins and other hydrolytic enzymes – all extremely potent chemicals harmful to the phagocytosed bacterium. If these measures are not sufficient to contain infection, bacterially-derived peptides from the phagolysosome are loaded onto MHC class II molecules and shuttled to the surface of the host cells to be presented to antigen-specific lymphocytes. Once these peptides are recognized by lymphocytes cell-mediated

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immunity ensues in which cytokines including IL-12, TNF-α, and IFN-γ are produced that greatly amplify the ability of the phagocytic cell to destroy engulfed pathogens. Taken together, these activities of the host normally constitute sufficiently effective means to combat encountered pathogens. Yet it is a fact that people remain latently infected and reactivate decades after initial infection. Punctuating this is the fact that chemotherapeutic regimens require six to nine months of drug treatment to be successful.

Acidification of the phagosome represents one of the first lines of defense against engulfed pathogens and evidence suggests that pH ranges outside of 6.1 – 7.6 are less than conducive to *M. tuberculosis* survival [361]. To deal with this potentially deadly host defense the bacilli have developed a means of excluding the proton ATPase pump from the membranes of the phagosome thus disenabling the acidification of the vesicle [158, 362].

ROI are typically very toxic to bacteria and *M. tuberculosis* has developed numerous methods of neutralizing this attack, as well. One of these mechanisms is the complement-mediated uptake in a manner to circumvent normal stimulation of ROI production by the macrophage [39, 363]. Further implication for the contribution of this mechanism to disease was provided by Schlesinger when he demonstrated that only virulent strains of *M. tuberculosis* are phagocytosed in a mannose-receptor and complement-receptor dependant fashion [220]. Various mycobacterial products have also been implicated in the neutralization of ROI, including lipoarabinomannan [34], glycolipids [247], and sulfolipids [27]. Finally, the genome of *M. tuberculosis* encodes several powerful antioxidants such as superoxide dismutase (*sodA*) and

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catalase/peroxidase (*katG*) [40], both shown to offer protection from destructive oxygen species.

In addition to the ability to produce compounds and proteins that ameliorate and defuse toxic agents within the phagosomal environment, the ability of the pathogen to directly subvert the normal physiological functions of the phagosome is remarkable. As has been described, one of the most potent weapons is the IFN-γ- and TNF-α-induced activation of nitric oxide synthase 2 (NOS2) which produces NO, and other RNI that are highly toxic to engulfed bacteria. The protective role of this ability against *M. tuberculosis* infection was shown by Chan, et al., in 1992 and was later confirmed by others [154, 364]. However, the tubercle bacilli have likewise developed means of overcoming this threat to some degree allowing persistence of bacteria. Several gene products have been implicated in this resistance, including *ahpC* (Rv2428), *lpd* (Rv0462), *sucB* (Rv2215), and *ahpD* (Rv2429).

The *M. tuberculosis* gene Rv2428, encoding the peroxiredoxin alkyl hydroperoxide reductase subunit C protein (AhpC), belongs to a family of peroxidases that function to protect a broad range of bacteria from oxidative and nitrogen-mediated stresses [365]. This gene product has been shown to catabolize a potent oxidant produced in activated macrophages, peroxynitrite anion ($ONOO^-$) [366]. The association of $ONOO^-$ susceptibility and virulence is demonstrated in the fact that avirulent BCG and *M. smegmatis* are susceptible to the antimicrobial effects of $ONOO^-$ while *M. tuberculosis* and *M. bovis* are not [367]. While it has been demonstrated that this protein confers protection from reactive nitrogen compounds [366], the overt role of this gene in virulence is a matter of debate as it has been shown that *ahpC* transcription in the virulent

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Erdman strain of *M. tuberculosis* is repressed under macrophage growth conditions while avirulent BCG demonstrates a constitutively high expression of this protein [368]. On the other hand, the redundancy of peroxiredoxins in the *M. tuberculosis* genome could account for this observation. Also, it is interesting to note that this gene repression is removed *in vitro* when bacterial growth reaches the stationary phase, implying a possible role during chronic infection *in vivo* [368].

In conjunction with AhpC, the gene products of *lpd, sucB,* and *ahpD*, lipoamide oxidoreductase, dihydrolipoamide succinyltransferase, and alkyl hydroperoxide reductase D protein, respectively, form an antioxidant complex that exhibits peroxidase and peroxynitrite reductase activity [369]. Full peroxynitrite activity was seen to be dependant upon the presence of all four of these proteins together. Interestingly, one of these components, Lpd, also plays an essential role in intermediary metabolism as the E3 portion of pyruvate dehydrogenase and α-ketogluterate dehydrogenase, both of which are essential for the synthesis of acetyl-coenzyme A from endogenous precursors. This ability is necessary for the function of the glyoxylate cycle known to be critical for the establishment of chronic infection [168].

Another possible mechanism of resistance to nitrosative activity is through *noxR1* and *noxR3.* These genes have been demonstrated to confer protection from RNI across multiple species when transformed into the RNI-sensitive *M. smegmatis* [370] as well as *S. typhimurium* [371]. However, when *noxR1* was deleted from the H37Rv strain of *M. tuberculosis* no attenuation of virulence was observed as would have been expected [372]. These data alone cannot dismiss the role of this gene in protection from killing

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mechanisms of infected macrophages since the molecular basis of resistance conferred by either of these genes is currently unknown.

Other mechanisms developed by the bacillus are the several means by which it can inhibit phagosomal maturation, specifically phagosome-lysosome fusion. That this ability is an active function of live bacteria was demonstrated in seminal studies showing that only living *M. tuberculosis* can inhibit fusion of the lysosome with the bacteria-containing phagosome [373-376]. In addition to inhibiting the acidification of phagosomes, evidence that *M. tuberculosis* prevents phagosomal maturation is offered by molecular markers on the surface of the endosome. Rab5, a GTPase associated with early endosomes exclusively, is released from the membrane as the phagosome matures whereas Rab7 is a GTPase associated with late endosomal vesicles. These GTPases confer the ability of phagosomal-lysosomal fusion. Research in several labs demonstrated that phagosomal maturation arrest resulted in the retention of Rab5 in the endosomal membrane [377, 378]. While it was shown that Rab7 was absent from arrested endosomes in one experiment [378] and present in the other [377] it is clear that GTPase-mediated membrane fusion is aberrant in *M. tuberculosis* infection.

Another mechanism of endosomal maturation arrest that has received much attention is the retention of TACO (tryptophan asparagines-rich coat protein) on the surface of the endosome. As with the GTPases describes earlier, TACO retention appears to be an active process exhibited by living bacilli exclusively [379]. The research demonstrated that TACO-negative macrophages were able to rapidly destroy BCG, while transfecting TACO into a TACO-negative cell line resulted in truncated phagolysosomal fusion and the subsequent inability to clear BCG infection. It should be

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noted, however, that the true significance of these data are a matter of debate based upon the method of infection [380]. It is clear, however, that the multitude of mechanisms by which the *M. tuberculosis* bacilli is able to grow and persist within the hostile environment of the host macrophage are complex.

1.8.4 *Subversion of acquired immunity*

Central to the acquired immune response is the presentation of pathogen-derived peptides to lymphocytes in the context of MHC molecules on the surface of professional antigen presenting cells (APC) with the resulting production of key cytokines such as IFN-γ and TNF-α. In order to persist within the host, a pathogen must be capable of neutralizing the effects of this process. In this respect, too, *M. tuberculosis* has proven equal to the task in a variety of ways. It was first observed by Chan and collegues in 1991 that LAM, an abundant glycolipid on the surface of *M. tuberculosis*, inhibits IFN-γ-dependant expression of MHC on the surface of human APCs [38]. A few years later multiple groups published work confirming these results, demonstrating that *M. tuberculosis* infection resulted in defective antigen presentation [381-384]. This attenuated antigen presentation was shown to be caused by an inhibition of MHC class II expression in infected cells. Glycolipids were not the only culprits implicated in this phenomenon; the 19kDa lipoprotein gene product was revealed to inhibit MHC class II expression over time through Toll-like receptor 2 (TLR2) stimulation [385-387]. It has been hypothesized that the delayed expression of this phenotype (truncated expression of MHC molecules) allows for a population of bacilli to outlast the initial burst of antimicrobial forces and persist within the host in a subclinical fashion.

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1.9 *Concluding remarks*

Most research to date focusing on bacterial physiology during animal infection has proceeded with the goal of determining metabolic processes and gene transcription patterns within the pathogen. On the other side of the issue, immunologists have largely looked at elucidating cellular mechanisms of the immune response and defining cellular subsets relevant during infection. Where these disciplines have converged, most research has focused on the stimulatory potential and immunologic relevance of dominant antigens during the first days and weeks after infection. Furthermore, most of the antigens evaluated thus far have been selected based on abundant production in standard *in vitro* growth conditions. However, the evaluation of multiple stress-induced gene products during the acute and chronic stages of infection in order to determine their ability to stimulate an immune response has not yet been performed. This lack of research leaves critical questions unanswered about the true relevance of stress-induced proteins and those produced exclusively during *in vivo*-like conditions. This research, therefore, seeks to determine the stimulatory capacity of several proteins believed to be produced during acute and chronic infection, based on hypoxic and alternate carbon-source models, in order to establish diagnostic and protective potential. I hypothesize that in response to stressed growing conditions *in vitro* the individual proteins produced by *M. tuberculosis* will approximate that which occurs within the host. In addition, since the pathology of disease is progressive and dynamic over the course of infection, I hypothesize that antigen-specific T cell activation to these differentially-produced proteins will by dynamic as well, reflecting the progressive nature of pathology and the

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response of the bacillus to the host environment. Finally, I put forth that vaccination with the stress-induced mycobacterial proteins will afford protection to the host against subsequent challenge, especially during the chronic stage of infection. This is the first work to identify such gene products, evaluate the kinetics of the immune response to these proteins throughout an animal infection, and assess the protective potential of these proteins in a vaccine.

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CHAPTER 2: Identification of proteins with altered secretion profiles in the SecA2 mutant of *M. tuberculosis*

(Presented in Braunstein, M., B.J. Espinosa, J. Chan, J.T. Belisle, W.R. Jacobs, Jr.. 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis.* Mol. Micro. **48**(2), 453-464.)

The aim of this study was to:

1. identify proteins with altered secretion patterns in a *secA2* deletion mutant of *M. tuberculosis*
2. determine the subcellular localization of proteins manifesting altered secretion patterns due to *secA2* deletion

Abstract

With the dramatic increase in the global burden of tuberculosis the need for a greater understanding of the mechanisms of pathogenesis as well as more effective vaccines and diagnostic reagents is greater than ever. Most of the exported proteins of *M. tuberculosis* contain a signal sequence and are translocated via the Sec-dependent secretion machinery. Several key virulence factors and antigens of *M. tuberculosis,* however, do not contain a classical signal sequence and yet are found in great abundance in the medium of actively growing bacilli, suggesting the utilization of an alternative translocation pathway. *M. tuberculosis* contains a homologous, but non-redundant *secA* gene called *secA2,* the deletion of which results in truncated virulence of the organism and

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increased survival time of mice infected with the mutant. This research describes the identification of proteins with an altered secretion profile in the *secA2* mutant of *M. tuberculosis.* Using 2D gel electrophoresis (2DE) followed by MS and MS/MS on the culture filtrates of log- and stationary-phase cultures, this research identified three proteins with decreased secretion in the mutant compared to the wild type: SodA (Rv3846), KatG (Rv1908c), and Rv0390. Both SodA and KatG are involved with the detoxification of destructive oxygen compounds and are believed to protect the organism from oxidative damage due to immune responses of the host. Interestingly, it was observed in the mutant by Western blot analysis of the whole cell lysates and the culture filtrates of the wild type and *secA2-/-* mutant that SodA not only exhibited decreased abundance in the culture filtrate of the mutant, but showed an increasing buildup within the cell, attesting to a defect in the translocation of this protein. The deficiency in KatG translocation was only observed during the first 5 days after inoculation of the culture and did not build up within the cells. The identification of alternate modes of protein export will augment our understanding of the virulence mechanisms of this pathogen and aid in the development of therapies to treat and prevent tuberculosis.

2.1 Introduction

How a pathogen interacts with its environment determines much of how disease will progress within the host. Most of the identified and characterized immunodominant antigens of *M. tuberculosis* are those that are found in the culture medium of *in vitro* cultures (Table 1.1). Many of the original transcripts of those proteins contain an amino acid signal sequence and are thus exported through the plasma membrane either to be

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inserted into the membrane or wall of the bacterial cell or else to be released as a soluble protein into the extracellular environment. On the other hand, there are a surprising number of bacterial products that are found outside of the bacilli in the absence of bacterial lysis that do not contain a secretion signal sequence. Much research has been done to explain this phenomenon.

The genome of *M. tuberculosis* possesses nearly all the components of the classical Sec-dependant secretion pathway found in Gram-positive bacilli, including *secA* (Rv3240c), *secY* (Rv0732), *secE* (Rv0638), *secG* (Rv1440), *secD* (Rv2587c), *secF* (Rv2586c), and *yajC* (Rv2588c). While not found in mycobacteria many gram-negative organisms also possess *secB*, a gene coding for a chaperone involved in the docking of precursor proteins with the SecA homodimer. Other secretion factors found in mycobacteria include: *secE2* (Rv0379), *ffh* (Rv2916c), *FtsY* (Rv2921c), *lepB* (Rv2903c), and *lspA* (Rv1539). It was recently discovered that *M. tuberculosis,* as well as other *Mycobacterium* spp., possess two *secA* homologues [1]. At the time of its discovery, mycobacteria was the only known prokaryote genus to exhibit this feature, but research has revealed other Gram-positive pathogens that also contain multiple homologues of this gene, including *Staphylococcus aureus* [2], *Streptococcus gordonii* [3], *Streptococcus parasanguis* [4], *Streptococcus pneumoniae,* and *Listeria monocytogenes* [5]. Work to elucidate the precise function of this *secA* paralogue is on-going, but increasingly, evidence is revealing the essential nature of this gene to the viability and virulence of these organisms.

In *M. tuberculosis,* the inefficiency of transformation and homologous recombination has hampered efforts to examine the function and nature of individual

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genes. More effective tools and methods have been recently developed to allow for transformations with integrating plasmids [6, 7] and efficient transposon mutagenesis [8-10]. The question of the functional nature of the *secA* homologue has benefited greatly as a result of these advances. Work of our collaborators (Drs. Braunstein and Jacobs) demonstrated that a mutant strain of *M. tuberculosis* (MC^2-3112) lacking *secA2* (Rv1821) exhibited no difference in growth rates when compared with the wild type strain grown *in vitro*. However, the length of survival of mice infected with the mutant was significantly longer than those infected with the wild type strain. The mutant also showed marked attenuation of virulence measured in bacterial load in the organs of infected mice during the first 20 days post infection. The subsequent course of infection, with the normally observed homeostasis between the host response and bacterial persistence are seen to parallel wild-type infection. These observations indicate that *secA2* could play a role in protection against the innate immune response of the host. Further evidence of this is the observation that SCID mice infected with the mutant lived significantly longer than those infected with the wild-type strain.

In this work, we demonstrate that SecA2 is essential for the optimal secretion of several proteins including superoxide dismutase (SodA) and catalase-peroxidase (KatG) both of which are implicated in immune subversion mechanisms of the bacterium by countering the oxidative attack of the host [11, 12]. Interestingly, both of these proteins, though observed in the medium of growing cultures, lack a traditional amino acid signal sequence motif.

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2.2 Material and Methods

2.2.1 Bacterial strains and growth conditions

M. tuberculosis $H_{37}Rv$ was obtained from American Type Culture Collection (Manassas, VA) and strain MC^2-3112 (*ΔsecA2* mutant of *M. tuberculosis*) was provided by Jacobs WR (Howard Hughes Medical Institute, Albert Einstein College of Medicine.) *ΔsecA2* was generated by allelic exchange whereby gene Rv1821 was deleted from the wild type strain $H_{37}Rv$. The *ΔsecA2* mutant and $H_{37}Rv$ were grown in 100 ml Sauton medium [0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.4% L-Asparagine 0.005% ferric ammonium citrate, 0.2% citric acid, 4.76% (v/v) glycerol, 0.00001% $ZnSO_4$] to mid- to late-log stage (14 days). 1 ml stocks were made from these cultures and frozen at –80°C. In three separate batches, four cultures were established using two stock samples of each strain inoculated into 10 ml of glycerol/alanine salts (GAS) medium [0.04% Bacto Casitone (Difco, Sparks, MD), 0.005% ferric ammonium citrate, 0.4% K_2HPO_4, 0.2% citric acid, 0.1% L-alanine, 0.12% $MgCl_2$-$6H_2O$, 0.06% K_2SO_4, 0.2% NH_4Cl, 0.072% NaOH, and 1.0% (v/v) glycerol. pH=7.0] [13]. These cultures were passed twice, increasing the volume of each culture 10x for each passage; the final culture volume being 1 L. The two cultures for each strain were incubated for 7 or 14 days and the CFP harvested.

In addition to the above, one stock vial of each strain was used to inoculate one 7H10 agar plate. The plates were incubated at 37°C for two weeks allowing a confluent lawn to form. About 50 mg of cells were transferred from the plates to 100 ml of GAS medium and incubated for 14 days. 10 ml aliquots from these cultures were used to inoculate four 100 ml GAS cultures for each strain. These final cultures were grown at

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37°C with gentle agitation for 3, 5, 7, and 14 days. Upon harvesting these cultures, the culture supernatants were separated from the cells by centrifugation for 20 minutes at 2000 x g.

2.2.2 Preparation of CFPs and whole cell lysates.

The CFP from all cultures were prepared as described [14]. The whole cell lysate (WCL) fractions were generated by inactivating the *M. tuberculosis* with 24,000 Gy of γ–irradiation, then suspending the cells in breaking buffer (PBS containing 0.06% DNase, 0.06% RNase, 0.07% pepstatin A, 0.05% leupeptin, and 20mM PMSF) and passing the mixture through a French Press (American Instrument Company, MO) 10 times at 1,500 psi. The lysate volume was then doubled with breaking buffer, mixed and centrifuged at 3,000 x *g* for 5 min. The supernatant was decanted and stored at –20°C.

2.2.3 2D-PAGE of CFPs.

Dried aliquots of CFPs from each strain were dissolved in a solubilization buffer containing 8.5 M Urea, 2% CHAPS, 20 mM DTT and 0.5% ampholytes (pH 4-7) by bath sonication and vortexing. The solution was allowed to sit at 4°C overnight to allow complete solubilization. First dimensional focusing was performed by rehydrating the solubilized samples into 13 cm immobilized pH gradient (IPG) strips (pH 4-7) and applying current across the strip using the IPGphor system (Amersham Pharmacia Biotech, Piscataway, NJ) with the following program: 50 v for 12 h, increase to 500 v over 1 h, step to 1,000 v and hold for 1 h, increase to 4,000 v over 1.5 h and hold for 2 h,

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increase to 8,000 v over 2 h and hold for 6 h. The IPG strips were then equilibrated in 5 ml of an SDS equilibration buffer (50 mM Tris-HCl, 6 M Urea, 30% glycerol, 2% SDS and 65 mM DTT) for 20 min at room temperature. The strips were subsequently placed on 12% SDS polyacrylamide gels and sealed with an agarose solution (14.4 g glycine, 3.03 g Tris-base, 1 g SDS and 1 g agarose). Second-dimension separation by electrophoresis was performed using 15 mA per gel for approximately 8 h. Proteins were visualized by Coomassie brilliant blue R-250 or silver nitrate.

2.2.4 Identification of proteins.

Protein spots of interest were selected and excised from the 2D-PAGE gels and subjected to in-gel digestion with modified trypsin (Roche Diagnostics, CA) as follows: first, the gel pieces were cut into small (0.5 mm^3) pieces to allow for more efficient trypsin digestion and subsequent peptide extraction from the gel. Next, Coomassie stain was removed from the protein spots using about 50 μl of a solution of 60% acetonitrile and 0.1% TFA in 0.2 M ammonium bicarbonate. Samples were incubated at 37°C for 30 min after which the liquid was removed. If the gel piece still exhibited a blue tint then this step was repeated. Decolored gel pieces were dried by speed-vac and rehydrated with 6 μl of a 0.008% modified trypsin solution followed by 10 μl additions of 0.2 M ammonium bicarbonate until the gel pieces were completely rehydrated. Sample were then incubated for 16-24 h at 37°C. The reaction was terminated by addition of 5 μl 10% TFA to each sample and the peptides were extracted using 200 μl 60% acetonitrile in 0.1% TFA. The peptides were dried by speed-vac and applied to a 0.2 x 50 mm C18 capillary reversed phase (RP) column (Michrom BioResources, CA) and eluted with an

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increasing acetonitrile gradient using a MicroPro capillary HPLC system (Eldex Laboratories). The RP effluent was introduced directly into a Finnigan LCQ (Thermoquest) electrospray ion-trap mass spectrometer, and the peptides were analysed by MS and MS/MS. The ESI needle was operated at 4 kV with a sheath gas flow of N_2 at 40 p.s.i. and a capillary temperature of 200°C. MS/MS data of the peptides were matched to *M. tuberculosis* proteins using the SEQUEST software. The SEQUEST software was set to consider the oxidation of methionine (+16.0) and the acrylamide modification of cysteine (+71.0).

2.2.5 Western Blot Analysis

CFPs and WCL from each strain were separated by SDS-PAGE and transferred to nitrocellulose membrane. To produce an immunoblot that could be effectively analyzed the CFP and WCL protein amounts used for each blot were as follows: 15 μg CFP and 15 μg WCL for α-SodA, 12 μg CFP and 20 μg WCL for α -KatG, 10 μg CFP and 10 μg WCL for α-PhoS1, 12 μg CFP and 20 μg WCL for α-DnaK, 20 μg CFP and 20 μg MCL for α-Acr, and 15 μg CFP and 15 μg WCL for α-GroEL. The antibodies used were CS-18 (α-SodA), IT-20 (α-Acr), IT-23 (α-PhoS1), IT-41 (α-DnaK), IT-42 (α-KatG), and IT-56 (α-GroEL) at dilutions of 1:50 [15, 16]. Secondary antibody used was rabbit α-mouse IgG conjugated with alkaline phosphatase at a dilution of 1:1000. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and NitroBlue Tetrazolium (Sigma Chemical Company, MO).

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2.3 Results

2.3.1 CFP profiles of $H_{37}Rv$ and MC^2-3112.

Culture filtrate proteins (CFP) from 7- and 14-day cultures of $H_{37}Rv$ and MC^2-3112 were analyzed by 2DE and stained with either Coomassie Brilliant Blue R-250 or silver nitrate for visualization of proteins. Protein spots were chosen for identification and further analysis based on apparent differences in abundance between the two strains. Throughout four consecutive batches a majority of proteins exhibited the same relative abundance regardless of the mutation while only 6 protein spots manifested consistent differences in abundance (Fig. 2.1). In-gel trypsin digestion, MS and tandem MS/MS analysis of these spots revealed 4 proteins (Acr, MPT63, RplL and CspA) that were present in greater abundance in the CFP of the mutant while 4 proteins (SodA, Acr, and Rv0390) were found in greater abundance in the wild type (Table 2.1). One spot (spot 3) was composed of 2 co-migrating proteins (Acr and MPT63) and the individual contribution of each protein to the observed difference was not discernable. Only MPT63 contained a classical signal sequence motif, but was found in greater abundance in the CFP of the mutant strain thus ruling out SecA2 contribution to its secretion. Some divergences in protein quantity between the strains were discernable in 7-day culture while others were only detectable after 14 days, suggesting differences in expression kinetics or mechanisms of secretion. Several protein spots, marked with an asterisk (Fig. 2.1), displayed differences between strains but were not considered in our analyses because of inconsistencies from one batch to another.

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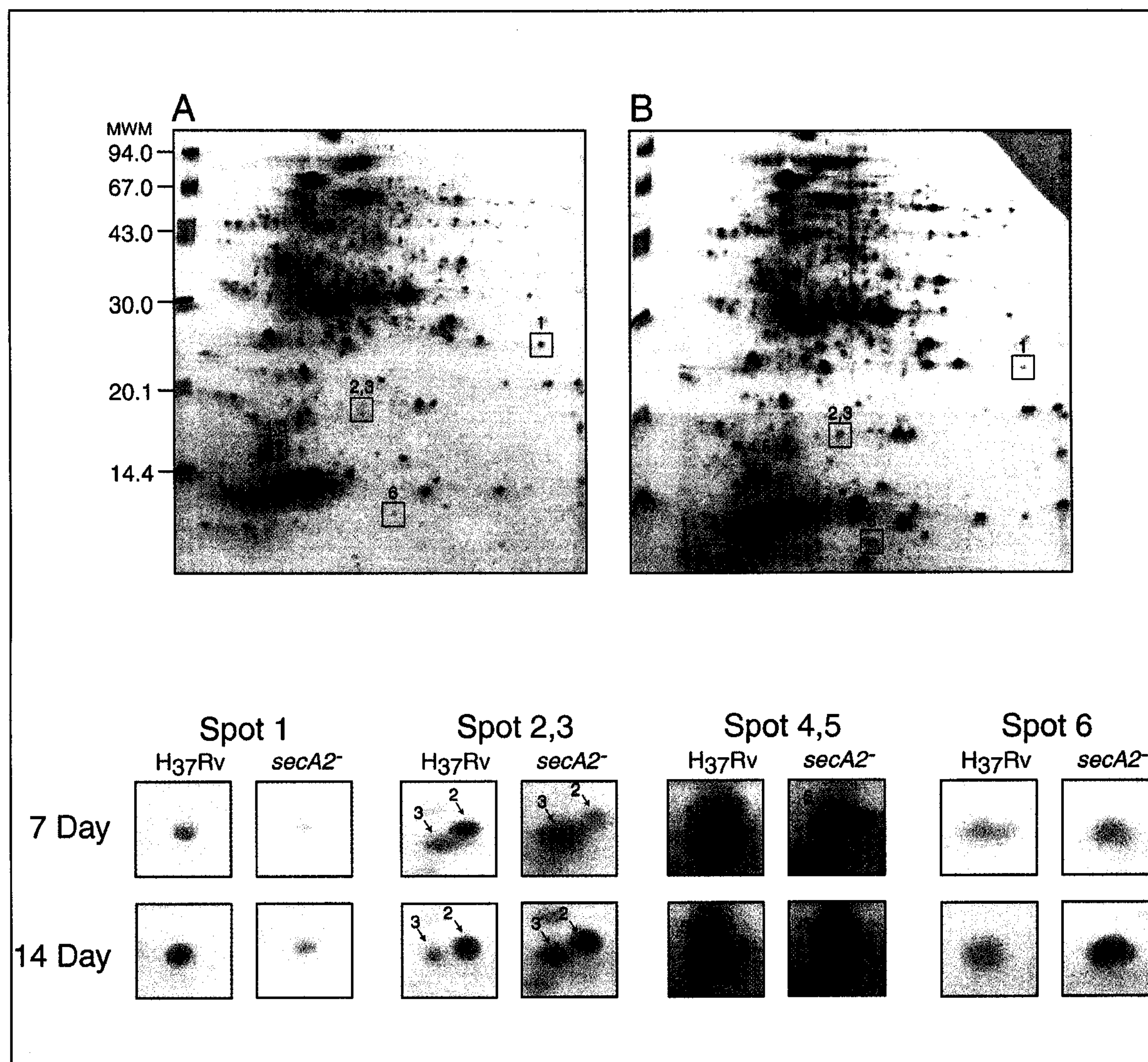
Figure 2.1 2D-PAGE analysis of culture filtrate proteins of $H_{37}Rv$ and Δ*secA2* mutant.



Culture filtrate protein of (A) $H_{37}Rv$ and (B) Δ*secA2* mutant from 14-day cultures were isolated and analyzed. Spots labeled 1-6 were extracted, identified, and analyzed. Areas of interest were enlarged to show subtle changes between protein abundance in the different samples. Spots labeled with an asterisk denote differences between pictured gels that were not consistent through all batches and therefore were not subjected to further evaluation.

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Table 2.1. Differentially released proteins of *M. tuberculosis*

Spot	Gene	Protein	Abundance in Culture Filtrate	Sec signal sequence in pre-protein
1	*sodA*	Superoxide dismutase	Greater in H37Rv	No
2	*acr*	α-crystallin (HspX)	Greater in H37Rv	No
3	*acr/rv1926c*	α-crystallin/MPT63	Greater in *ΔsecA2*	No/Yes
4	*rplL*	Ribosomal protein L12	Greater in *ΔsecA2*	No
5	*rv0390*	Conserved hypothetical protein	Greater in H37Rv	No
6	*cspA*	Cold Shock Protein	Greater in *ΔsecA2*	No

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2.3.2 SecA2 functions in the secretion of SodA and KatG

To further investigate the apparent differences observable between the wild type and *ΔsecA2* strains Western blot analyses were performed on cultures grown for 3, 5, 7, and 14 days *in vitro*. To answer the question of whether the *secA2* gene product is actually involved with translocation of proteins or if differential protein abundance is caused by transcription or translation perturbations in the mutant, CFP and WCL fractions of each strain and culture were evaluated to determine the localization of individual proteins of interest. Proteins evaluated in this assay include Acr, SodA, DnaK, PhoS1, GroEL, and KatG. Immunoblot for Rv0390 was not performed because of a lack of MAb for this protein. To determine the scope of SecA2 function DnaK and PhoS1 were evaluated, as well. DnaK, while lacking a signal sequence, has been observed in the CFP as well as the WCL of cultures [16-18]. On the other hand, PhoS1, found in both the CFP and WCL fractions of *in vitro* cultures, contains an amino acid signal sequence. Similar to the action of superoxide dismutase (SodA) in the reduction of superoxide radicals to hydrogen peroxide and water, catalase/peroxidase (KatG) functions in the reduction of bactericidal hydrogen peroxide to water and oxygen. Therefore, KatG was also measured in this assay. The cytoplasmic heat shock protein GroES has been used previously as a sentinel for bacterial lysis [16, 19].

CFP and WCL fractions were loaded onto SDS-PAGE single dimension gels, blotted onto nitrocellulose membranes and developed using MAbs to individual proteins and alkaline phosphatase conjugated antibodies. Results are shown in Figure 2.2. In

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each culture (3, 5, 7, and 14 days) SodA was consistently observed in greater abundance in the CFP of the wild type strain, while exhibiting higher abundance in the WCL of the mutant strain validating the hypothesis that *secA2* functions in the active translocation of SodA. Equally intriguing is the observation that KatG export is also affected by deletion of *secA2*. Marked differences were only seen at 3 days culture growth followed by slight, but discernable, divergences in 5 to 14 day cultures (Table 2.2). DnaK and PhoS1 protein levels were consistent through all samples indicating that SecA2 does not function in the secretion of all signal sequence-deficient (DnaK) or signal sequence-containing (PhoS1) proteins in general. The exclusive localization of GroEL to the WCL fraction in all culture times indicates that bacterial lysis is not a contributor to the abundance of CFP proteins.

Table 2.2. Relative fold difference of proteins* in CFP and cell lysate fractions of *ΔsecA2* cultures as determined by densitometry and normalized to $H_{37}Rv_{1.00}$

		Days of Growth			
Protein	Sample	Day 3	Day 5	Day 7	Day 14
SodA	CFP	0.05	0.19	0.03	0.04
SodA	Cell lysate	2.45	2.74	3.25	3.17
KatG	CFP	0.07	0.34	0.32	0.33
KatG	Cell lysate	0.74	0.88	0.86	0.92
PhoS1	CFP	1.09	1.07	0.82	0.93
PhoS1	Cell lysate	1.07	1.00	0.88	0.95

*Immunoblots in Figure 2.2 were analyzed by densitometry. Protein amount detected in the *ΔsecA2* mutant samples was normalized to corresponding samples from the wild-type strain. Values represent a ratio of mutant:wild-type where equal amounts of protein is given as 1.0.

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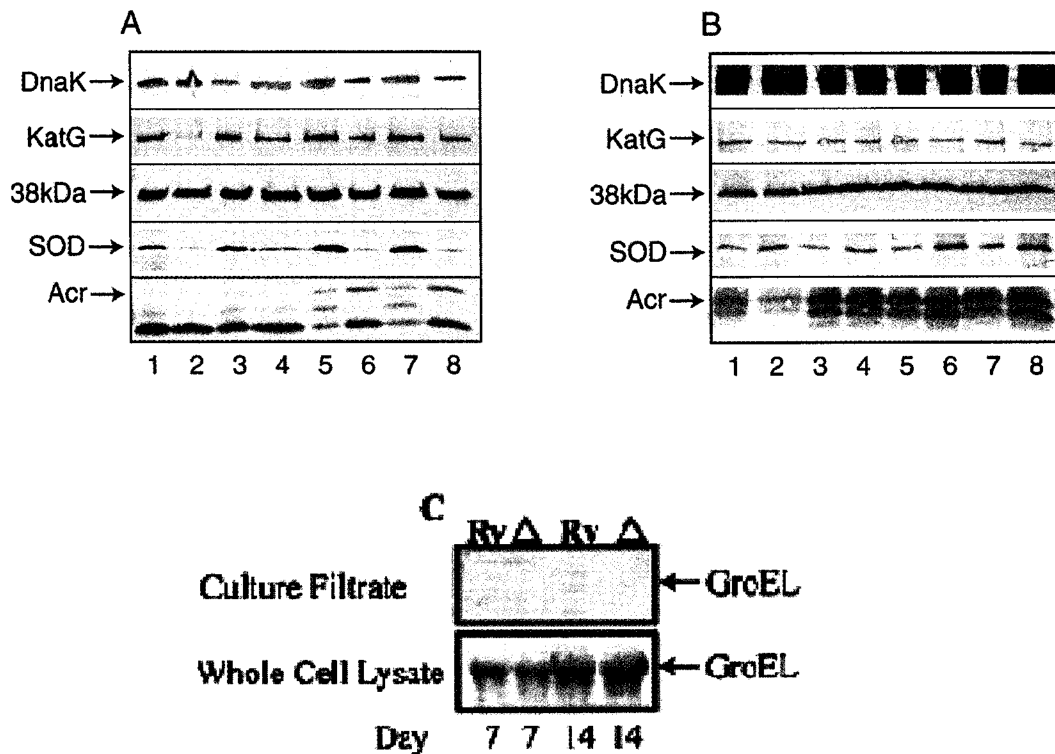
Figure 2.2 Location of selected proteins in $H_{37}Rv$ and Δ*secA2* mutants over 14 days of growth.



The abundance of select proteins from $H_{37}Rv$ (lanes 1, 3, 5, and 7) and the Δ*secA2* mutant (lanes 2, 4, 6, and 8) located in the (A) culture filtrates and (B) whole cell lysates was determined by Western blot immunoassay. Cultures were analyzed at 3 days (lanes 1 and 2), 5 days (lanes 3 and 4), 7 days (lanes 5 and 6), and 14 days (lanes 7 and 8). C) Equal amounts of proteins from the CFP and WCL fractions from day 7 cultures were subjected to immunoblot with α-GroEL antibody.

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2.4 Discussion

Translocation of precursor proteins across and into the bacterial membrane is mediated by components of the general secretory pathway. The components of this translocase are termed Sec proteins [20]. The highly dynamic protein, SecA, is unique in that it functions in several critical capacities within this pathway. First, it interacts directly with the signal sequence of precursor proteins slated for export [21, 22]. Second, it functions as a homodimeric structural component in the protein translocation channel binding with phospholipids in the cell membrane and with the essential, membrane-bound secretory protein SecY [23]. Third, as a membrane component this protein cycles proteins through the cytoplasmic membrane in an ATP-dependant manner [24], and finally, it interacts with the cytosolic SecB protein chaperone [25, 26].

This protein export pathway is essential for survival of the bacterium and also plays a critical role in the virulence of the organism as the interaction of the pathogen with the host requires export of protein factors. As such, this pathway is highly conserved throughout the bacterial kingdom and has been implicated in the virulence of many pathogens [27]. It has been shown that in order for a pre-protein to be translocated through this machinery it must contain an amino acid signal sequence which is recognized by the SecA protein [28, 29].

The discovery of SecA and a second SecA paralog in *M. tuberculosis* opened the possibility of an undiscovered pathway of protein secretion or, at least, a related, but unique, branch of classical Sec-dependant protein secretion. Since its discovery in *M. tuberculosis* [1], many other Gram-positive bacteria have been found to possess multiple *secA* gene homologues. In order to elucidate the precise role of *secA2* in the pathogenesis

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of *M. tuberculosis,* this work focused on observing and identifying proteins existing in different amounts in the medium of actively growing cultures of the *ΔsecA2* mutant compared to wild-type, $H_{37}Rv$. Since the *ΔsecA2* mutant demonstrated truncated bacterial growth *in vivo* we hypothesized that virulence factors would be found in lower quantities in the culture medium of the mutant, and that this difference would be observable early, corresponding to attenuation of virulence in the animal.

Most of the proteins observed in the CFP of each culture were represented in identical amounts at 7 and 14 days of growth (Figure 2.1). Only two proteins were identified from the 2DE gels as being released in reduced amounts from the *ΔsecA2* mutant: SodA and Rv0390. SodA, superoxide dismutase, functions in the reducing of bactericidal superoxide radicals to hydrogen peroxide and water. Rv0390 has no known function, but is shown to be upregulated under nutrient starvation, indicating a possible role *in vivo* during stress conditions [30]. Building from the 2DE gel observations and the known function of SodA, the amount and localization of SodA and KatG was analyzed using immunostaining with antibodies specific for these proteins (Figure 2.2). Using PhoS1 and DnaK as total protein concentration controls, and GroEL as a cell lysis control, we observed that not only was SodA differentially released in greater abundance from the wild-type, but it accumulated in the cytoplasm of the mutant. This observation argues against the possibility of varied levels of transcription between the mutant and the wild-type, but rather is evidence that SecA2 actively participates in secretion of the protein. Also of interest is the observation that KatG presented the same pattern of decreased abundance in the medium in *in vitro* growth of the mutant at 3 and 5 days. This trend, however, did not continue at 7 and 14 days as dramatically as at early time

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points and there was no observed accumulation of KatG in the cytosol like there was for SodA. One reason for this could be a transcription feedback inhibition limiting the concentration of catalase in the cytosol. This phenomenon has been well characterized in numerous eukaryotic [31-33] and prokaryotic systems [34-36].

The mechanisms by which these proteins are recognized for translocation, and the part SecA2 plays in their secretion have yet to be elucidated. Essential to the export of proteins through the general sec-dependent secretion pathway is the presence of a cleavable amino acid signal sequence at the N-terminal region of the translated protein. If it is true that these proteins are secreted through sec-dependent mechanisms, the manner in which these proteins are recognized and slated for translocation, and their insertion into the membrane-bound secretion apparatus is an enigma as neither SodA, KatG, nor Rv0390 contain classical signal sequences. Moreover, none of these proteins shared sequence homologies that would indicate possible sites of recognition by components of the Sec machinery. The presence of an unrecognized motif shared by this subset of proteins, however, cannot be ruled out. Another scenerio could be that these proteins are transported in their mature, folded state and recognition could be dependant upon the secondary or even tertiary structures of these molecules. This type of secretion has been observed in other bacterial species [37], but even then the recognition of precursor proteins depends upon a signal sequence motif.

Another area of ambiguity is the precise role assumed by SecA2 in protein secretion in general. Since this protein retains homology in a motif corresponding to the ATP binding site in SecA1 it can be assumed that this component is necessary for SecA2-mediated protein translocation. One possible function of SecA2 could be that it functions

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like SecA1 in a homodimeric fashion interacting with the other members of the Sec pathway to shuttle signal sequence-lacking proteins through the plasma membrane. Another possibility is the formation of a SecA1:SecA2 heterodimer that facilitates the export of this subset of proteins. A third possibility is that SecA2 serves as a cytosolic chaperone for leaderless proteins to help them effectively dock with the membrane-bound SecA1 homodimer. A final role could be that SecA2 functions as an intermediary component of a larger secretion system where it is involved with the membrane anchoring of other molecules essential for the translocation of leaderless proteins. In support of this idea, it has been observed in *L. monocytogenes* that *secA2* resides in a locus with other genes necessary for the transport of virulence factors [38]. This locus includes a homologue of SecY2, another essential component of the Sec pathway, among other uncharacterized genes (*orf1, orf2,* and *orf3*), all of which are essential for virulence [33].

The observed increase of proteins RplL (50S ribosomal protein), CspA (cold shock protein A) and the different migration pattern of Acr in the culture medium of the *ΔsecA2* mutant may simply reflect an increased state of stress in the bacilli since these proteins have all been characterized as stress-induced [39-42]. Thus their increased presence may have no correlation to the specific mutation, or altered mechanisms of secretion and cell component trafficking as much as a general response to an abnormal physiological state.

With the knowledge that ROI represent a significant bactericidal component of the immune response, and that superoxide specifically has been shown to play a role in initial containment of infection with *M. tuberculosis* [43], it is tempting to speculate that

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the proteins affected by *secA2* deletion represent a subset of gene products necessary for bacterial subversion of host defense mechanisms. It has been shown that these gene products confer protection in a broad range of bacterial species against ROI produced during the oxidative burst of the host [44-46]. This could serve to explain the observations that bacterial growth is truncated only during the first 20 days *in vivo* after infection with the mutant compared to the wild-type, thus corresponding to initial, innate mechanisms of infection containment before the arrival of the acquired immune response. Additional evidence for the role of SecA2-secretion partners involved in virulence *in vivo* can be seen in other bacterial systems. In *S. gordonii* the SecA homologue is associated with secretion of an adhesin, GspB, that is critical to virulence of the organism [3]. Even more interesting is research showing that the *secA2* gene of *L. monocytogenes* mediates the secretion of 17 distinct proteins independent of SecA1 essential for the subversion of the host inflammatory response [47].

As an intracellular pathogen, *M. tuberculosis* is required to confront many hostile environments including extremes in pH, oxidative stress, nutrient deprivation, toxic enzymes and free-radicals. Specialized secretion mechanisms that control the translocation of specific subsets of virulence factors provide this organism with the ability to strictly control its response to the environment thus ensuring its survival within the host.

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Chapter 3: An *in vitro* approach to the search for new antigens of *M. tuberculosis* through the mimicking of *in vivo* conditions

The aims of this study were to:

1. evaluate the profile of proteins found in the culture filtrate of *M. tuberculosis* grown under *in vitro* conditions believed to mimic animal infection,
2. produce recombinant *M. tuberculosis* proteins found to be expressed at higher levels in mimicked *in vivo* conditions.

Abstract

It is believed that after initial containment by the host the *M. tuberculosis* bacilli survive within a granuloma in a microaerophilic environment. As the pathophysiology of disease changes over the course of infection, I hypothesize that the bacilli must change in order to survive within the host and, therefore, antigens required to provide protection to the host during late stages of infection will differ from those that elicit early protection. As such, the identification of *M. tuberculosis* proteins produced during infection is an important step toward unraveling the kinetics of the immune response to tuberculosis. In order to identify proteins produced by the bacilli under conditions found in the host during the chronic stage of infection, *M. tuberculosis* was cultured under microaerophilic, anaerobic, and alternate carbon-source conditions. The culture filtrates of these cultures were then analyzed by 2DE and individual protein spots observed to be in greater

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abundance were identified by MS and MS/MS. This research identified α-crystallin (Acr, Rv2031c), single-stranded binding protein (Ssb, Rv0054), pyrophosphatase (Ppa, Rv3628), and bacterioferritin (BfrB, Rv3841c) as hypoxia-induced proteins that were present in the culture medium. The proteins identified in this work as well as several proteins identified by others as induced by hypoxic stress (Fba, Icl, Rv1738, Rv1813c, Acg, Rv2624c, and Rv2626c) were cloned into *E. coli* and produced in a recombinant form. Exported repetitive protein (Erp, Rv3810) was also produced because of its strong antigenic properties. These proteins were utilized in the next chapter of this work to assess the immune response over the time course of infection.

3.1 Introduction

Tuberculosis is the second leading cause of death by a single etiologic agent worldwide and remains the leading killer of women in the world [1]. Nearly 9 million people contract this disease and 2 million people die from it each year. The enormity of this pandemic is illustrated in the estimation that one third of the world's population, over 2 billion people, are believed to be infected with *M.* tuberculosis in a latent state. Many cases of clinically active tuberculosis develop from reactivation of this latent condition, reflecting emergence of an actively growing population from bacilli in a dormant or nonreplicative state [2, 3]. This is especially true among elderly patients and those with compromised immune systems, such as those with diabetes or AIDS [4]. The successful diagnosis and treatment of this disease depends upon the ability to recognize active as well as latent infection.

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Even though BCG has been in use for over 80 years, it is clear that more effective preventative measures are essential if we hope to conquer this disease. The current BCG vaccine used throughout most of the world has several major drawbacks. First, BCG is a live organism that must be cleared by a healthy host response, and those with compromised immune systems, such as HIV-positive individuals, have been observed to develop disseminated BCG infection when given the vaccine [5, 6]. Second, it has been shown that BCG only gives significant protection during early adolescence while its efficacy wanes in adulthood. Third, the overall protective efficacy varies widely from 0-80%, depending on geographical location and type of study. Presumably this is because continuous exposure to high levels environmental mycobacteria (EM) raise the baseline in non-vaccinated groups resulting in an apparent decrease in the protective efficacy of BCG. Additionally, constant exposure to EM can deplete the T cell memory pool in vaccinated individuals, thus reversing the protection conferred by BCG [7]. Lastly, this vaccine interferes with the diagnostic PPD test, yielding false positives in those that have received the vaccine. In light of these problems, the development of a more effective vaccine that does not interfere with current diagnostics and is safe to administer to the immunocompromised population is a major focus of study throughout the world.

To better understand the host immune response to this organism it is becoming increasingly essential to study the bacterium itself. Historically, our knowledge of the physiology of *M. tuberculosis* has come by observing the organism as it is grown in the laboratory while difficulty of performing biochemical and analytical tests on the small number of bacilli isolated from an animal infection has hampered efforts to elucidate true characteristics of *in vivo* infection. However, metabolic and regulatory processes need to

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be considered in light of their environmental conditions and stimuli. Based on the premise that bacilli exist within the host in an environment that is significantly different than that found in a culture flask, various models have been developed over the years in order to determine the precise metabolic and physiological state of *M. tuberculosis* during the course of an animal infection. Recent research has focused mainly on the gene and protein profiles of bacilli grown under a variety of conditions believed to better emulate true animal infection in hopes of developing vaccine candidates, discovering new drug targets and exposing novel diagnostic markers. Bloch and Segal observed gross physiological differences between *in vitro* cultures and bacilli retrieved from a mouse (e.g. cell wall constituents) [8]. This was accompanied by a distinct increase in virulence in the mouse-derived bacilli. More recently, bacilli grown *in vitro* under conditions reflective of the host environment were evaluated for their gene transcription [9-14] and protein profile changes [12, 14].

Perhaps the most famous of these models is the Wayne model of dormancy put forth in the late 1970s and early 1980s [12, 15]. Based on evidence that the lesions within an infected host were restricted in oxygen availability, Wayne demonstrated that *M. tuberculosis* was indeed capable of survival under conditions of moderate and extreme anoxia. In the initial version of the Wayne model, cultures of *M. tuberculosis* were allowed to settle by gravity through a self-generated oxygen gradient to an anaerobic state at the bottom of an unshaken culture flask. Wayne observed that these bacilli became dormant and non-replicative while exhibiting increased expression of isocitrate lyase and glycine dehydrogenase. Subsequent passage of these bacteria in oxygen-rich cultures resulted in the synchronous resumption of replication, indicating a stage-specific

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cell cycle arrest. In a subsequent revision of this model, *M. tuberculosis* was grown in slowly agitated, sealed cultures with a limited amount of oxygen. This allowed the examination of the bacteria and bacterial byproducts at defined oxygen concentrations as the available oxygen was gradually used up. Later work showed that this anaerobic-induced non-replicative state resulted in the bacteria becoming refractory to common antimycobacterial drugs used to target cellular processes [13]. These data may help paint a picture of a dormant state in which the tubercle bacilli can escape antibiotic sterilization and persist latently within a host for years.

Of particular interest in recent years has been the observation that specific metabolic changes are likely to be necessary for *in vivo* growth. In the metabolic pathways of bacteria, there is a key branch point between the tricarboxylic acid (TCA) and glyoxylate cycles determined by the available carbon source. When glucose or other 6-carbon sugars are utilized by the organism the TCA cycle converts acetyl-CoA, derived from the oxidation of pyruvate, to carbon dioxide and several other phosphorylated molecules to provide energy and reducing power for cellular processes. Alternatively, acetyl-CoA can also be produced by the β-oxidation of available fatty acids in the absence of sugars. However, with each cycle of the TCA pathway, two carbon atoms are lost in the form of carbon dioxide resulting in the need to replenish the dicarboxylic acids that are drained from the TCA cycle for other biosynthetic processes. In the absence of glucose this is accomplished by the bacteria utilizing the glyoxylate shunt which converts isocitrate to malate and succinate thus bypassing the steps where loss of carbon occurs. In *E. coli,* it has been demonstrated that production of the enzymes of the glyoxylate

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shunt, isocitrate lyase and malate synthase, is induced when acetate or fatty acids are the sole carbon source [16].

In 1982, Wayne discovered that the isocitrate lyase (Icl) enzyme in *M. tuberculosis* was present in higher abundance when the bacterium was grown under oxygen limiting conditions [12] indicating a possible role for this metabolic pathway during infection. Subsequent work has revealed that the glyoxylate shunt not only plays a role in infection, but is essential to the persistence of the organism. McKinney, et al. demonstrated through a series of experiments using *M. tuberculosis* mutants that Icl is essential for survival of the organism within activated macrophages and during chronic stages of infection within the mouse [17]. Interestingly, no difference is observed between the mutant and the wild-type in non-activated macrophages nor during the first two weeks of the mouse infection before the establishment of acquired immunity [17]. Therefore, this pathway could mark a protective response to the onset of acquired immunity and the subsequent activation of infected macrophages, however, the mechanism by which the glyoxylate shunt alleviates the effects of acquired immunity are not currently known. The significance of this pathway in microbial pathogenesis in general is illustrated in the fact that a wide range of organisms, from the human pathogenic fungus, *Candida albicans* [18, 19] to the plant fungus, *Magnaporthe grisea* [20] to *Mycobacterium tuberculosis* [17] all exhibit decreased virulence upon inhibition or mutation of enzymes involved in the glyoxylate cycle.

Building upon this hypoxia model of bacterial dormancy, other labs have undertaken to describe the gene transcription and protein expression patterns likely induced in infection [9, 11, 14]. In addition, true *in vivo* assays of gene transcription have

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also been developed. In one experiment, using a method to capture and identify genes transcribed in response to phagocytosis by human macrophages several alternative sigma factors and transcriptional regulators were observed to be induced *in vivo* [21]. While this model lacks such defining characteristics of true infection as activation of infected macrophages, with the ensuing production of RNI, and environmental changes due to the physiology of granuloma formation with the corresponding nutrient and oxygen deprivation, it does define a more representative set of factors likely encountered during animal infection. In an attempt to evaluate metabolic activities during true infection, Timm, *et al.* analyzed select *M. tuberculosis* genes for their levels of transcription in infected mouse and human lung samples [22]. They discovered, by comparison of *sigA*/16S mRNA levels, that bacterial growth characteristics during later stages of infection *in vivo* were similar to those observed *in vitro* when cultures entered the stationary phase, suggesting a metabolic shift to dormancy in a persistent animal infection. By further analysis of individual genes responsive to nutrient and oxygen depletion, these researchers found that upon the transition from acute to chronic infection in the mouse, environmental pressures such as oxygen limitation or increased nitric oxide concentration and decreased iron availability were present. They also demonstrated a catabolic shift to the metabolism of fatty acids by the bacteria during later stages of infection [22].

In order to define transcriptional and protein production patterns during an animal infection, bacilli was grown under *in vitro* conditions that mimic the hypothesized environment within an animal infection followed by the implementation of 2DE and MS/MS were employed for the identification of individual proteins. Based on research

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previously performed and corroborated by the work just described, the culture conditions utilized to mimic *in vivo* conditions include the following: the Wayne model's gradual shift-down to hypoxia, a rapid shift to hypoxia, and a normally aerated culture where the fatty acid dipalmitoylphosphatidylcholine replaces glycerol as the carbon source. While most of the proteins visualized by 2-DE appeared to remain constant regardless of the tested growth conditions this work did identify several proteins that were observed to be present in higher levels under the non-standard growing conditions. Those proteins observed to be up-regulated under hypoxic or alternate carbon source growth in this study as well as in the work of others [9, 11, 14] were produced using an *E. coli* expression system. These recombinant proteins were purified for use in a future assay of the kinetics of the immune response.

3.2 Material and methods

3.2.1 Bacterial growth

M. tuberculosis $H_{37}Rv$ (obtained from American Type Culture Collection, Manassas, VA) was used to inoculate Middlebrook 7H10 agar plates with OADC and grown to a confluent lawn (15 days). Subsequently, four cultures of 10 ml GAS medium [23]were inoculated from these plates and allowed to grow for 14 days at 37°C with rapid agitation using magnetic stir bars. These cultures were passed twice, increasing the volume of each passage 10x with the final culture volume being 1 L. After 14 days growth in 1 L cultures, the bacteria were collected by centrifugation at 2,500 rpm. The supernatants were discarded and the cell pellets were washed 2x with GAS medium **without glycerol**. Each cell pellet was added to one of four separate cultures prepared as

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described in Table 3.1 and allowed to grow at 37°C for 14 days. This procedure (inoculating 10 ml cultures from the same plates) was repeated three times to produce consecutive batches.

Table 3.1 Growth conditions of various stress conditions

Culture medium	Ingredients	Culture conditions
GAS - Standard	0.04% Bacto Casitone (Difco, Sparks, MD), 0.005% ferric ammonium citrate, 0.4% K_2HPO_4, 0.2% citric acid, 0.1% L-alanine, 0.12% $MgCl_2$-$6H_2O$, 0.06% K_2SO_4, 0.2% NH_4Cl, 0.072% NaOH, and 1.0% (v/v) glycerol. pH=7.0	I L CULTURE, FREE AERATION, RAPID AGITATION ON SHAKER TABLE (210rpm) (standard growth conditions)
GAS - Rapid hypoxia	0.04% Bacto Casitone (Difco, Sparks, MD), 0.005% ferric ammonium citrate, 0.4% K_2HPO_4, 0.2% citric acid, 0.1% L-alanine, 0.12% $MgCl_2$-$6H_2O$, 0.06% K_2SO_4, 0.2% NH_4Cl, 0.072% NaOH, and 1.0% (v/v) glycerol. pH=7.0	I L CULTURE OVERLAID WITH MINERAL OIL, NO AGITATION
GAS - Gradual hypoxia	0.04% Bacto Casitone (Difco, Sparks, MD), 0.005% ferric ammonium citrate, 0.4% K_2HPO_4, 0.2% citric acid, 0.1% L-alanine, 0.12% $MgCl_2$-$6H_2O$, 0.06% K_2SO_4, 0.2% NH_4Cl, 0.072% NaOH, and 1.0% (v/v) glycerol. pH=7.0	750 ml CULTURE IN 1 L BOTTLE (0.5 head-space ratio), VERY SLOW AGITATION WITH MAGNETIC STIR BAR (30-40 rpm)
GAS with phospholipid	0.04% Bacto Casitone (Difco, Sparks, MD), 0.005% ferric ammonium citrate, 0.4% K_2HPO_4, 0.2% citric acid, 0.1% L-alanine, 0.12% $MgCl_2$-$6H_2O$, 0.06% K_2SO_4, 0.2% NH_4Cl, 0.072% NaOH, and 0.5% ***phospholipid***. pH=7.0	1 L CULTURE, STANDARD GROWTH CONDITIONS

3.2.2 Protein preparation and gel electrophoresis

The CFP from all cultures above were harvested and prepared as described [24]. Briefly, cultures were vacuum filtered through a 0.2μm ZapCap® filter (ISCBioExpress, UT) and 5 g of sodium azide was added to each filtrate sample to ensure sterilization. For the "GAS – rapid hypoxia" culture, the culture medium was isolated by pipetting the liquid phase exclusively and leaving behind the mineral oil. CFPs were then concentrated through a 3,000 molecular weight cut off (mwco) Amicon® filter (Millipore, MA),

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washed 3x with ddH_2O, and concentrated to 30 ml. To eliminate background nucleic acid staining in the gels, CFP samples were incubated with 0.06% DNase and RNase for 30 min at room temperature. Protein concentrations were determined by the BCA (biconconinic acid) assay and CFP were aliquoted into 200 and 400 μg samples for 2D gel electrophoresis. Dried aliquots of CFP from each growth condition were dissolved in a solubilization buffer containing 8.5 M Urea, 2% CHAPS, 20 mM DTT and 0.5% ampholytes (pH 4-7) by bath sonication and vortexing. The solution was allowed to sit at 4°C overnight. Isoelectric focusing was performed by rehydration of the solubilized samples into 13 cm immobilized pH gradient (IPG) strips (pH 4-7) and applying current across the strip using the IPGphor system (Amersham Pharmacia Biotech, NJ) as described in Table 3.2.

Table 3.2 IPGhor program conditions

Step	Gradient or Constant (Step)	Voltage	Time (h)
1	Step	50v	12.0
2	Gradient	500v	1.0
3	Step	1000v	1.0
4	Gradient	4000v	1.5
5	Step	4000v	2.0
6	Gradient	8000v	2.0
7	Step	8000v	6.0

The IPG strips were then equilibrated in 5 ml of an SDS equilibration buffer (50 mM Tris-HCl, 6 M Urea, 30% glycerol, 2% SDS and 65 mM DTT) for 20 min at room temperature with gentle agitation on a rocking table. The strips were subsequently placed on 12% SDS polyacrylamide gels and sealed with an agarose solution (1.44% glycine, 0.303% Tris-base, 0.1% SDS and 0.1% agarose in ddH_2O). Second-dimension separation

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by SDS-PAGE was performed using 15mA per gel for approximately 8 h. Proteins were visualized by Coomassie brilliant blue R-250 (Sigma-Aldrich, WI) and silver nitrate stains. 2D gels were analyzed using the PDQuest 7.0 software (BioRad Laboratories, CA) by the combination and averaging of three individual gels from each growth condition. The resulting composite gel images were normalized against the total protein amount in all spots of each image and specific protein spots were compared. Histograms were generated for specific spots to display their relative increase or decrease in response to *in vivo* mimicking growth conditions. Histograms were also produced for three control proteins that appeared to exhibit the same abundance under all conditions were also analyzed.

3.2.3 Protein identification

Proteins spots of interest were identified as described in Chapter 2 of this work.

3.2.4 Cloning of $H_{37}Rv$ genes into E. coli expression strains

The corresponding gene sequences of proteins identified by MS-MS were obtained from Tuberculist (http://genolist.pasteur.fr/TubercuList). Genes encoding an N-terminal amino acid signal sequence were cloned after the point of cleavage of the secretion signal. Signal sequence-containing proteins and cleavage points were determined using SMART (Simple Modular Architecture Research Tool) (http://smart.embl-heidelberg.de/) [25, 26]. To PCR individual DNA sequences of interest 2 μl (25 ng/μl) $H_{37}Rv$ DNA (01.Rv.2.10.17.x obtained from), 4 μl of each primer (1μg/μl), and 1 Ready-to-go PCR Bead (Amersham Biosciences, NJ) were added to 15 μl

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sterile ddH_2O. PCR was allowed to progress as follows: heat to melting temperature of 96°C for one min, cool to elongation temperature of 72°C for three min, and cool to annealing temperature of 53-54.5°C for one min according to the melting temperature of each primer set (Table 3.3), and repeated through 25 cycles. PCR products were run on 1.5% agarose gels and purified by excision from the gel followed by gel extraction using QIAquick® Gel Extraction Kit (Qiagen Incorporated, CA). Purified PCR products were ligated into the TA pGEM vector and transformed by heat shock into the *E. coli* Top10 OneShot® bacterial strain (Invitrogen Corporation, CA). S.O.C. medium (200 μl) was added to each tube and bacterial cultures were incubated at 37°C for 30 min. LB agar plates containing 100 μg/ml ampicillin and coated with 80 μl of a 20 mg/ml solution of x-gal (dissolved in DMF and allowed to evaporate from plate surface for 60 min) were inoculated with one loop of each culture and allowed to grow at 37°C overnight. Positive (white) colonies were selected. From each ligation, three positive colonies were used to inoculate three separate LB broth cultures (5 ml). pET23 plasmid was isolated by growing a 100 ml culture of DH5α:pET23 followed by MaxiPrep® purification (Qiagen Incorporated, CA) of plasmid following manufacturer instructions. pGEM: $H_{37}Rv$ gene and pET23 plasmid were concurrently digested with restriction enzymes (Table 3.4) and 8 μl of each clone and 1 μl of pET23 plasmid were mixed with 1 μl of 10 mM ATP, 1.2 μl 10x AP Buffer, and 1 μl T4 DNA ligase and incubated for 16 h at 37°C. *E. coli* Top10 OneShot® bacteria were then transformed with pET23:$H_{37}Rv$ gene constructs by heat shock and allowed to grow for 1 h as before. Transformants were plated on LB agar plates with 100 μg/ml ampicillin and incubated overnight at 37°C. Single colonies were picked as before and inoculated into 5 ml LB^{amp} and incubated for 6 h. MiniPrep®

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purification (Qiagen Incorporated, CA) of plasmids was performed according to manufacturer instructions. Transformation of *E. coli* expression strains (Table 3.3) was performed identically to previous transformations. The pET expression strains utilized in this work were either pET (DE3) pLysS, pLysE, or Star pLysS. The pLys designation indicates the presence of a plasmid, which encodes T7 lysozyme to a small (S) or large (E) extent, thus inhibiting low levels of T7 polymerase activity before induction. The Star designation indicates that the strain encodes higher levels of GC-rich tRNA codons needed to translate high GC-rich genes.

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Table 3.3 Cloning of genes and production of recombinant proteins

Gene	Name	Sense Primer (3' to 5')	Antisense Primer (3' to 5')	Signal sequence removed	Restriction enzyme sites	*E. coli* expression strain	Annealing Temp. Used
Rv0363c	Fba	ATTCGCCATATGCCTATCGCAACGCCCGA	CTCGAGGTGGGTTAGGGACTTTCCGG	None	NdeI, XhoI	BL21 (DE3) Star pLysS	54.3°C
Rv0467	Icl	GGAATTCCATATGATGTCTGTCGTCGGCACCCC	CGGCATAAGCTTGTGGAACTGGCCCTCTTCGG	None	NdeI, HindIII	BL21 (DE3) pLysE	56.9°C
Rv1738		GGAATTCCATATGATGTGCGGCGACCAGTCGGA	CGGCATCTCGAGATACAACAATCGCGCCGGCT	None	NdeI, XhoI	BL21 (DE3) pLysE	56.9°C
Rv1813c		GGAATTCCATATGCATCTCGCCAACGGTTCGAT	CGGCATCTCGAGGTTGCACGCCCAGTTGACGA	Yes (residues 1-33)	NdeI, XhoI	BL21 (DE3) pLysE	55.6°C
Rv1860	MPT-32	GGAATTCCATATGGATCCGGAGCCAGCGCCCCC	CGGCATCTCGAGGGCCGGTAAGGTCCGCTGCG	Yes (residues 1-40)	NdeI, XhoI	BL21 (DE3) pLysE	60.8°C
Rv2032	Acg	GGAATTCCATATGATGCCGGACACCATGGTGAC	CGGCATCTCGAGCCGGTGATCCTTAGCCCGAA	None	NdeI, XhoI	BL21 (DE3) pLysE	56.9°C
Rv2624c		GGAATTCCATATGATGTCTGGGAGAGGAGAGCC	CGGCATCTCGAGGCGGCGAACGACAAGCACCG	None	NdeI, XhoI	BL21 (DE3) Star pLysS	56.9°C
Rv2626c		GGAATTCCATATGATGACCACCGCACGCGACAT	CGGCATCTCGAGGCTGGCGAGGGCCATGGGCG	None	NdeI, XhoI	BL21 (DE3)	56.9°C
Rv3628	Ppa	GGAATTCCATATGGTGCAATTCGACGTGACCAT	CGGCATCTCGAGGTGTGTACCGGCCTTGAAGC	None	NdeI, XhoI	BL21 (DE3) pLysE	54.3°C
Rv3810	Erp	GGAATTCCATATGTATTTTCTTGTCTACGAATC	CGGCATCTCGAGGGCGACCGGCACGGTGATTG	Yes	NdeI, XhoI	BL21 (DE3) pLysE	54.3°C
Rv3841	BfrB	GGAATTCCATATGATGACAGAATACGAAGGGCC	CGGCATCTCGAGGAGGCGGCCCCCGGCAGCGT	Yes	NdeI, XhoI	BL21 (DE3) pLysE	54.3°C

3.2.5 Production and purification of recombinant proteins

LB broth (2L) containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol was inoculated from 5 ml cultures of each clone and allowed to grow to an OD of 0.3 (A_{600}). Gene expression was induced by adding IPTG to a final concentration of 1.0 mM and cultures incubated for 4-6 h at 37°C. Bacterial pellets were isolated by centrifugation at 2,000 x g for 30 min. Cell pellets were suspended in 5-10 ml Binding Buffer (Table 3.4) containing protease inhibitors (0.025% Pepstatin A, 0.05% Leupeptin, 0.2% PMSF, 2.5% DNase, 2.5% Rnase, and 5% Lysozyme) and disrupted by sonication (5 X 60 seconds, pulsed). Lysed pellets were centrifuged at 3,000 x g for 15 min and supernatants were added to Ni-affinity columns (His-Bind Resin ®, Novagen, CA). Columns were washed by sequential addition of 15 column vol of Binding Buffer, 10

Table 3.4 Protein purification solutions

Solution	Recipe
Charge Buffer	1.3% $NiSO_4$ in ddH_2O
Binding Buffer	5mM Imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 7.9 in ddH_2O
Wash Buffer	60mM Imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 7.9 in ddH_2O
Tris Buffer	10mM Tris-HCl, pH 7.9 in ET-Free ddH_2O
Tris with ASB-14	10mM Tris-HCl, 0.5% ASB-14, pH 7.9 in ET-Free ddH_2O
Elute Buffer	1.0M Imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 7.9 in ddH_2O
Ammonium bicarbonate (AmBic)	10mM ammonium bicarbonate in ET-free H2O

vol of Wash Buffer, 5 column vol of Tris Buffer, 5 column vol of Tris with the detergent ASB-14 to remove LPS, and 5 column vol of TrisBuffer. Each buffer was allowed to pass through the column by gravity until the solution volume reached the top of the resin before the next buffer was added. Proteins were the collected by addition of 5 ml Elute

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Buffer. Protein solutions were dialyzed against 10 mM ammonium bicarbonate for 72 h, refreshing the solution each 24 h. Dialyzed protein solutions were then subjected to filtration over a 5,000 mwco membrane and washed 3 x with 15 ml endotoxin-free (ET-Free) H_2O to remove all ammonium bicarbonate. For the purification of Rv1813c, all buffers (Table 3.4), except the Charge Buffer, were made with the addition of urea to 6M final concentration. Once eluted from the column with 6M urea Elute Buffer, the solution was dialyzed against 6M urea, 10mM AmBic for 24 h, followed by 4M urea, 10mM ammonium bicarbonate for 24 h, followed by 2M urea, 10mM ammonium bicarbonate for 24 h, followed by 10mM ammonium bicarbonate for 24 h. Remaining solution was centrifuged at 14,000 x g for 10 min. Protein concentrations of all proteins were determined by BCA assay and aliquots of 20 μg were made and frozen at –80°C.

3.3 Results

3.3.1 The culture filtrate fraction of M. tuberculosis $H_{37}Rv$

CFP was isolated from a culture of $H_{37}Rv$ *M. tuberculosis* grown to stationary phase (14 days) at 37°C. Analysis has shown that a majority of mycobacterial proteins possess a pI of between 4-7 [27, 28]. Separation and visualization of CFP was, therefore, performed by 2D-gel electrophoresis using the pH range of 4-7 for isoelectric focusing, followed by molecular weight separation over a 15% SDS acrylamide gel in the second dimension. Over 350 individual protein spots were observed and 83 different proteins (covering 210 individual spots) were identified following in-gel digestion and analysis by MS and MS/MS (Figure 3.1; Table 3.5). In the past, abundant amounts of material has led to the ease of characterization of several proteins, while the relative scarcity of many

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proteins has hampered efforts to completely identify the entire composition of the CFP fraction. The utilization of the extremely sensitive method of ESI MS and MS/MS analysis has allowed the identification of even the most faintly visible proteins (e.g. spot #44). Since the method of protein identification includes the scoring of observed fragmentation patterns of enzymatically digested proteins based on theoretical peptides derived from the tuberculosis genomic sequence the failure to identify many of the remaining proteins visible by 2DE could be attributed to either post-translational modifications not included in the theoretical data set or the refractive nature of some proteins to enzymatic digestion.

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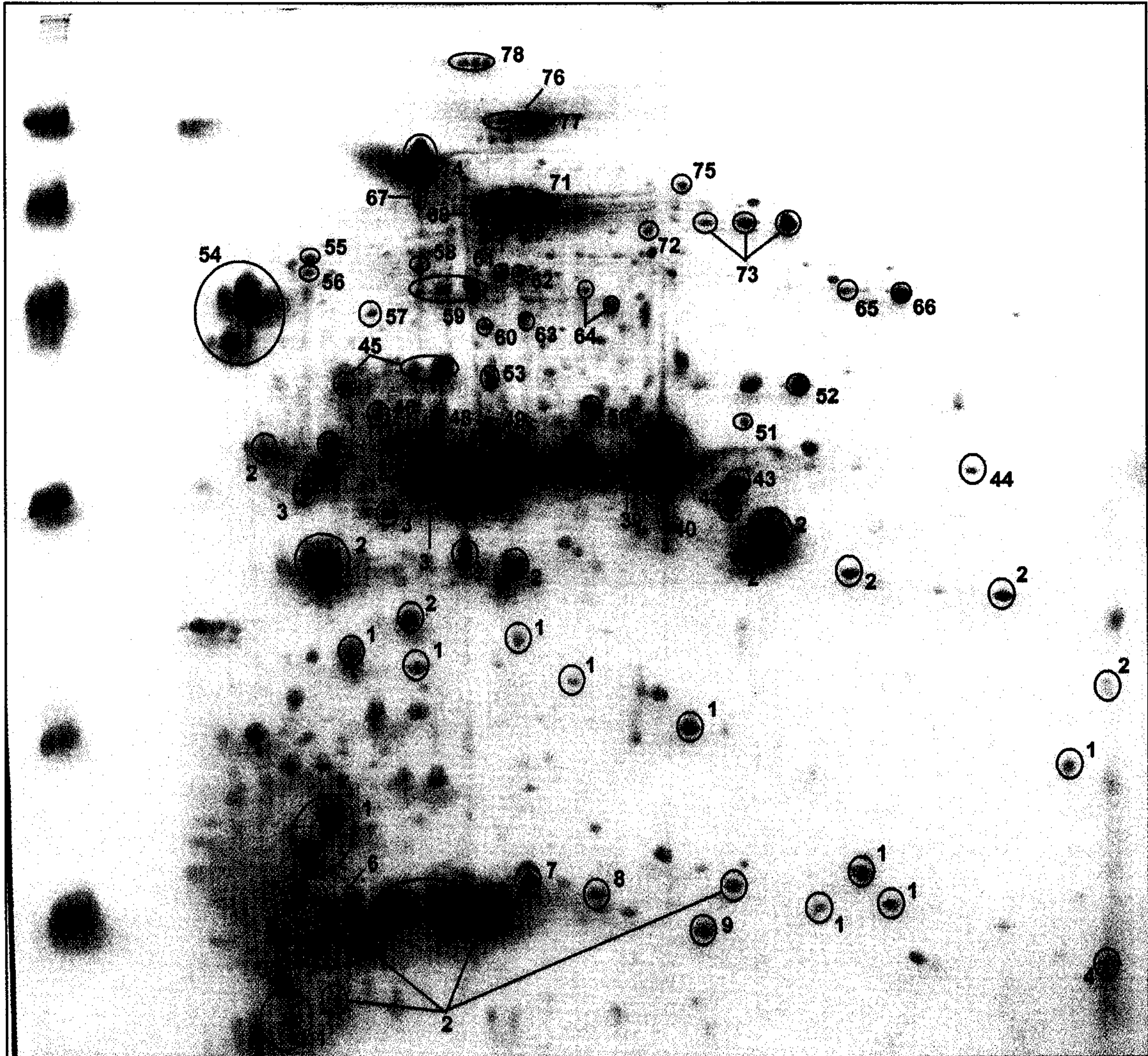

Figure 3.1 2DE gel of the culture filtrate fraction of *M. tuberculosis* H_{37}Rv.
The CFP was analyzed by 2DE in the pH range of 4 – 7. The proteins were separated by isoelectric point in the first dimension and molecular weight in the second dimension. The resultant gel was stained by Coomassie G-250 and individual spots were excised and subjected to ESI MS and MS/MS. Identified proteins are circled and numbered and identification is put forth in Table 3.5.

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Table 3.5 **ESI MS and MS/MS identification of protein spots from the CFP of *M. tuberculosis* $H_{37}Rv$.**

Spot	Protein	Gene	Peptides matched	AA % coverage	Spot	Protein	Gene	Peptides matched	AA % coverage
1	ESAT-6	Rv3875	3	76	40	MPT51	Rv3803c	2	14
2	GroES	Rv3418c	6	57	40		Rv1815	1	20
3	Acr	Rv2031c	5	30	41	Ag85A	Rv3804c	11	30
4		Rv0559c	4	16	43		Rv3310	7	54
5	MPT53	Rv2878c	6	24	44	EchA16	Rv2831	3	22
6		Rv3874	5	48	45	PstS1	Rv0934	5	29
7	TrxC	Rv3914	2	21	46		Rv2971	4	37
8	multiple homologous genes	Rv1038c, Rv3620c, Rv2347c, Rv1197, Rv1792	3	14	47	CelA	Rv0062	8	37
9	CspA	Rv3648c	3	63	48		Rv0315	4	20
10		Rv0983	5	13	49	EchA21	Rv3774	3	21
11		Rv0455c	7	32	50		Rv3389c	7	35
12		Rv0569	5	22	51	DapA	Rv2753c	2	14
13	MPT63	Rv1926c	8	57	52	Fba	Rv0363c	1	5
14		Rv2140c	6	55	53	FecB	Rv3044	4	24
15	Rpi	Rv2465c	2	15	54	MPT32	Rv1860	8	29
16		Rv1984c	3	26	55		Rv3881c	5	14
17	BfrA	Rv1876	5	55	56		Rv0020	3	9
18		Rv1885c	4	51	57		Rv2721c	6	21
19	Ssb	Rv0054c	3	22	58	NdkA	Rv2445c	4	51
20	PpiA	Rv0009	2	15	59	FadA3	Rv1074c	12	39
21	MPT64	Rv1980c	6	41	60	EchA9	Rv1071c	6	19
22	Ppa	Rv3628	2	24	61	FadA4	Rv1323	4	20
23		Rv3036c	9	43	62	KatG	Rv1908c	5	9
24	Adk	Rv0733	2	15	63	TrpS	Rv3336c	5	38
25	EchA3	Rv0632	7	21	64	ArcA	Rv1001	3	11
26	MPT51	Rv3803c	8	64	65	Ald	Rv2780	4	36
27	Frr	Rv2882c	3	23	66	Frr	Rv2882c	7	47
28	SodA	Rv3846	7	47	68	GabD2	Rv0234c	4	15
29		Rv0577	4	23	69	MetK	Rv1392	6	32
30	PepA	Rv0125	3	17	69	SerA	Rv2996c	7	51
30	ESAT-6	Rv3875	1	33	70	GabD2	Rv0234c	8	18
31	PepA	Rv0125	5	24	70	ESAT-6	Rv3875	3	51
31	MPT63	Rv3671c	3	16	71	GlnA	Rv2220	15	33
32	PepA	Rv0125	1	8	72	Fum	Rv1098c	3	25
33	PrcB	Rv2110c	2	9	73		Rv0462	7	28
33		Rv2660c	1	50	74	DnaK	Rv0350	8	20
34	Ag85A	Rv3804c	10	42	75	AldC	Rv2858c	2	9
35	CelA	Rv0062	3	16	76	LeuA	Rv3710	5	10
36	Ag85B	Rv1866c	3	14	77	GlcB	Rv1837c	14	25
37	Ag85A	Rv3804c	4	17	78	Acn	Rv1475c	14	27
37	Ag85B	Rv1866c	7	39	76	LeuA	Rv3710	5	10
38	Ag85C	Rv0129c	4	13	77	GlcB	Rv1837c	14	25
39	PrcA	Rv2109c	4	43	78	Acn	Rv1475c	14	27

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3.3.2 Differential culture filtrate protein composition of bacterial cultures

To evaluate the proteins within the CFP of $H_{37}Rv$ *M. tuberculosis*, 14 day cultures grown under standard and experimental conditions were isolated and visualized by 2D-gel electrophoresis. Throughout four consecutive batches, six protein spots exhibited consistently varied levels of abundance between the standard and altered growth conditions (Figure 3.2). Protein expression patterns and MS identification are put forth in Table 3.5. MPT-32, a glycoprotein thought to be involved in bacterial colonization and invasion into host cells [29], is found in reduced abundance in all stressed, or *in vivo*-mimicking culture conditions. Alternatively, Ag85A, a fibronectin binding protein involved in cell wall mycolylation, only displayed significantly decreased abundance in cultures of gradual oxygen limitation whereas rapid oxygen reduction and phospholipid carbon source cultures yielded no change from standard growth conditions. Four proteins, Acr (Rv2031c), Ssb (Rv0054), BfrB (Rv3841), and Ppa (Rv3628) all demonstrated increased expression under gradual oxygen limitation, while all but Ssb showed the same pattern under rapidly hypoxic conditions. Only Acr and BfrB exhibited increased abundance when grown in a phospholipid-containing medium (Figure 3.3). Gel images from four batches were averaged and normalized against total protein in each sample and compared between all culture conditions. Graphical representation of this analysis is put forth in Figure 3.4. Inset histograms depict relative protein abundance from averaged batches.

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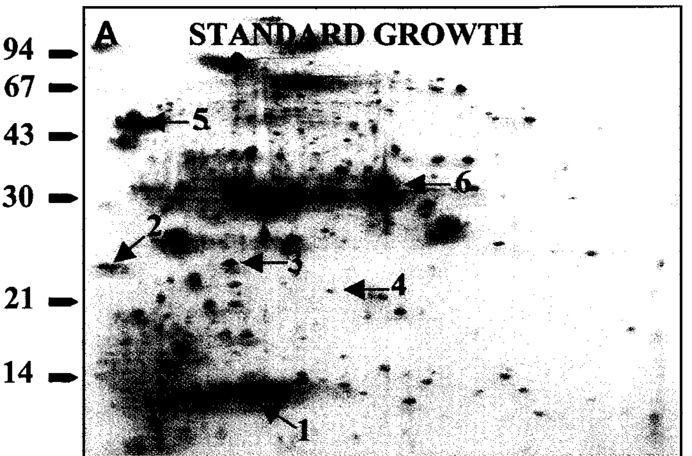

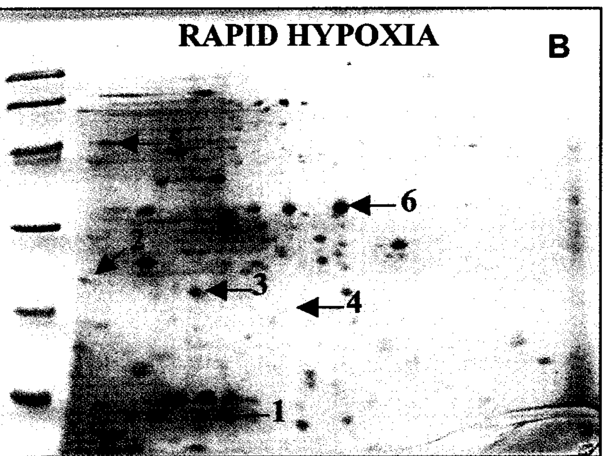

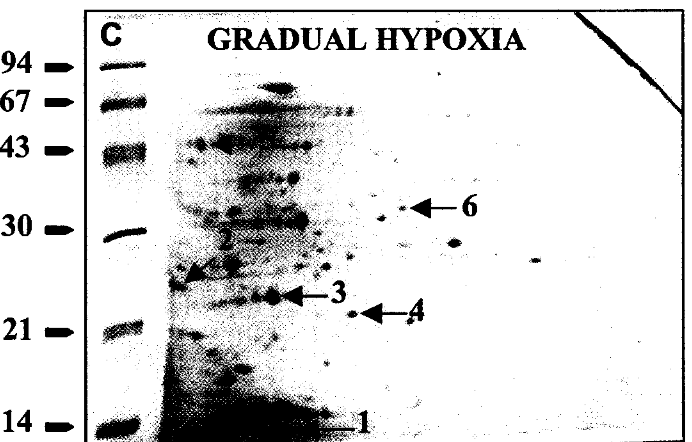

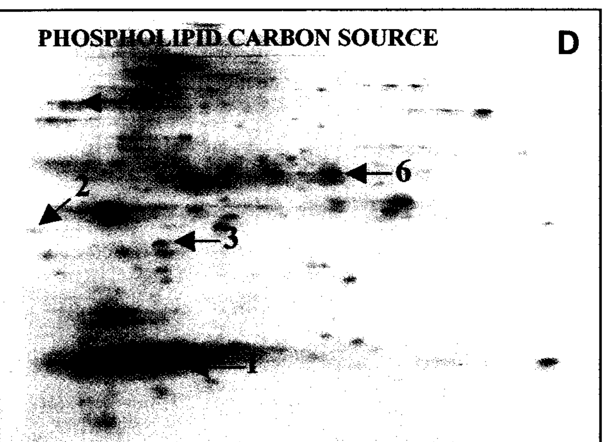

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Figure 3.2 2D gel electrophoresis on the CFPs obtained from different growth conditions.
M. tuberculosis H37Rv was cultured in (A) GAS medium with standard aeration, (B) GAS medium in a standing flask with mineral oil applied to the surface of the culture, (C) GAS medium with a 0.5 ratio head-space in a sealed bottle with slight stirring, and (D) GAS medium with dipalmitoylphosphatidylcholine replacing glycerol and standard aeration. CFP of each culture was analyzed as before and proteins were identified by ESI MS and MS/MS analysis. Protein spots indicated are those that are seen to be consistently different between growth conditions throughout three consecutive batches. Proteins with greater abundance under alternative conditions were identified as (1) Acr (Rv2031c), (2) BfrB (Rv3841), (3) Ppa (Rv3628), and (4) Ssb (Rv0054). Proteins with decreased abundance under alternative conditions were identified as (5) MPT32 (Rv1860) , and (6) Ag85A (Rv3804c).

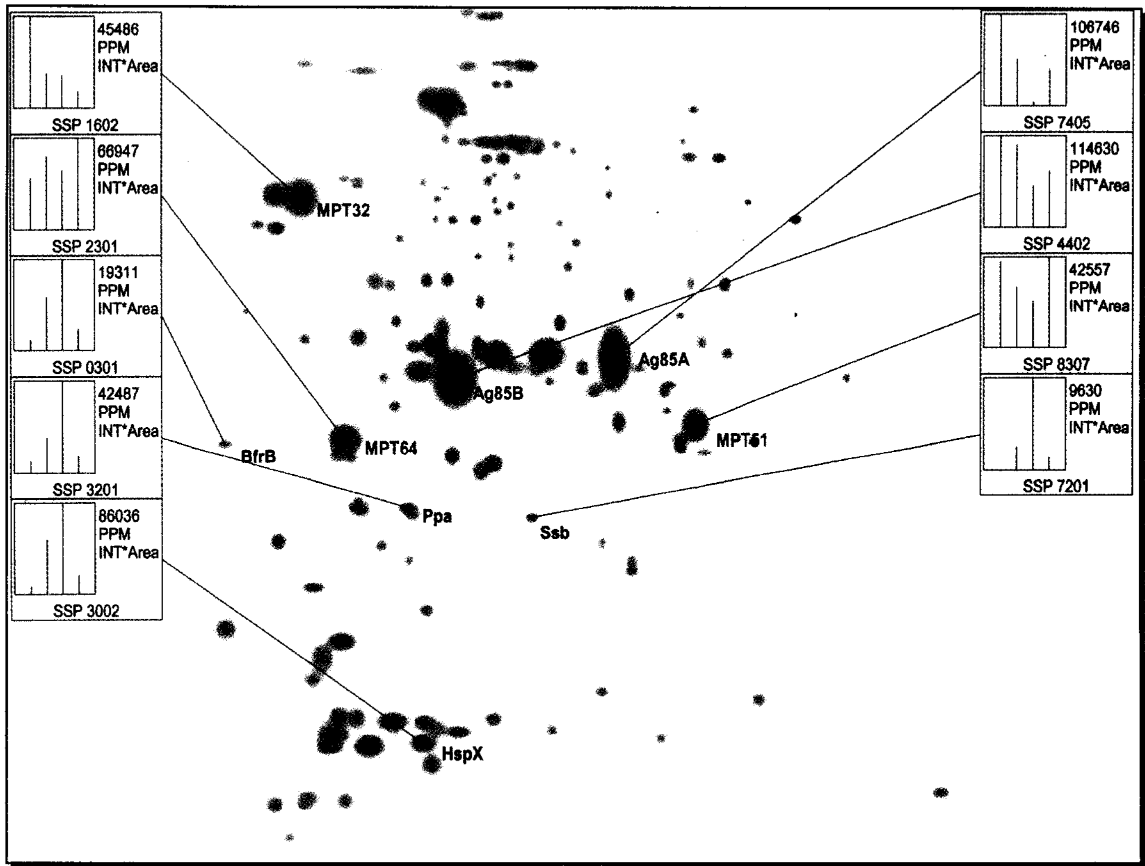

Figure 3.3 **Gaussian image of a composite gel of all growth conditions.**
2D gels were analyzed using the PDQuest 7.0 softward from BioRad Laboratories. The gel images were normalized against the total protein amount in all spots in the gels and specific protein spots were compared. Histograms were generated for specific spots of interest including those proteins observed to be induced (blue) or reduced (red) in response to *in vivo* mimicking growth conditions. Three control proteins (green) were also analyzed. The histograms show the relative abundance of each protein (from left to right) in standard growth, rapid hypoxia, gradual hypoxia and phospholipid carbon source conditions.

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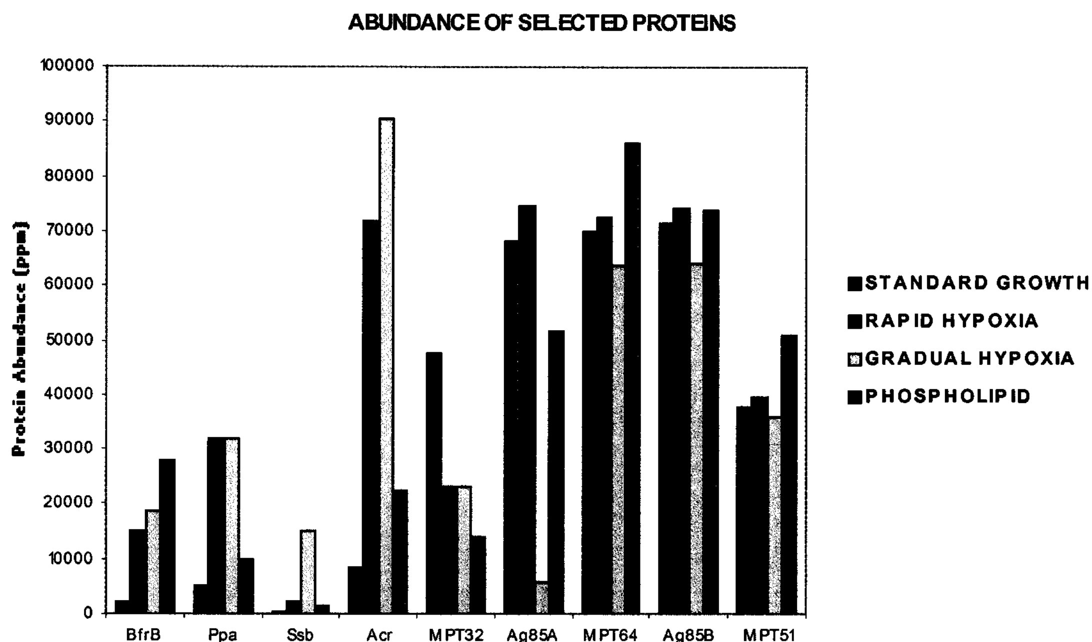

Figure 3.4 Relative abundance of select proteins observed in different growth conditions. Protein abundance was calculated from averaged data of normalized gels from three separate experiments. Standard deviations for all values are less than 8.5%.

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3.3.3 Production of recombinant M. tuberculosis proteins

In order to assess the significance of individual proteins during the course of an animal infection, four *M. tuberculosis* genes exhibiting increased protein production levels during hypoxic and nutrient starvation conditions in this work as well as seven other genes of similar induction characteristics in the work of other laboratories [9, 11, 14] were chosen for further evaluation. These genes were cloned into the pET23b expression vector and transformed into various BL21 *E. coli* strains for the production of recombinant proteins (Table 3.3). Verification of the correct construct for each clone was performed by PCR at each step and re-digestion of the pET23b clones after incorporation of each gene using the same restriction nucleases used to construct the clones. Plasmids were subjected to nucleotide sequencing and determined to contain the mycobacterial gene of interest in the correct orientation and in frame with the T7 promoter. Transformed bacterial cultures were induced by 0.5-1.0mM IPTG and recombinant protein was purified by Ni-affinity column chromatography. Protein identity and purity was verified by SDS-PAGE followed by mass spectrometry (Figure 3.5 and Table 3.6). Because of the ability of endotoxin to alter dendritic cell and lymphocyte activities in culture, LPS levels were evaluated and determined to be less than 13 U/mg by the (LAL) assay (Table 3.6).

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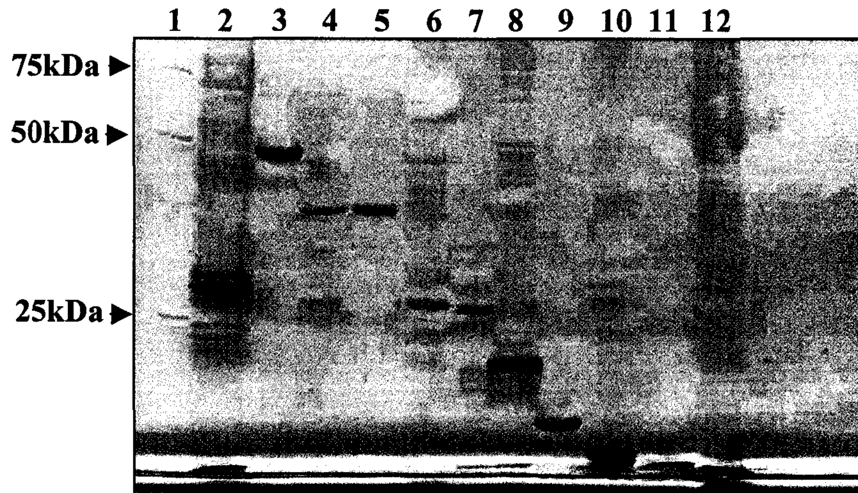

Figure 3.5 **SDS-PAGE of cloned proteins after column purification.** CFP (mg) and individual proteins (5mg) were loaded onto a 12.5% SDS gel and 30 mA was applied to the gel for 1.5 h. The gel was subsequently stained with Coomassie R250 for 1 h. Lanes 1) Molecular weight marker, 2) CFP, 3) Rv0467 (Icl), 4) Rv2032 (Acg), 5) Rv0363c (Fba), 6) Rv2624c, 7) Rv3810 (Erp), 8) Rv3841 (BfrB), 9) Rv3628 (Ppa), 10) Rv2626c, 11) Rv1813c, 12) Rv1738.

Table 3.6 **Recombinant protein identification and endotoxin content.**

Gene	Protein	Peptides Matched (MS/MS)	AA % Coverage	Endotoxin (EU/mg)
Rv0363c	Fba	6	20.3	2.65
Rv0467	Icl	19	58.3	5.33
Rv1738		5	34.7	6.81
Rv1813c		3	16.1	9.26
Rv2032	Acg	15	50.3	2.68
Rv2624c		5	15.1	8.61
Rv2626c		6	46.1	3.56
Rv3628	Ppa	10	55.8	12.61
Rv3810	Erp	4	24.2	4.21
Rv3841c	BfrB	4	34.3	6.49

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3.4 Discussion

The fact that *M. tuberculosis* exhibits different growth characteristics reflected in altered gene expression when exposed to a variety of environmental conditions has been well established for over 50 years [8] but the elucidation of what drives specific "transcriptional shifts" and what those changes specifically are is critical to the study of host-pathogen interaction and understanding the progression of disease and has been the subject of intense research efforts. Efforts to establish a model representative of environmental conditions during infection stemmed from early research demonstrating that the tuberculosis granuloma, which is present in the murine lung from approximately 30-200 days post infection, exhibits lower pO_2. This understanding, in addition to the knowledge that phospholipids comprise the dominant carbon source within a mammalian infection [30], allow the creation of an *in vitro* model that more accurately mimics *in vivo* conditions.

Based on these models, four proteins, bacterioferritin (BfrB, Rv3841), α-crystallin (Acr, Rv2031c), pyrophosphatase (Ppa, Rv3628), and single-stranded DNA binding protein (Ssb, Rv0054) were seen to be present at significantly higher levels when grown under at least one of the non-standard conditions. BfrB was seen to be increased at least 5 times when cultures were grown in all three *in vivo*-mimicking conditions. The highest level of BfrB, a 9-fold increase over standard growth, was observed in the phospholipid culture. Acr demonstrated the greatest increases under rapid hypoxia and gradual hypoxia conditions with levels rising to 9 and 13 times normal, respectively. Under phospholipid culture conditions Acr exhibited a 3-fold increase in abundance. Ppa also

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displayed modest increases under all non-standard conditions with a 6-fold increase manifested in both hypoxic cultures. The extremely low levels of Ssb in standard growth cultures is so minimal as to make fold-increase comparisons difficult and perhaps irrelevant, but a strong increase in protein abundance was observed in cultures grown under gradual hypoxia.

BfrB

Infecting bacteria must acquire iron from the host in order to survive and one defense mechanism employed by the host is to limit intracellular concentration of iron through the iron chelating properties of host-derived transferrins. In response to this nutritional deprivation, the *M. tuberculosis* bacillus produces iron-chelating molecules of its own. One such molecule is BfrB, or bacterioferritin [31] which is proposed to function as an iron repository during intracellular growth [32]. The mature BfrB molecule is a spherical homopolymer made up of 24 chains around a central hollow core that can hold up to 5000 FeIII atoms [33] in a soluble, nontoxic and readily available state. In addition to this work, others have observed an increase both in BfrB protein abundance [14] and gene expression [11] when the bacillus is grown under hypoxic conditions. Additionally, this molecule has been linked to protection against oxidative stress in *Neisseria gonorrhoeae* [34], thus alluding to its potential for ameliorating the effects of the oxidative burst encountered within infected macrophages. As an extracellular molecule found in the culture filtrates of log- and stationary-phase cultures, the potential for uptake, digestion and presentation to immune mechanisms of the host is extremely high. Indeed, Covert, et al. identified this protein as an antigen of the cytosol

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and CFP capable of inducing secretion of IFN-γ by activated T cells [35]. Taken together, its up-regulation in response to stress, role in intracellular survival of the bacterium, extracellular localization, and antigenicity describe a very attractive target for recognition by the immune response during persistent infection.

Ppa

Inorganic pyrophosphatase (PPase) is an essential enzyme that catalyzes the hydrolysis of pyrophosphate formed by the utilization of ATP to two orthophosphate molecules. While the homology between different phylogenic kingdoms is small, variants of this protein are found in all bacterial, plant and animal species in which its presence has been investigated. However, within bacterial families highly conserved structural integrity domains and catalytic domains are observed. [36]. For example, PPase from *E.coli* shares 80% sequence similarity to *L. pneumophila*, 74% to *M. tuberculosis*, and 78% to *B. subtilis.*

Legionella pneumophila, a ubiquitous aquatic organism that normally parasitizes protozoa, is the cause of Legionnaires' disease in humans. Pathogenesis in man is dependant on its ability to infect and multiply within alveolar macrophages [37] and alveolar epithelial cells [38]. Because of its distinctive pathogenic niche within humans, parallels in the physiological response to intracellular survival as well as general stress responses are likely to exist between this organism and *M. tuberculosis*. In *L. pneumophila,* expression of PPase has been shown to be induced by intracellular infection of a macrophage cell line, while the same induction was not observed in response to *in vitro* stress, including hypoxia [39]. The inability to artificially induce this

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heightened expression has been attributed to the rapid generation time of intracellular versus *in vitro*-grown bacilli. While this protein is not predicted to be secreted, nor does it possess a signal sequence, the current finding that the *M. tuberculosis* pyrophosphatase, Ppa, is found in greater abundance in the culture filtrates of hypoxic cultures could be indicative of a stress-induced increase in biosynthetic processes within the cell, such as DNA, RNA or protein biosynthesis.

One group, however, has previously explored the possibility of stress- and intracellular-induction of the BCG *ppa* gene and failed to observe any increased gene transcription in response to these stimuli [40]. The discrepancy in observations can be easily attributed to experimental approach. The work by Triccas, et al. [40] observed *ppa* promoter activity fused to a GFP reporter when exposed to lowered pH, osmotic shock, oxidative stress, and nutrient deprivation as well as engulfment by murine macrophages, while the Ppa protein itself was not assayed in any form. My work, on the other hand, quantifies the amount of the protein in each sample when exposed to decreased oxygen levels, a stress condition not tested in the previous work. Additionally, it is conceivable that an increase in protein abundance could be observed in the absence of increased promoter activity through release of internal protein stores or shedding of the protein into the culture medium.

Ssb

Another protein seen more abundantly in cultures grown under hypoxic conditions is Ssb, a single stranded DNA binding protein. Activities within the bacterial cell involving DNA, such as replication, recombination and repair, require the conversion

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of dsDNA to ssDNA. Ssb functions by binding to the transiently-formed ssDNA to protect it from degradation, nuclease activity and aberrant recombination. The action of this protein is highly conserved among bacterial species and it is listed as a member of the minimal gene set necessary to maintain life [41]. In light of its obvious intracellular function, it is interesting to note that *ssb* in mycobacteria codes for a putative signal sequence and is known to be found in the culture filtrate of actively growing bacilli [35]. Additionally, in *M. tuberculosis,* Ssb has been identified as a T-cell antigen, inducing the production of IFN-γ [35]. Unfortunately, efforts to express this protein in a recombinant form were not successful. While it was possible to clone the gene sequence into the pET23b expression vector and subsequently transform that vector into the *E. coli* expression strains BL21 (DE3), BL21 (DE3) pLysE, pLysS and STAR, no protein was produced after induction with IPTG concentrations ranging from 0.2-1.0 mM and temperatures ranging from 30-37°C. This inability could be due to toxicity of this protein to *E. coli* arising from the functional activity of this protein. It is conceivable that high concentrations of this protein could interfere with normal transcriptional activities within the cell by excessive binding to DNA and incompatibility with other enzymatic processes within the cell.

Acr

All proteins within a cell must exhibit the correct tertiary structure in order to be functional. When cellular proteins become non-functional due to conformational aberrations the degredation of these proteins is critical. Also, many secreted proteins must be kept in an unfolded conformation in order for translocation to occur. This

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policing and maintenance of gene products is performed by specialized proteins called chaperones. Chaperones act in a multitude of ways to ensure proper folding of translated proteins within the cell. One of these chaperones, a member of the "small heat shock protein" family is an α-crystallin lens protein homologue, HspX or Acr [42]. Within mycobacteria, this protein is only found in the highly related tuberculosis complex. The increase of this gene product in *M. tuberculosis* during anoxic growth was first determined by Wayne in 1979, when it was observed that this protein was a major antigen recognized by sera from patients with progressive cavitary tuberculosis [43]. Since that time, work done to characterize the transcription patterns of this gene have demonstrated that this protein is strongly up-regulated during stationary growth and in old cultures [11, 13, 44, 45]. Additional research has proven the necessity of this protein during intracellular growth in macrophages [46, 47].

The increase of these proteins (BfrB, Ppa, Ssb, and Acr) under hypoxic and phospholipid growth conditions detail a logical scenario of general response to stress and possible preparation for interaction with the host during intracellular habitation. It is reasonable to conclude that the bacilli, when confronted with conditions to which it has evolved responses capable of ensuring life, express these proteins in greater abundance. Of primary concern to the bacilli would be structural integrity of existing cellular components, therefore expression of chaperones, heat shock proteins and repair mechanism components would increase. These components would almost exclusively be represented in the somatic compliment of bacterial proteins and would not be seen in this research, however it is a well-documented phenomenon to observe Acr in the extracellular environment [14, 28, 48]. The next response to stress and potential *in vivo*

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conditions could reasonably be the production of proteins necessary only during these specific conditions, e.g. alternative sigma factors, nutrient acquisition molecules, immune evasion components, and cell wall biogenesis, repair and/or alteration molecules. This increased transcriptional activity would necessitate the greater abundance of single stranded DNA binding protein to ensure efficient and normal gene transcription. Also, pyrophosphatase would be expected in greater abundance due to the sudden shift in metabolic and physiological biogenesis pathways. Finally, as just mentioned, nutrient acquisition in the new environment would be a major focus of cellular energies and since iron is central to many enzymatic functions as a co-factor and also involved in proper functioning of oxygen-transferring enzymes, cytochromes, oxygenases, and hydroxylases the need for a readily available source of this molecule is critical. As such, the greater abundance of iron acquisition and storage molecules, such as BfrB, would be expected under stressed *in vitro* conditions as well as during *in vivo* growth. It is expected that if the somatic protein fraction of these cultures were analyzed many more changes in protein production would be observable, however this research is primarily interested in proteins that would be readily available for processing and presentation to the host immune system.

One argument to explain the increased abundance of these proteins in the culture filtrates could be greater lysis of bacteria grown under hypoxic conditions. This position, however, is not supported by the observation of other strictly somatic proteins (e.g. GroES) in the 2-DE gels as would be expected if global autolysis were a factor [28, 49].

In order to evaluate the immune response to these proteins during a chronic infection the genes *bfrB, ppa, ssb,* and *acr* were cloned into *E.coli* for expression as

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recombinant proteins. Additionally, seven stress-induced genes of *M. tuberculosis,* chosen from the literature [9, 11, 14], were likewise cloned. These include the following: Rv0363c (*fba*), Rv0467 (*icl*), Rv1738, Rv1813c, Rv2032 (*acg*), Rv2624c, and Rv2626c.

Fba, fructose bisphosphate aldolase, is a key enzyme of glycolysis converting fructose 1,6-bisphosphate to 3-phosphoglyceraldehyde. Old and recent studies alike have demonstrated increased gene product levels and enzymatic activity of this protein under hypoxic conditions [14, 50]. The hypoxic-induced regulation of this gene does not appear to be unique to *M. tuberculosis* as increased levels are also observed in *B. subtilis* and *L. brevis* under low oxygen growth [51, 52].

Isocitrate lyase (Rv0467, Icl) is of particular interest in this study because of its observed necessity during the persistent phase of infection and because of its role during hypoxic growth and fatty acid metabolism as has been described before [12, 17].

The *M. tuberculosis* genes Rv1738, Rv1813c, Rv2032 (*acg*, for *acr* co-regulated gene), Rv2624c, and Rv2626c encode conserved hypothetical proteins with no known functions. With the exception of Rv1813c and Rv2626c none of these genes, or their cognate proteins, have been characterized except for their increased mRNA levels under hypoxic growth conditions. The fold-increase of Rv1738, Rv1813c, Rv2032, Rv2624c, and Rv2626c mRNA in low oxygen growth is 63.3, 14.7, 43.9, 44.3, and 37.4, respectively [11]. The protein product of Rv1813c contains a sec-dependant signal sequence motif and a strong hydrophobic (possibly transmembrane) portion at its post-secretion N-terminus while the gene product of Rv2626c was identified as a T-cell antigen in a murine IFN-γ assay [35]. In the microarray study the average induction of those genes observed to be induced under hypoxia was 13.9 fold. Due to the greater-

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than-average induction of those genes listed here, along with the other considerations stated for Rv1813c and Rv2626c, these gene products were chosen for cloning.

In addition to these stress-induced gene products, a known T-cell antigen and virulence factor was cloned, as well. The exported repeated protein (Erp or PirG), Rv3810, is a 36kDa protein that is unique to the genus *Mycobacterium*. It was first described in 1988 as a dominant antibody target present in patients with multibacillary leprosy [53, 54]. Recently, microarray studies have shown that mRNA levels of this gene are up-regulated in response to starvation conditions [9]. While *erp* is present in all tested mycobacteria species, it is not found in closely related *Corynebacterium diphtheriae* nor seen in other members of the order *Actinomycetales* [55]. Interestingly, in *M. tuberculosis,* the *erp* gene is found between the cell wall genes *glf* and *csp*, both of which are contiguous in *C. diphtheriae.* The gene product of *erp* is a cell membrane- or cell wall-associated protein that is secreted via the classical sec-dependant protein translocation pathway [56, 57]. The *erp* gene product from all tested species contains three regions: a highly conserved amino-terminal domain containing a sec-related signal sequence, a conserved proline and alanine rich carboxyl-terminal domain, and a central region containing PGLT/PGLTS repeats which vary in number between two and six repeats. Erp's role in virulence was illustrated by deletion of the gene in BCG and *M. tuberculosis.* Both of these strains showed dramatic attenuation in the ability to infect both macrophage cultures and mice; $H_{37}Rv$ and BCG respectively demonstrating 3 and 4 logs fewer CFUs in the lungs of mice 40 days post infection [58]. While the function of this gene product has not been identified, the striking attenuation in virulence of strains

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lacking this gene, its up-regulation under stressed conditions, and its immunodominance observed in leprosy patients make this protein particularly intriguing for further analysis.

In conclusion, this research illustrates the utility of combining 2-DE analysis and ESI MS/MS peptide analysis to investigate how *M. tuberculosis* responds to environmental change. In addition to supporting the findings of other studies in regard to the mycobacterial hypoxic response, we add two more proteins, Ssb and Ppa, to the list of stress response proteins and further characterize this physiological state of the bacterium. In order to enhance our understanding of mycobacterial latency and interactions between the bacterium and the host during early as well as late infection the host response to these stress induced proteins, individually, must be evaluated. This evaluation is the focus of the next chapter.

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Chapter 4: Kinetics of the T cell response to recombinant *M. tuberculosis* proteins during pulmonary infection with *M. tuberculosis*

The aim of this study was to:

1. evaluate the kinetics of the T-cell response to individual *M. tuberculosis* proteins
2. evaluate the cytokine profile produced in response to individual proteins throughout infection
3. determine the protective efficacy of different hypoxia- and alternate carbon source-induced protein preparations against subsequent infection when given as a subcutaneous vaccination

Abstract

To evaluate the kinetics of T cell activity over the course of an experimental animal infection C57BL/6 mice were infected via low dose aerosol and lymphocytes from lungs and spleens were isolated at various time points throughout the course of infection. Native and recombinant proteins were added to bone marrow-derived dendritic cells (BMDC) and overlaid with lymphocytes. INF-γ production was assessed via flow cytometry as a measure of lymphocyte activation. The kinetics of the immune response to this array of mycobacterial proteins was markedly diverse over 195 days of infection and depended on the origin of the lymphocytes, the T-cell phenotype ($CD4^+$ or $CD8^+$),

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and length of infection. The T cell response can be divided into four categories: (1) those that induce a significant response early (Icl, Acr, and Ag85A), (2) those that produce a response early as well as late (CFP, ESAT-6, Rv1738, Rv1813c, Rv2624c, Erp, and BfrB), (3) those that produce a response late only (Fba and Acg), and (4) those that induce no significant response at all (MPT-32, Rv2626c, and Ppa).

In addition, the supernatants from these T cell overlays were analyzed for the concentrations of cytokines IL-12p70, TNF-α, IFN-γ, MCP-1, IL-10, and IL-6. The cytokine profiles detail a complex interplay between signaling molecules at each time point of infection that is highly dynamic and antigen specific. Roughly the same biphasic pattern observed in the lymphocytic activation assay was reflected in the concentration of pro-inflammatory (IL-12p70, TNF-α, and IFN-γ) cytokines, although the times at which peaks of activation and highest cytokine concentrations are observed do not correlate between the assays. Also, the inhibitory activity of IL-10 on inflammatory processes can be observed as high IL-10 concentration generally correlates to a lower concentration of IFN-γ. Another interesting correlation is an inverse relationship between IL-10 and IL-6 concentrations over time, with IL-10 peaking early and declining thereafter and IL-6 steadily increasing over the course of infection, highlighting the possibility of using cytokine profiles as a diagnostic tool for the identification of acute versus recrudescent infection, and as a marker for reactivation.

As the ultimate goal of this research is to identify protein candidates for use in a vaccine formulation protective against acute and chronic infection as well as reactivation disease, C57BL/6 mice were vaccinated with a pool of hypoxia-induced proteins with adjuvant alone and as a boost to initial BCG vaccination. The hypoxic proteins alone did

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not confer protection significantly over saline, but when given in a prime-boost scheme with BCG afforded 0.6 and 1.8 logs protection in the lung over BCG and saline, respectively, at 30 days post infection (pi), increasing to 1.1 and 1.9 logs of protection over BCG and saline, respectively, at 60 and 90 days pi. Further research determining the kinetics of the T-cell and cytokine response to individual *M. tuberculosis* antigens will identify novel diagnostics and skin-test antigens as well as facilitate the fine tuned development of a vaccine strategy utilizing antigens recognized throughout the infection optimized to confer protection against acute and chronic disease and potentially recrudescence.

4.1 Introduction

As has been discussed previously, one of the most intriguing aspects of *M. tuberculosis* is its ability to survive and persist within the host for extended periods of time even in the face of a robust and active immune response. Hypotheses to explain this pathogen's ability to establish an asymptomatic carrier state are extremely varied emphasizing our lack of understanding in this regard. Theories have focused on genetic factors and gene transcription patterns of the pathogen, physiology and metabolism of the bacterium, host-pathogen interaction, and the immunopathology, architecture and environment within the host.

It is clear from our current knowledge that the components involved with this phenomenon are complex, inter-related and multifactorial, and a solid understanding is still years away. Several aspects of disease, however, during the chronic stage of

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infection are understood. The initial response to *M. tuberculosis* infection is IL-12 production by infected APCs, whereupon MHC class II-restricted $CD4^+$ T cells secrete IFN-γ which in turn activates the APC to become more bactericidal. These $CD4^+$ T cells are essential for initial control of infection [1-3] as well as control of the reactivation of latent disease [4]. This is perhaps most convincingly demonstrated by the reactivation of tuberculosis in people whose T cell population is depleted by concomitant HIV infection [5-8]. Even though the evidence for the $CD8^+$ T cell contribution to protection against primary infection is limited, this cell type has been shown to confer a level of protection against later stages of infection albeit to a lower efficiency when compared to that conferred by $CD4^+$ T cells [2, 9].

IFN-γ is the key determinant produced by these cells in response to infection. IFN-γ activates macrophages in which the pathogen resides and, in the mouse, stimulates expression of the antimicrobial enzyme nitric oxide synthase (NOS2) resulting in the generation of NO [10]. While not essential for control of *M. tuberculosis* infection early, NOS2 KO mice show an increased bacterial load within the lungs manifested during the chronic stage of infection (50-180 days p.i.) and a more rapid time-to-death compared with a wild-type infection [11]. The central role for IFN-γ in control of *M. tuberculosis* infection is illustrated by the extreme sensitivity of IFN-γ knock out (GKO) mice to infection [12, 13], and increased susceptibility to mycobacterial infection in humans lacking the gene for IFN-γR [14].

Because of its central importance, the research reported in this chapter utilizes IFN-γ production by $CD4^+$ and $CD8^+$ T cells to evaluate the activation of the host immune mechanisms in response to specific proteins of *M. tuberculosis* that are believed

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to be produced during the chronic stage of *in vivo* infection. In addition to IFN-γ, the concentrations of cytokines IL-6, IL-10, MCP-1, TNFα, and IL-12p70 were assayed in order to gain a greater understanding of the cellular mediators involved in the establishment of persistence. In order to evaluate the immune response throughout the mouse infection, this assay was performed at days 15, 30, 45, 60, 90, 120, 150, and 195 post infection.

In summary, the lymphocytic response to these proteins can be divided into three separate stages. The $CD4^+$ T cell response was elevated to many of the proteins tested and appeared to peak at 30 days post infection. Between 60 and 90 days post infection, a sharp decrease in the response to these proteins was observed, followed by a second increase of IFN-γ positive $CD4^+$ T cells beginning at 120 days post infection. Alternatively, the percentage of IFN-γ-secreting $CD8^+$ T cells appeared to peak at about 45 days post infection. While the same general pattern of decreased IFN-γ producing cells during the chronic stage of infection can be observed in both lymphocyte populations, $CD8^+$ T cells demonstrate a much more varied response to the tested proteins and exhibited a relatively smaller percentage decrease than did the $CD4^+$ T cell population. In addition, the tested proteins all yielded distinct profiles of T-cell recognition demonstrating the dynamic and multifactorial interaction between *M. tuberculosis* and its host. Characterization of the kinetics of the T cell response to *M. tuberculosis* antigens through all stages of infection is of critical importance to the development of new diagnostic reagents and novel vaccine strategies aimed at protecting against primary infection as well as recrudescence.

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4.2 Material and Methods

4.2.1 Mice

Female C57BL/6 mice at 6-8 weeks old were purchased from Charles River Laboratories (Worchester, MA) and maintained under specific pathogen-free conditions at the Bioenvironmental Hazards Research Building at Colorado State University. The mice were provided sterile water, mouse chow, shaved wood bedding, and enrichment throughout the duration of the research.

4.2.2 Animal infection

Mice were infected aerogenically with the $H_{37}Rv$ strain of *M. tuberculosis* by using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN), such that ~100 bacilli were deposited in the lungs of each animal. Bacterial numbers were enumerated at 1 day post infection by individually plating entire homogenized lungs from 5 mice.

4.2.3 Bone marrow-derived dendritic cells (DC)

Naïve mice, 10-20 weeks old, were euthanized by asphyxiation with CO_2 gas and their femurs and tibia bones were removed and placed in ice-cold tissue culture media RPMI-1640 (Life Technologies, Gaithersberg, MD) supplemented with 1% L-glutamine (Sigma-Aldrich), 0.1 mM nonessential amino acids (Life Technologies), 1% HEPES (Sigma-Aldrich), 1% penicillin-streptomycin, 1% sodium pyruvate (Sigma-Aldrich), and 50 μM 2-ME (Sigma-Aldrich). Each bone was excorporated and the proximal terminus of each bone trimmed to allow insertion of a 26.5-gauge needle to flush out the bone

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marrow with 10.0 ml cold cRPMI. The solution was passed through a 10 ml pipette multiple times to achieve a single-cell suspension. Cells were counted and suspended in cRPMI to a concentration of 2 X 10^5 cells/ml in cRPMI supplemented with 2 ng/ml of granulocyte/macrophage colony stimulating factor (GM-CSF, Peprotech Inc. NJ). An aliquot (10 ml) of this cell suspension was added to tissue culture petri dishes and incubated at 37°C in a CO_2 incubator. Media with GM-CSF was added at every 48 h. On day 8 post-harvest (158 h), the cells were harvested by vigorous pipetting and were centrifuged at 200 x g for 5 min. The cell pellet was suspended in cRPMI without GM-CSF at a concentration of 2 X 10^5 cells/ml and 100 µl/well was added into 96-well plates.

4.2.4 Isolation of lung and spleen cells from infected mice

Infected and age-matched naïve mice were euthanized, as before, at 15, 30, 45, 60, 90, 120, 150, and 195 days post infection (n=4 infected, n=2 naïve mice for each time point). Lungs were perfused through the right ventricle of the heart with 10 ml cold PBS containing 30 U/ml heparin (Sigma-Aldrich) followed by removal of spleens and lungs to 5 ml cold cRPMI without FBS. Lungs were then cut into ~3 mm^3 pieces with a razor blade and incubated in 5 ml cRPMI (without FBS) containing 0.7 mg/ml collagenase IX (Sigma-Aldrich) and 30 µg/ml DNase (Sigma-Aldrich) at 37°C for 30 min. Single-cell suspensions of spleens and digested lungs were obtained by passing organs through a 70 µm cell strainer. Resident macrophages and monocytes were depleted from organ homogenates by incubation of cell suspension in tissue culture plates at 37°C for 1 h, thus allowing for selective adherence of APCs to the culture plates. The non-adherent cells

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were gently removed and centrifuged at 1000 x g for 5 min. RBCs were lysed by suspension of cell pellet in Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$) for 3 min.

4.2.5 Protein pulsing of DC culture

Freeze-dried recombinant *M. tuberculosis* proteins Rv0363c, Rv0467, Rv1738, Rv1813c, Rv1860, Rv2032, Rv2624c, Rv2626c, Rv3628, Rv3810, Rv3841 (produced in this work), Acr (Rv2031c) and ESAT-6 (Rv3875), native Ag85A (Rv3804c) and CFP, and ovalbumin were separately suspended in cRPMI to a concentration of 10 μg/ml and 100 μl of each sample was added to individual DC culture wells. Cultures were incubated for 24 h at 37°C in a CO_2 incubator.

4.2.6 T-cell overlay

Remaining lymphocytes were suspended in cRPMI with 20 ng/ml IL-2 to a concentration of 4 X 10^6 cells/ml and 50 μl was added to pulsed DC cultures. Overlays were incubated at 37°C for 24 h; during the last 5 h of incubation cells were stimulated with 1 μg/ml anti-CD28 (clone 37.51) and monensin. After 24 h incubation, supernatants were collected and frozen at –80°C for subsequent analysis, following which the cells were suspended in 200 μls RPMI (Irvine Scientific, Santa Ana, CA) supplemented with 1% glutamine, 1% HEPES, and 0.1% N_3Na (Sigma-Aldrich) and incubated at 4°C for 20 min.

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4.2.7 *Flow cytometry*

Following incubation with RPMI, cells were stained with 25 μg/ml specific antibody (BD PharMingen, San Diego, CA) for 30 min at 4°C in the dark. Cells were stained with anti-CD4 PerCP (peridinin chlorophyll protein-labeled clone GK1.5) and anti-CD8 APC (allophycocyanin-labeled clone 53-6.7). For intracellular IFN-γ staining, cells were permeabilized by 20 min incubation at 4°C with BD Cytofix/Cytoperm (BD PharMingen, San Diego, CA), followed by 15 min incubation at 4°C with Perm/Wash. Cells were then stained with anti-IFN-γ FITC (fluorescein isothiocyanate-labeled clone H1.2F3) for 30 min at 4°C in the dark. Control cell samples labeled with isotype antibodies (anti-mouse IgG1-PerCP, anti-mouse IgG1-APC, and anti-mouse IgG2a-FITC) were also prepared. Cells were washed twice and suspended in dRPMI with N_3Na for flow analysis. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA) dual laser flow cytometer with excitation wavelengths at 488 nm and 633 nm, and data were analyzed with CellQuest software (Becton Dickinson, San Diego, CA). Lymphocytes were gated based on characteristic forward- and side-scatter profiles. 10,000 total events were read for lung samples and 30,000 total events were read for spleen samples.

4.2.8 *CBA analysis*

96-well plates of 24 h T cell overlays were centrifuged at 200 x g for 5 min and culture supernatants were isolated by decanting 200 μl from each well. These supernatants from each time point were immediately frozen at –80°C to allow concurrent analysis of all samples. Once all overlays were performed, supernatants were allowed to

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thaw and 25 μl from each sample was transferred to new 96-well plates. Inflammatory cytokines including IL-12, TNF-α, IFN-γ, MCP-1, IL-10, and IL-6 were quantified simultaneously using an inflammatory cytokine cytometric bead array (CBA) kit. The CBA kit and CBA software were purchased from BD Pharmingen. The CBA kit provides a mixture of six microbead populations which exhibit distinct fluorometric intensities and are coated with antibodies specific for individual cytokines. Equal volumes (25 μl) of each supernatant sample and PE detection reagent antibody were added to mixed microbeads in 96-well plates. After 2 h incubation at room temperature the beads were centrifuged at 500 x ***g*** for 5 min and washed with 300 μl CBA Wash Buffer. The beads were again centrifuged at 500 x ***g*** for 5 min and suspended in 180 μl Wash Buffer. The FACSCalibur flow cytometer was calibrated with setup beads (provided in the CBA kit) and 1800 gated events were acquired for each sample according to product instructions. Cytokine concentrations were quantified based on their fluorescent intensities (FL2) and were computed using a standard reference curve generated by cytokine reference standards provided with the kit. Lower limits of detection varied for each cytokine based upon standard curves as follows: IL-12 (4 pg/ml), TNF-α (12 pg/ml), IFN-γ (36 pg/ml), MCP-1 (55 pg/ml), IL-10 (9 pg/ml), and IL-6 (16 pg/ml).

4.2.9 Vaccine testing

Ten- to twelve-week-old C57BL/6 mice were vaccinated by subcutaneous injection as detailed in Table 4.3. Vaccinated mice were then infected by LDA with the $H_{37}Rv$ strain of *M. tuberculosis* (lot # TMC-102) using a Glas-Col aerosol generator

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(Glas-Col, Terre Haute, IN). Initial infection was verified by harvesting and homogenizing the lung from 1 mouse at 24 h post infection and plating on 7H11 Middlebrook Agar plate. The plate was counted after 22 days of incubation and found to contain 107 CFU. At 30, 60, and 90 days post infection, lungs and spleens were harvested and viable counts performed. Each group originally contained 4 mice. Because of ulcerative dermatitis occurring in the BCG and the two HPP groups, several mice had to be euthanized before the end of the experiment. Total mice in each group are given in Table 4.3.

Table 4.1 Hypoxia-induced Protein Pool (HPP) Vaccination of C57BL/6 Mice

Group	Vaccinate (Day 1)	Boost (Day 21)	Boost (Day 42)	LDA (Day 72)	Day 30 Viable Count (VC) lung & spleen	Day 60 VC lung & spleen	Day 90 VC lung & spleen
Saline	Saline (200µl) SC	Saline (200µl) SC	Saline (200µl) SC	10^2 cfu/mouse 4 mice each group	4 mice	4 mice	4 mice
BCG	10^6 CFU SC	—	—		4 mice	3 mice	3 mice
HPP*	11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC	11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC	11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC		3 mice	3 mice	2 mice
BCG + HPP	10^6 CFU BCG + 11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC	11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC	11µg protein pool in 25µg MPL-SE + 250µg DDA in 200µl solution. SC		3 mice	3 mice	2 mice

* Hypoxia-induced protein pool includes 1µg per injection of each of the following: Rv0363c (Fba), Rv0467 (Icl), Rv1738, Rv1813c, Rv2031c (Acr), Rv2032 (Acg), Rv2624c, Rv2626c, Rv3628 (Ppa), Rv3810 (Erp), and Rv3841 (BfrB).

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4.3 Results

4.3.1 T lymphocyte activation profiles display a dynamic, antigen-specific response that changes in a characteristic pattern over the time-course of infection.

In order to determine the strength of a specific lymphocyte response to individual proteins, lung and spleen lymphocytes from naïve and infected animals were incubated with DC which had been pulsed with recombinant *M. tuberculosis* proteins for 24 h and analyzed by flow cytometry according to their FSC and SSC profile as well as for their expression of cell surface molecules CD4 and CD8 (Figure 4.1). Based on their production of IFN-γ, these T lymphocyte subsets were analyzed for protein-specific activation through the time-course of infection up to 195 days post challenge. While each protein elicited an individually unique level of activation at each time point, in most cases the strongest IFN-γ responses were observed in the lung during the first 30 days post infection in $CD4^+$ T cells and 45 days post infection in $CD8^+$ T cells (Figure 4.2). Known T cell antigens CFP, Acr, Ag85A, ESAT-6, Erp and BfrB induced high percentages of activated $CD4^+$ T cells at 30 days post infection at 22.7, 22.2, 13.2, 13.0, 12.1, and 11.8%, respectively, while conserved hypothetical proteins Rv2624c, Rv1738, and Rv1813c also elicited significant responses of 12.6, 10.9, and 8.4%, respectively, at the same time point. Interestingly, known T cell agonists MPT-32 and Rv2626c [15, 16] failed to induce significant T cell activity throughout the course of this experiment. This flurry of initial activity was followed by a dramatic decrease in T cell response to all proteins during the time points of 60-90 days. Following this abatement in lymphocytic activity, a number of proteins elicited a renewed production of IFN-γ by the lung and/or spleen lymphocytes starting at 120 days post infection and increasing through the end of

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the experiment (Figure 4.2). These proteins include CFP, Ag85, ESAT-6, Fba, Rv1738, Rv1813c, Acg, Rv2624c, Erp, and BfrB. On the other hand, proteins, Acr, MPT-32, Icl, Rv2626c, and Ppa failed to induce IFN-γ production in $CD4^+$ or $CD8^+$ T cells during the later stages of infection.

Evaluating the CD4 data in the lungs and based on activation patterns throughout the infection it is possible to group these proteins into four categories: (A) proteins that elicit a significant response early, but fail to do so late; (B) proteins that invoke a response both early as well as late; (C) proteins that elicit a response only during late stages of infection; and (D) proteins that failed to induce a response at any time during this assay. Group A consists of Icl and Acr; group B consists of CFP, Ag85A, ESAT-6, Rv1738, Rv1813c, Rv2624c, Erp, and BfrB; group C consists of Fba and Acg; group D comprises MPT-32, Rv2626c, and Ppa. A more detailed report of CD4 and CD8 activation in the lung and spleen is put forth in Table 4.4.

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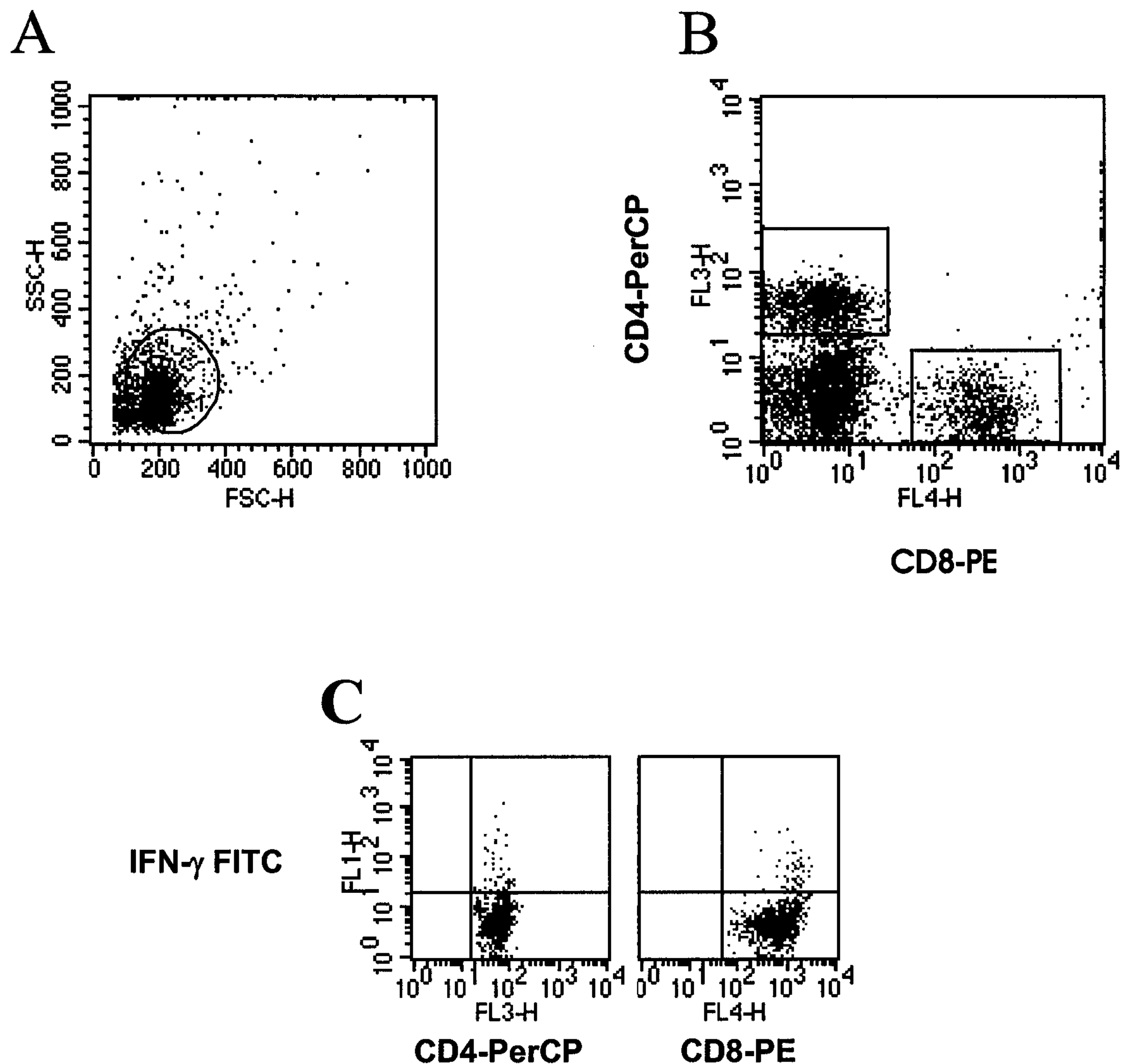

Figure 4.1 Gating and flow cytometry analysis of CD4$^+$, CD8$^+$, and intracellular IFN-γ stained lymphocytes. After incubation with protein-pulsed dendritic cells, lung and spleen lymphocytes were stained with fluorescent antibodies and analyzed by flow cytometry. (A) Lymphocytes were identified and gated by their characteristic scatter profile. (B) and (C) CD4$^+$ (R2) and CD8$^+$ (R3) lymphocytes were evaluated separately for their production of intracellular IFN-γ. Percentages of activated CD4$^+$ and CD8$^+$ lymphocyte populations were calculated based on the presence of intracellular IFN-γ.

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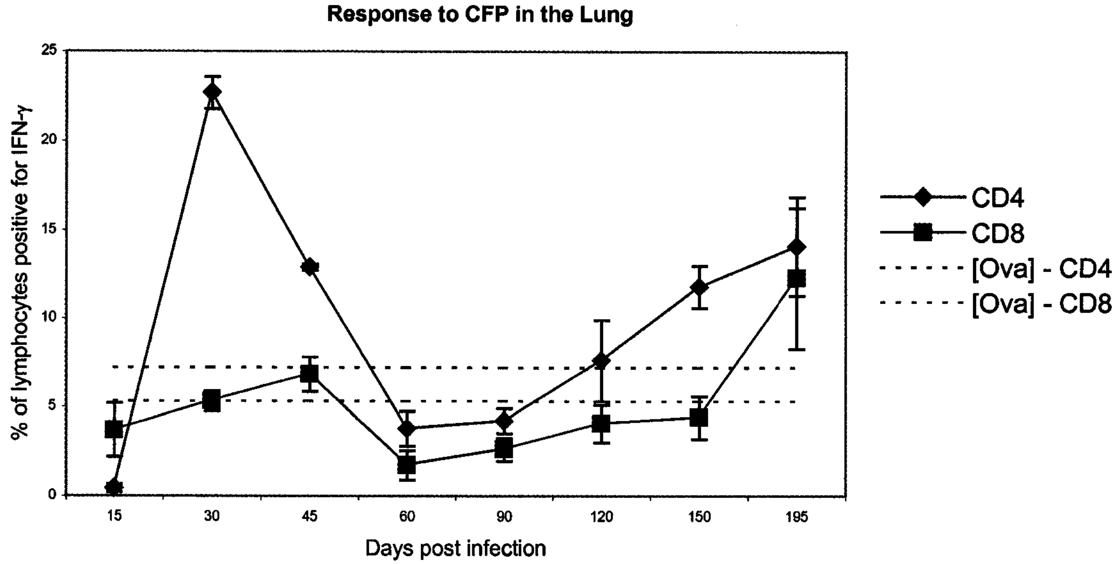

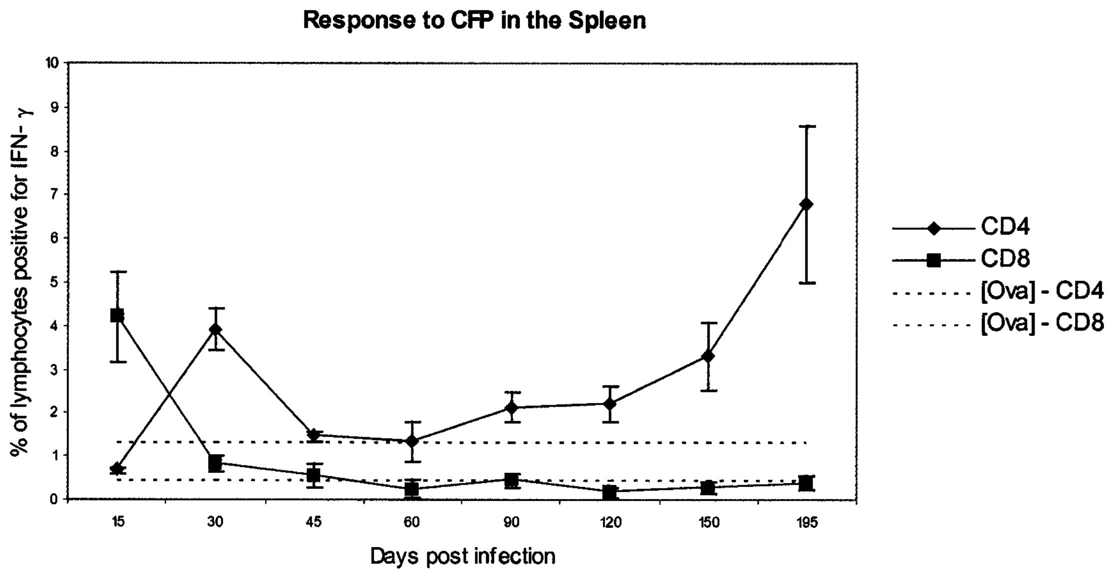

Figure 4.2 CD4^{+} and CD8^{+} response as measured by IFN-γ production in the lungs and spleens of infected C57BL6 mice. Intracellular IFN-γ, CD4^{+}, and CD8^{+} staining of lung and spleen lymphocytes overlayed onto CFP-pulsed dendritic cells were analyzed by flow cytometry. Percentages of individually gated CD4^{+}, and CD8^{+} cells positive for IFN-γ are graphed. Dotted lines represent the average response to ovalbumin + 3 standard deviations.

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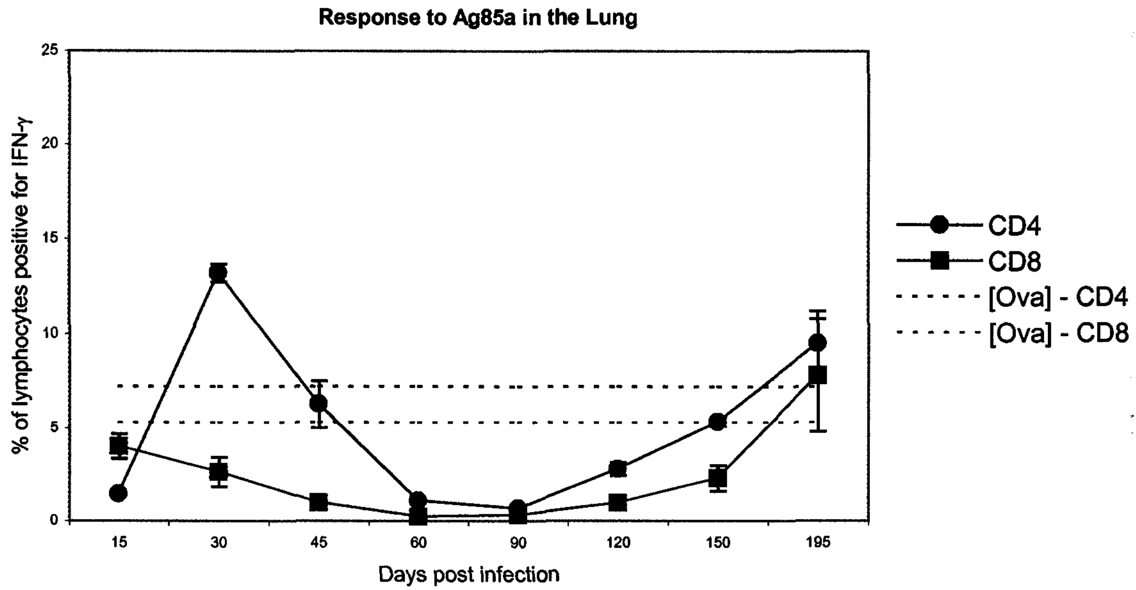

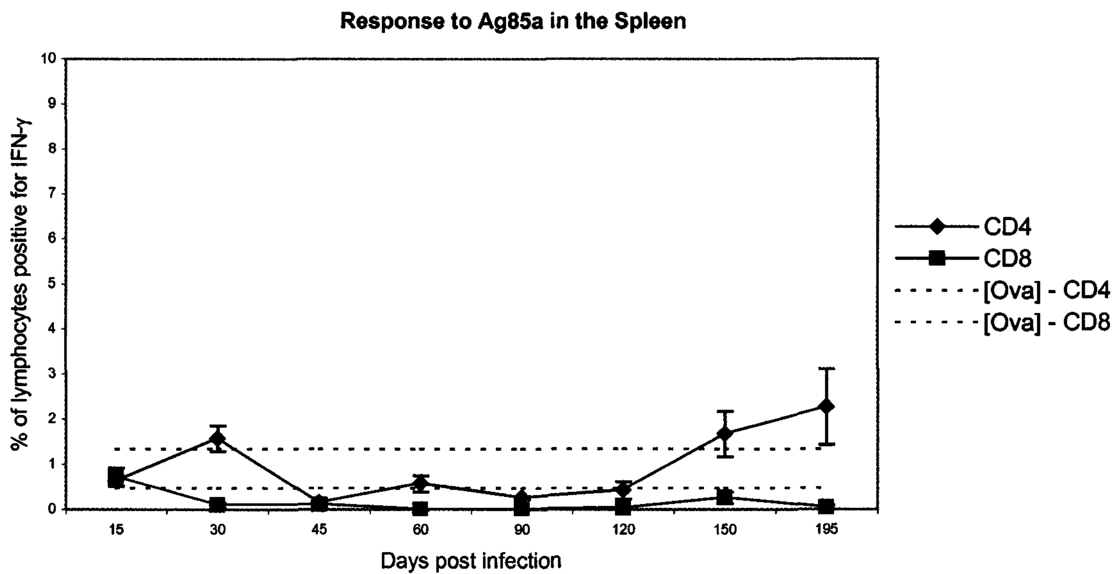

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Ag85a-pulsed dendritic cells.

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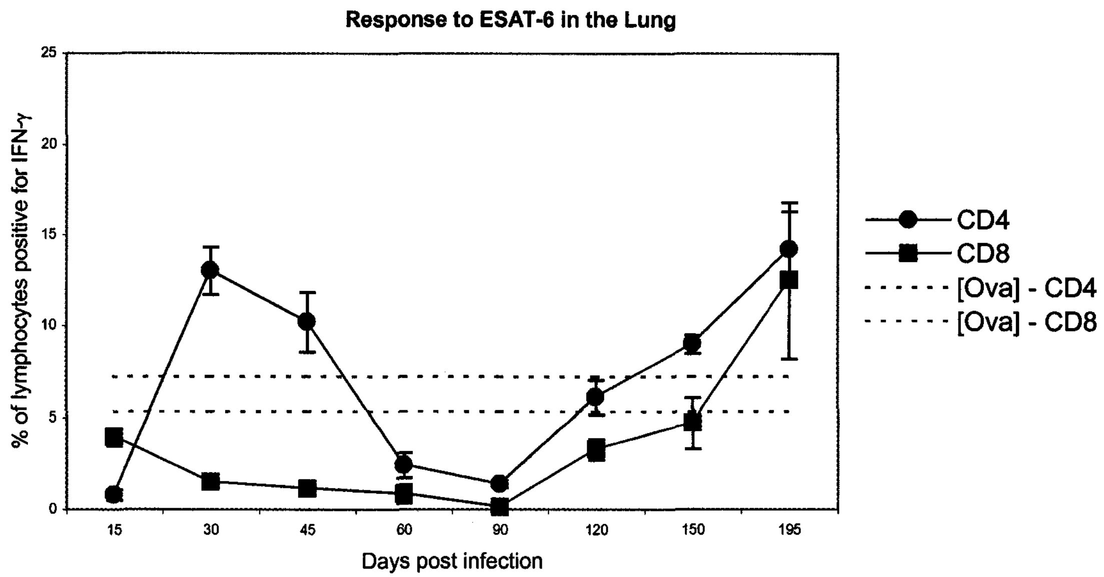

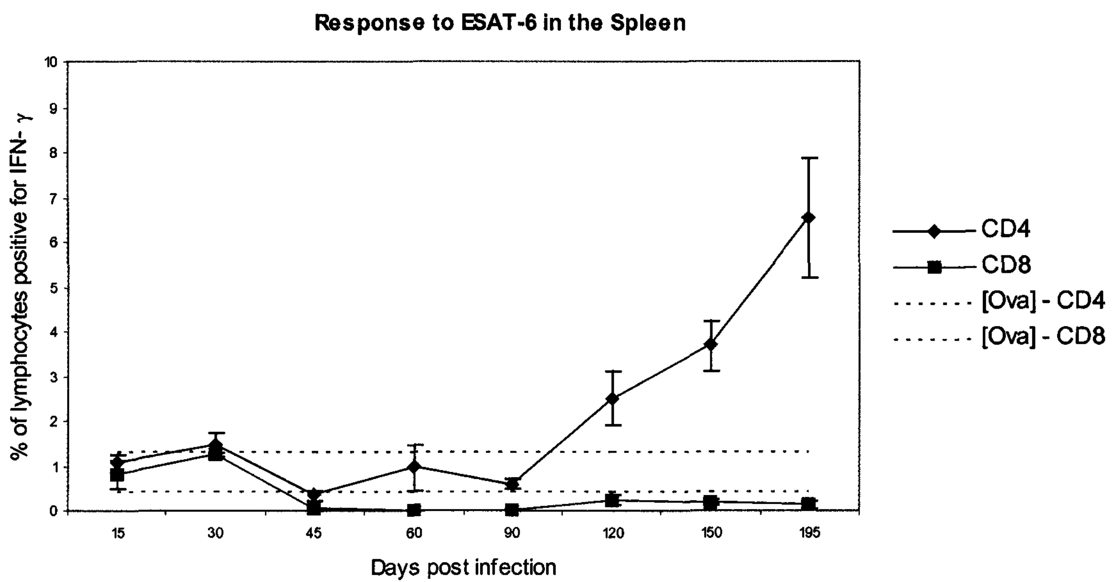

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on ESAT-6-pulsed dendritic cells.

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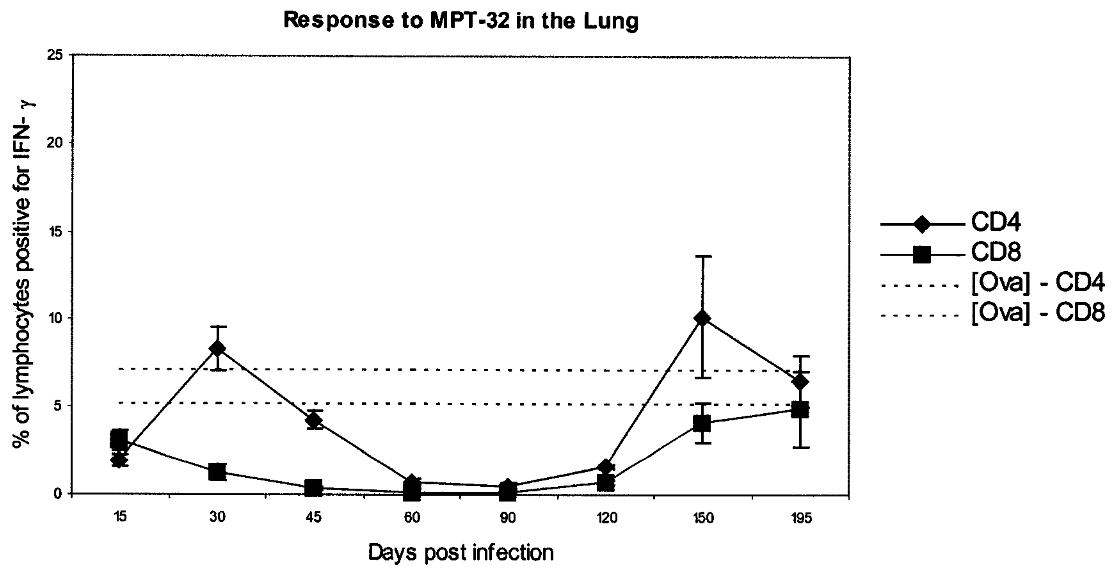

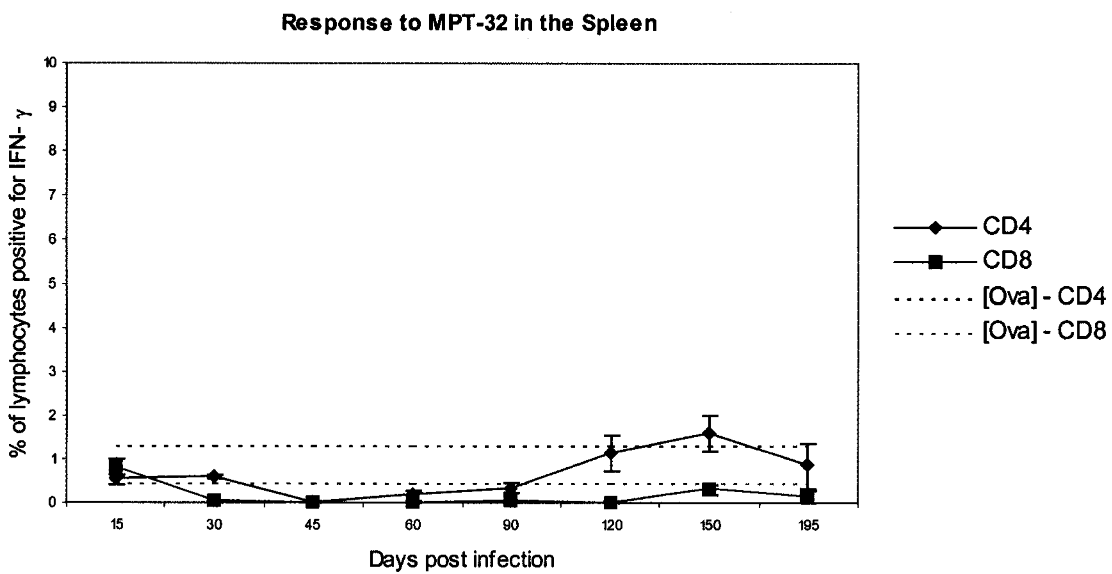

Figure 4.2 (cont.) Percentage of IFN-γ positive CD4$^+$ and CD8$^+$ lung and spleen cells after overlay on MPT-32-pulsed dendritic cells.

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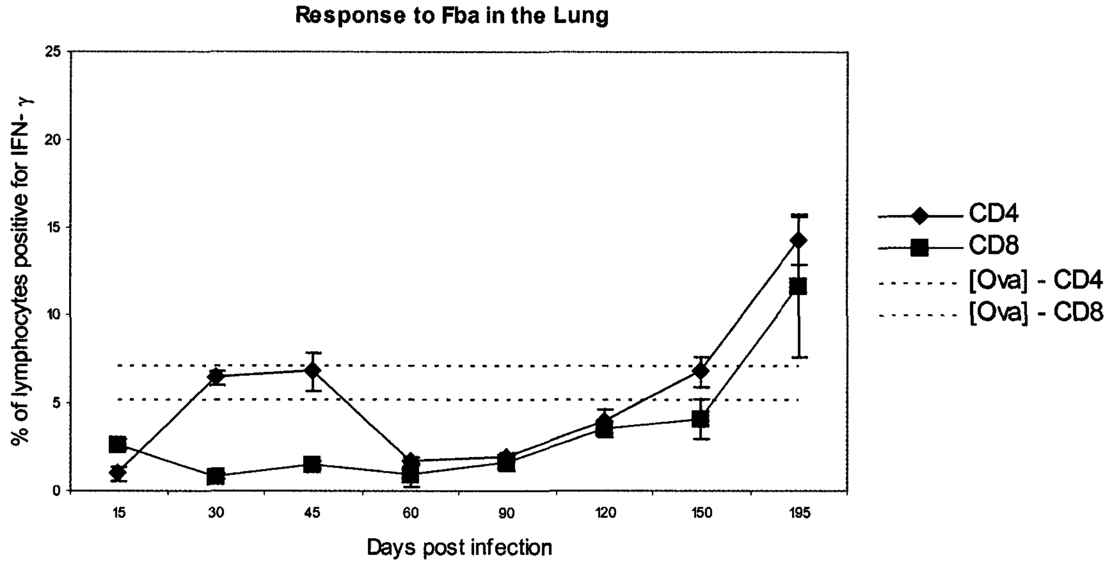

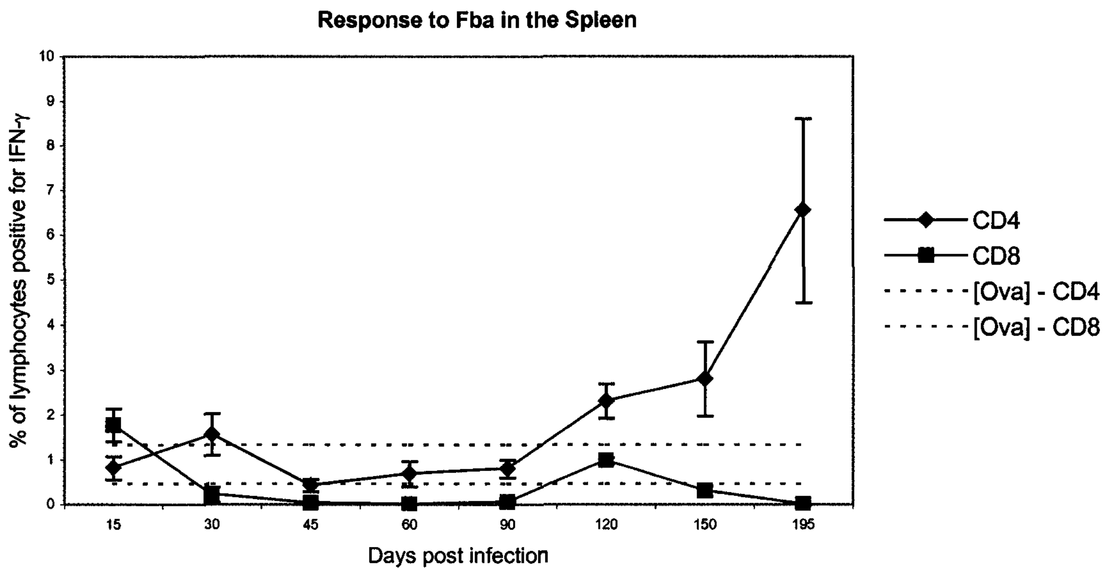

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Fba-pulsed dendritic cells.

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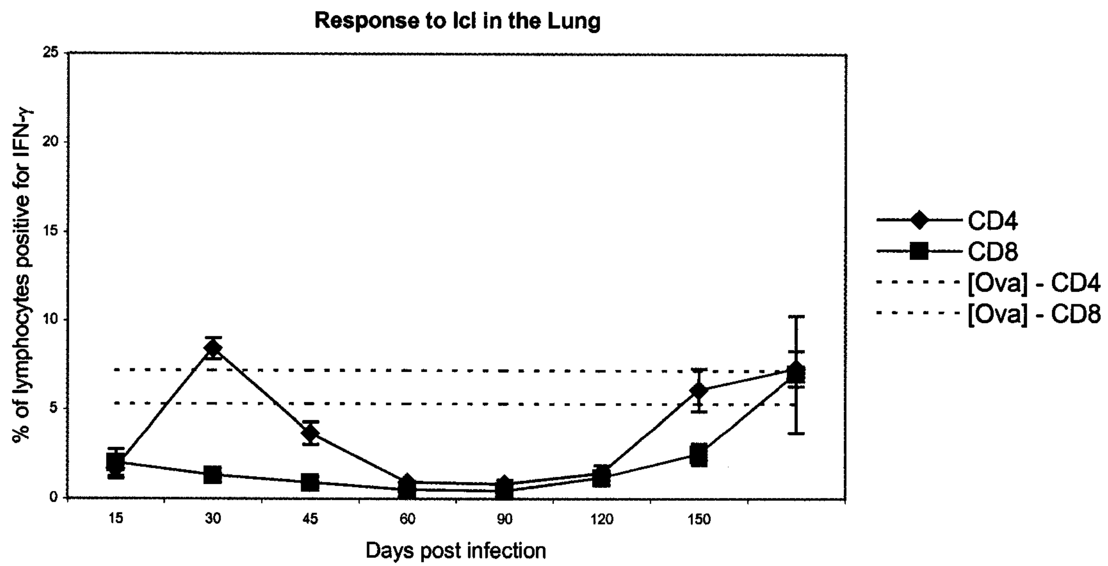

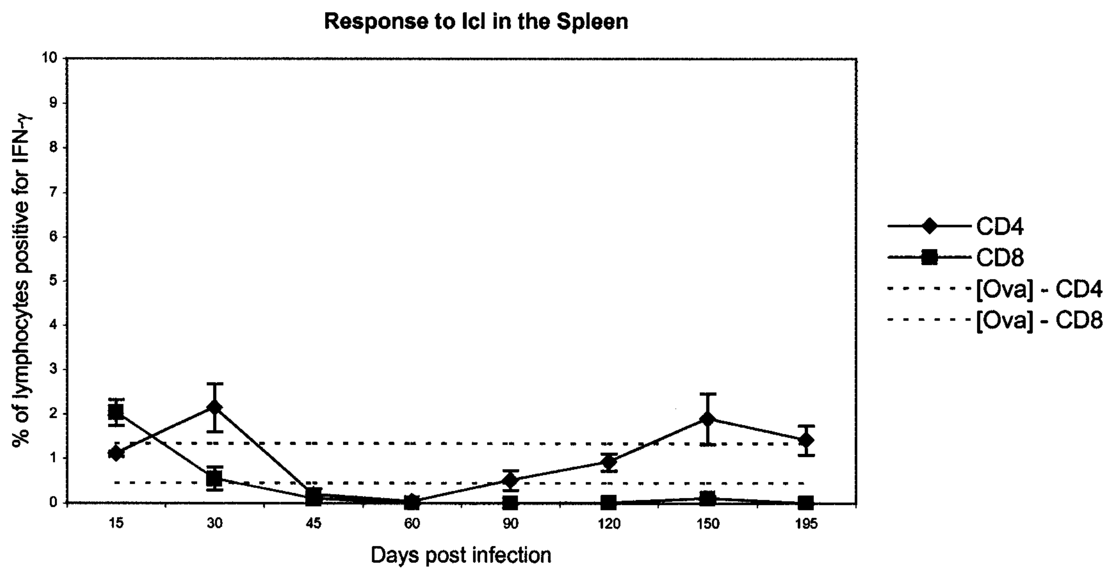

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Icl-pulsed dendritic cells.

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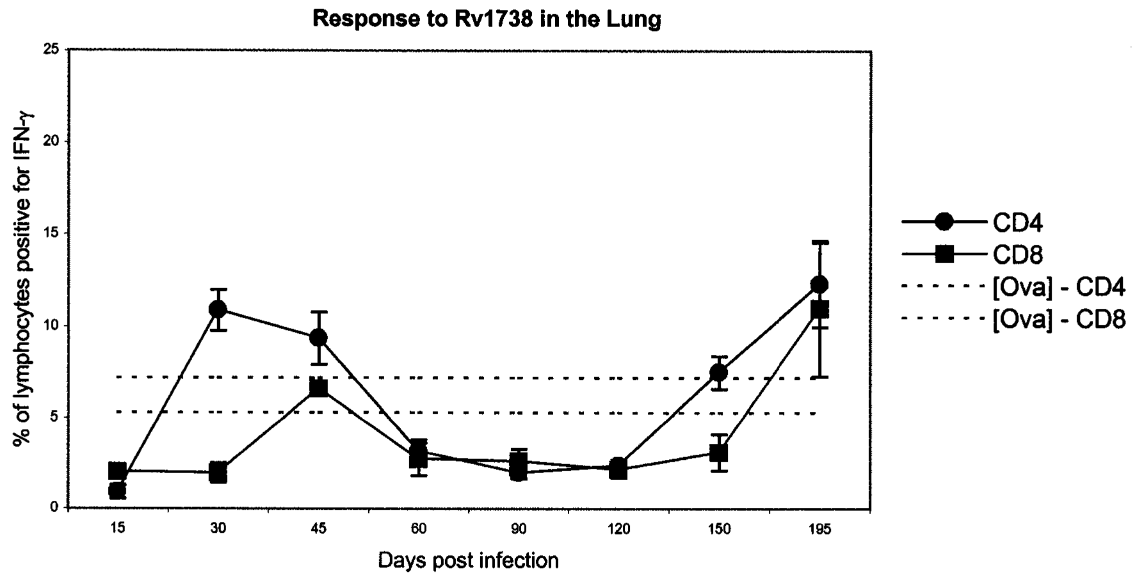

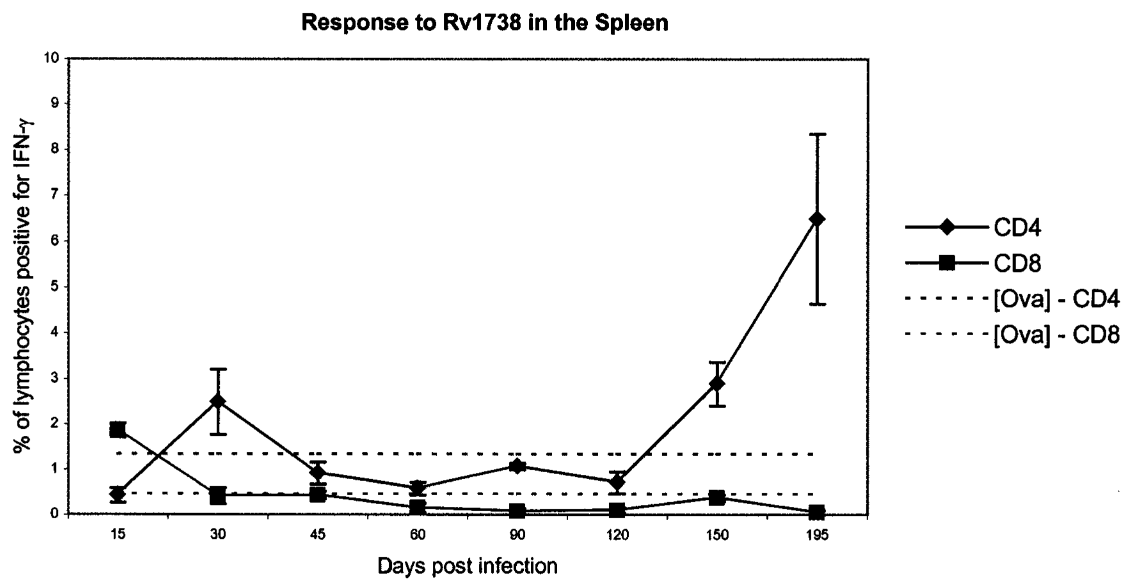

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Rv1738-pulsed dendritic cells.

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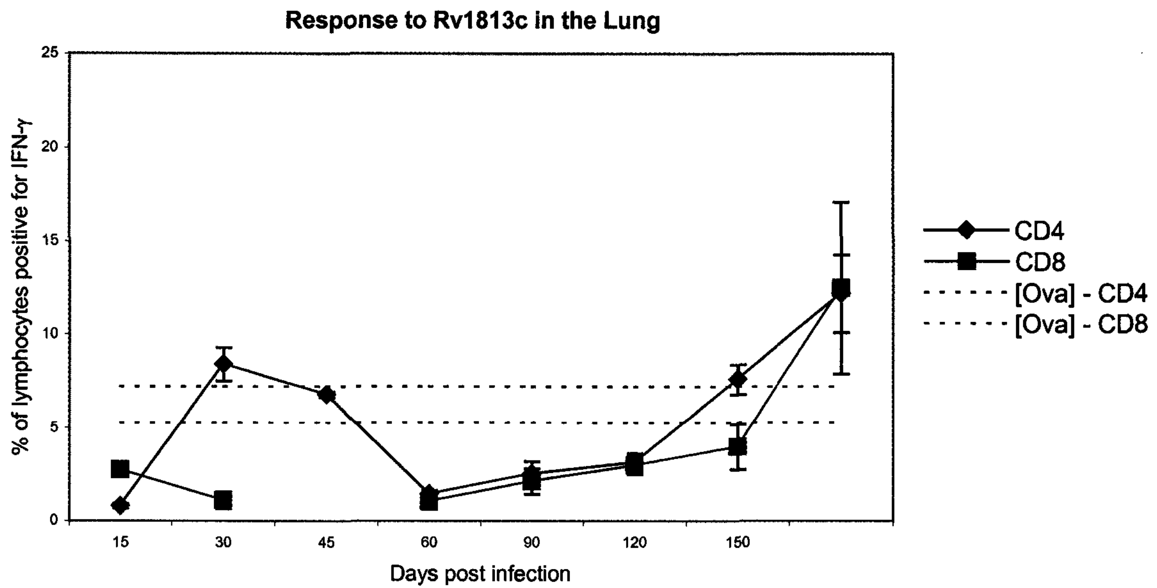

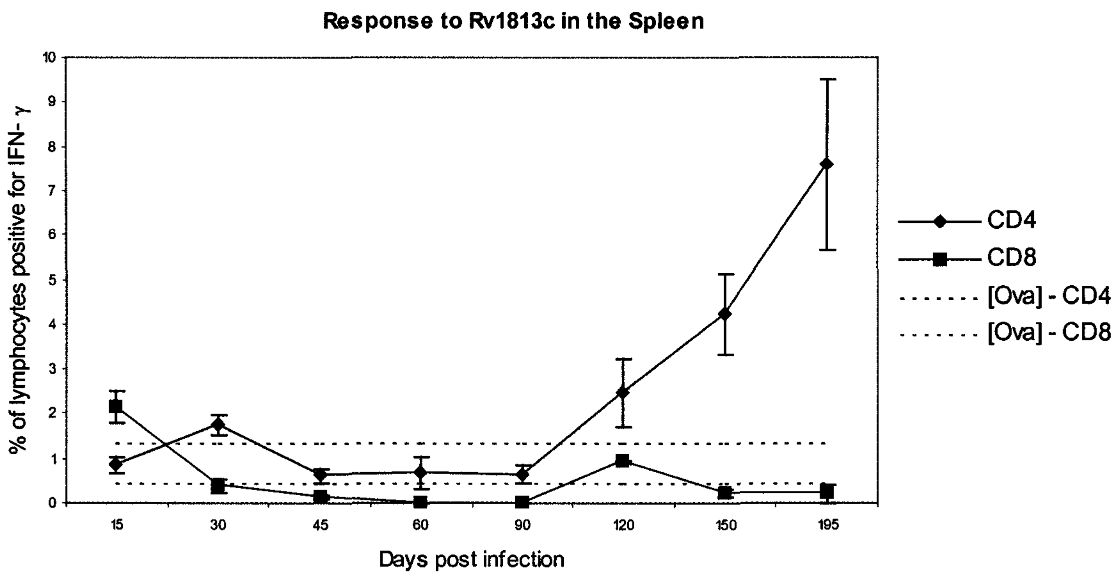

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Rv1813c-pulsed dendritic cells. Day 45 lung cells were not stained for CD8 by mistake in this assay and no data was generated at this timepoint.

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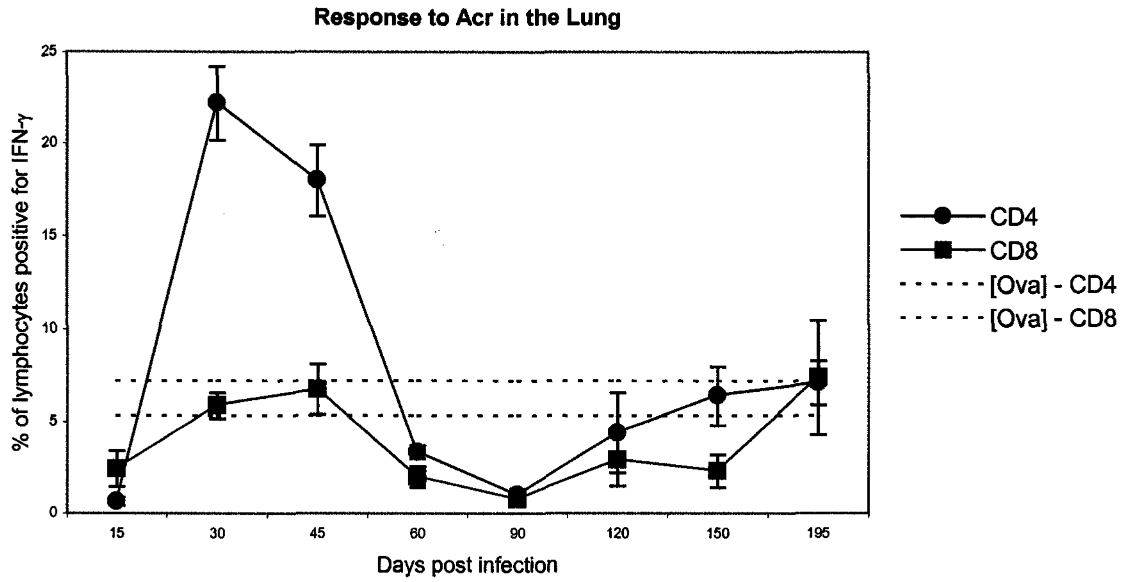

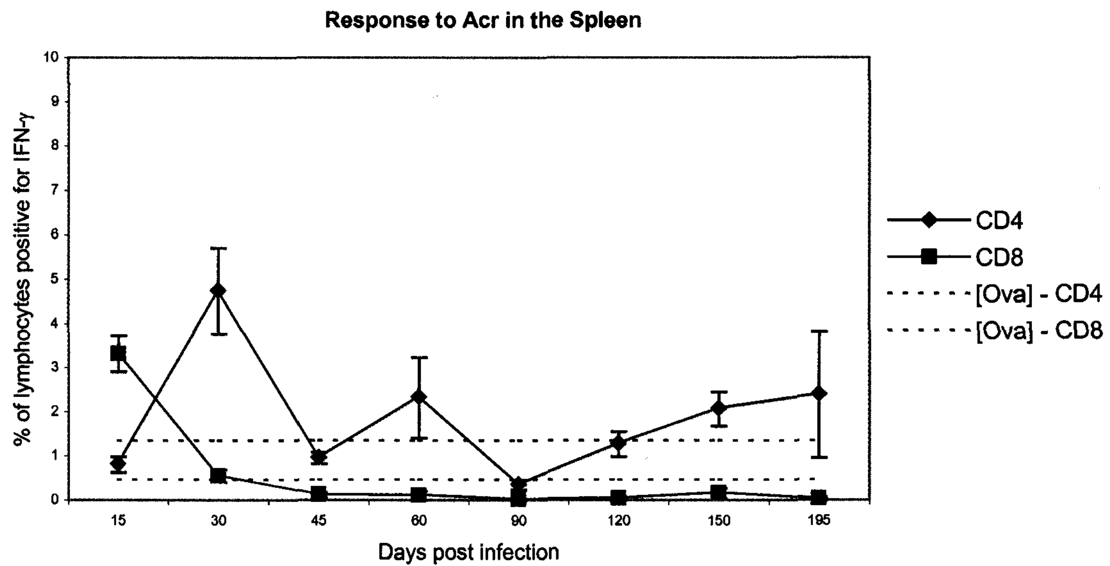

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Acr-pulsed dendritic cells.

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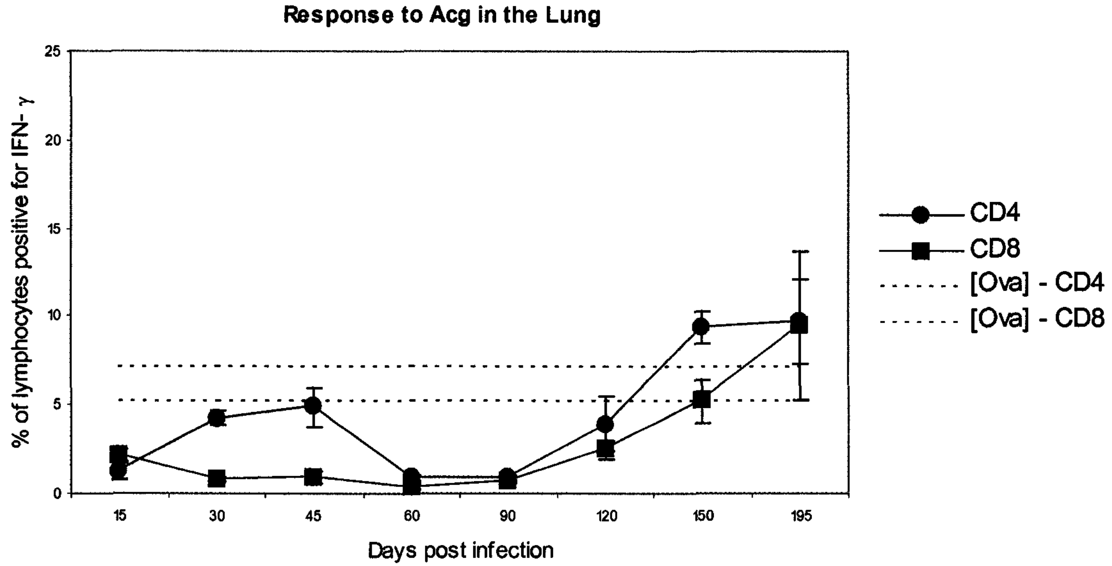

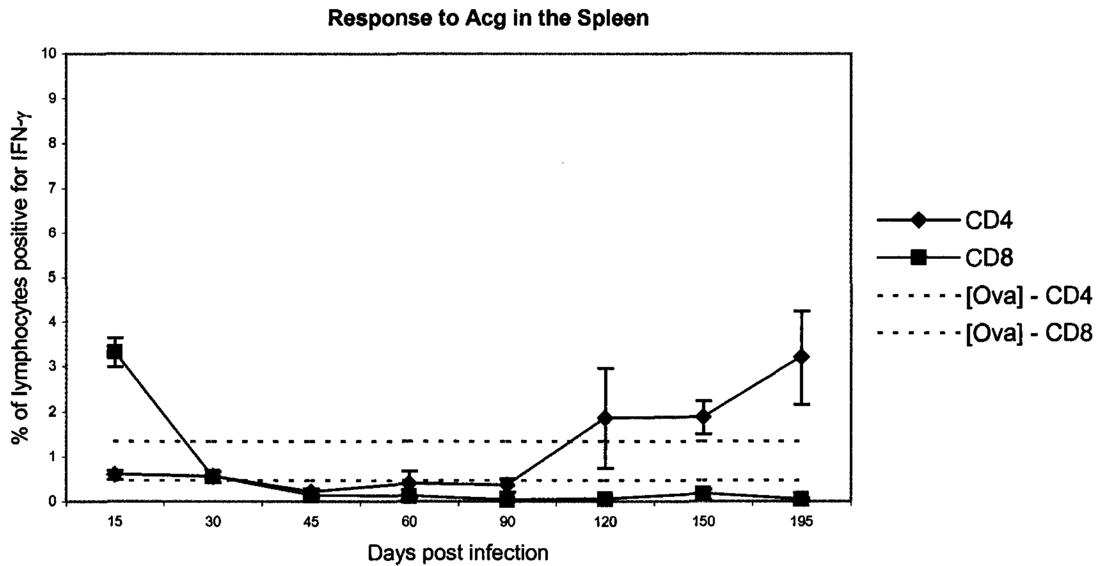

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Acg-pulsed dendritic cells.

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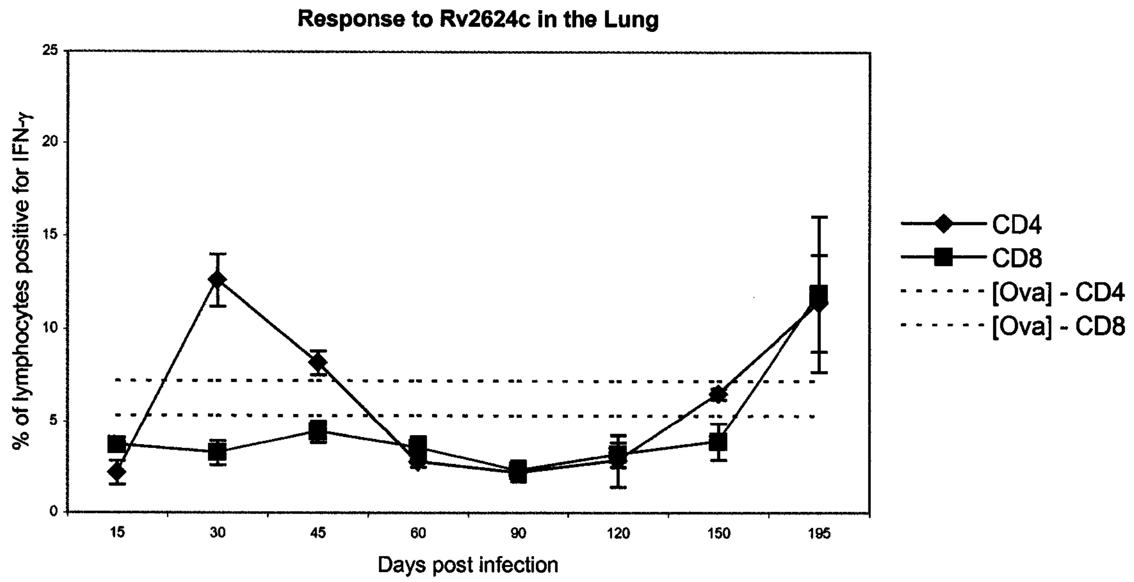

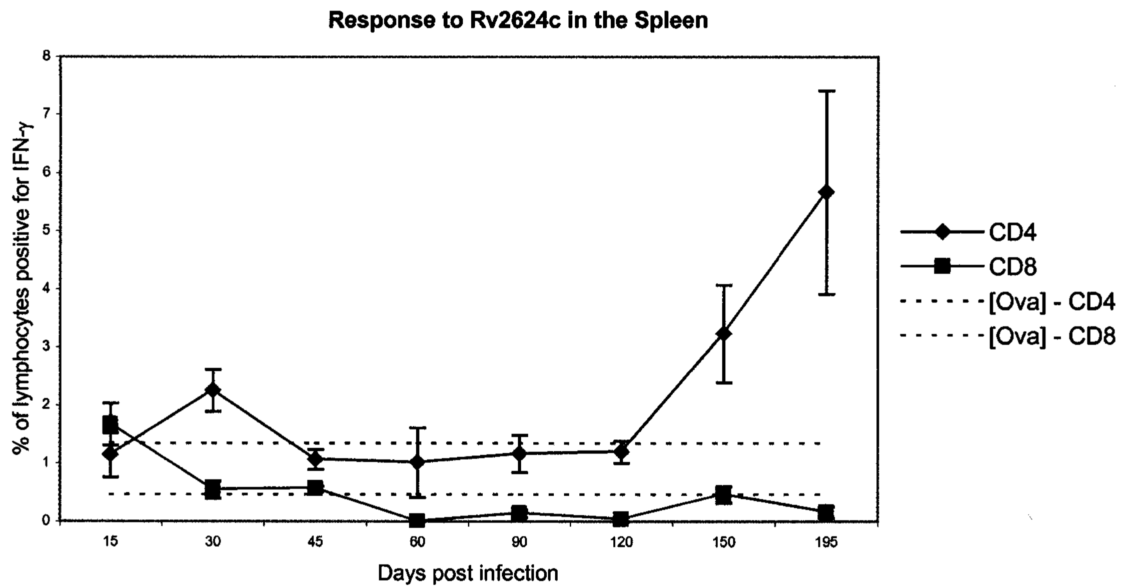

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Rv2624c-pulsed dendritic cells.

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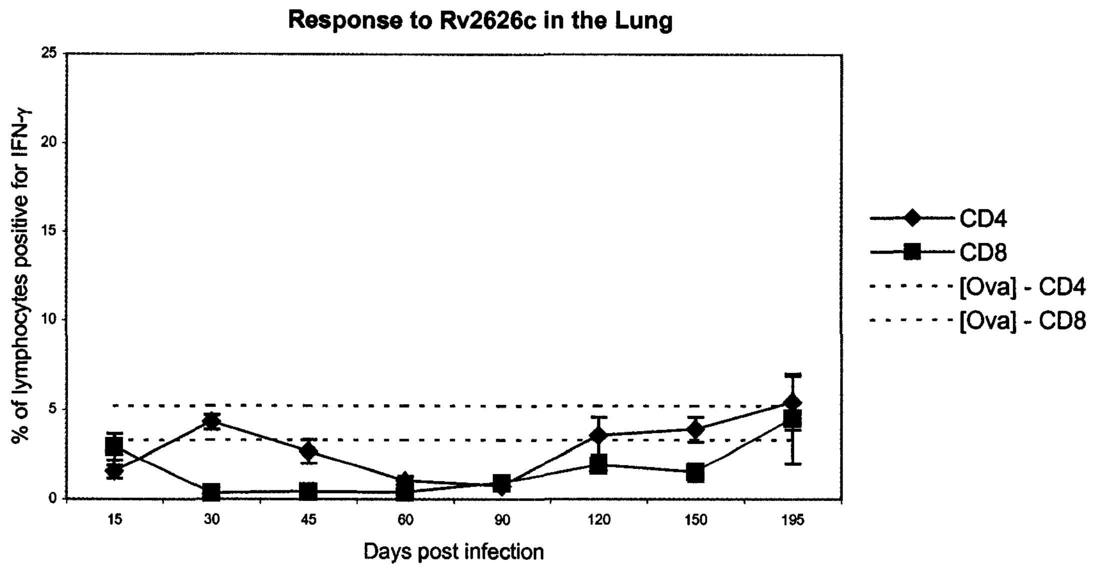

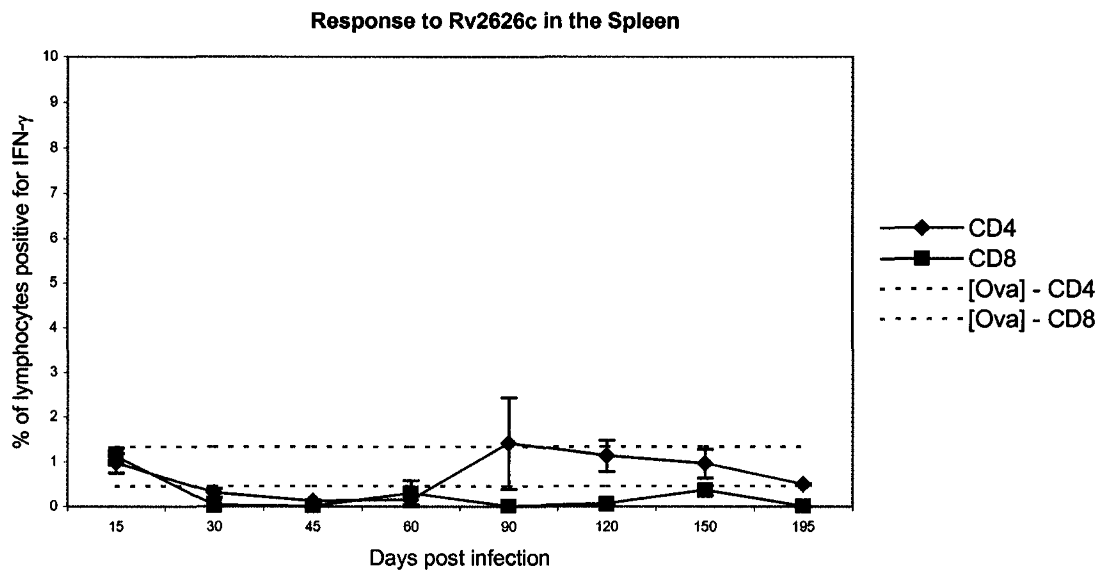

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Rv2626c-pulsed dendritic cells.

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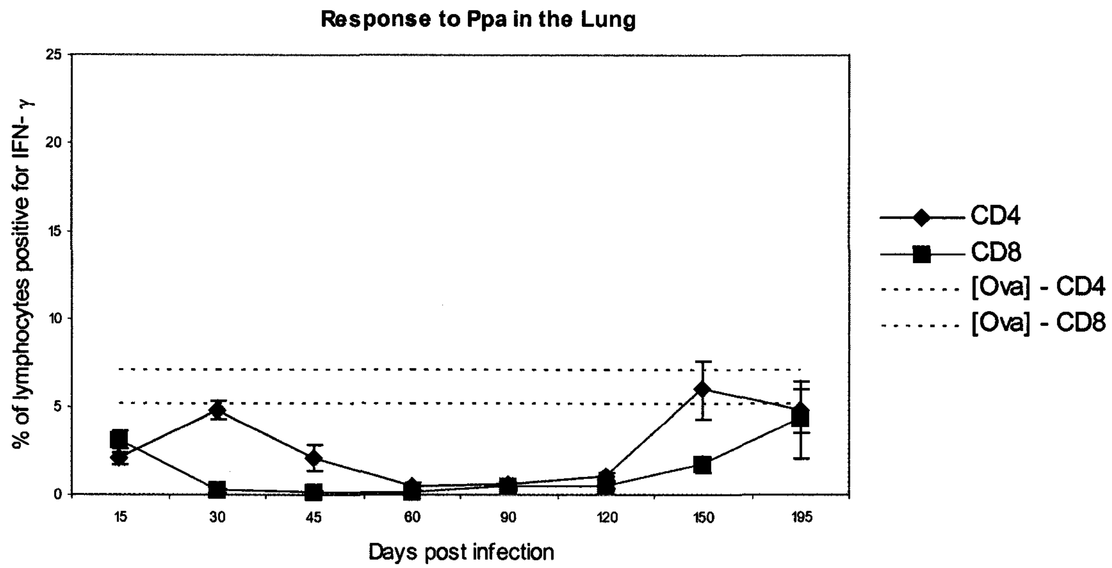

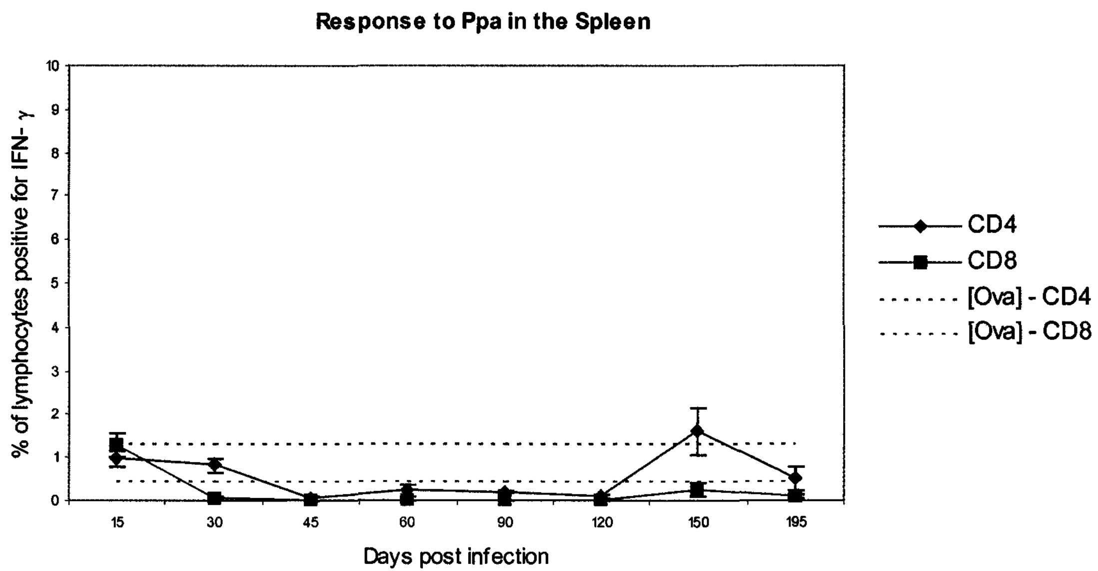

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Ppa-pulsed dendritic cells.

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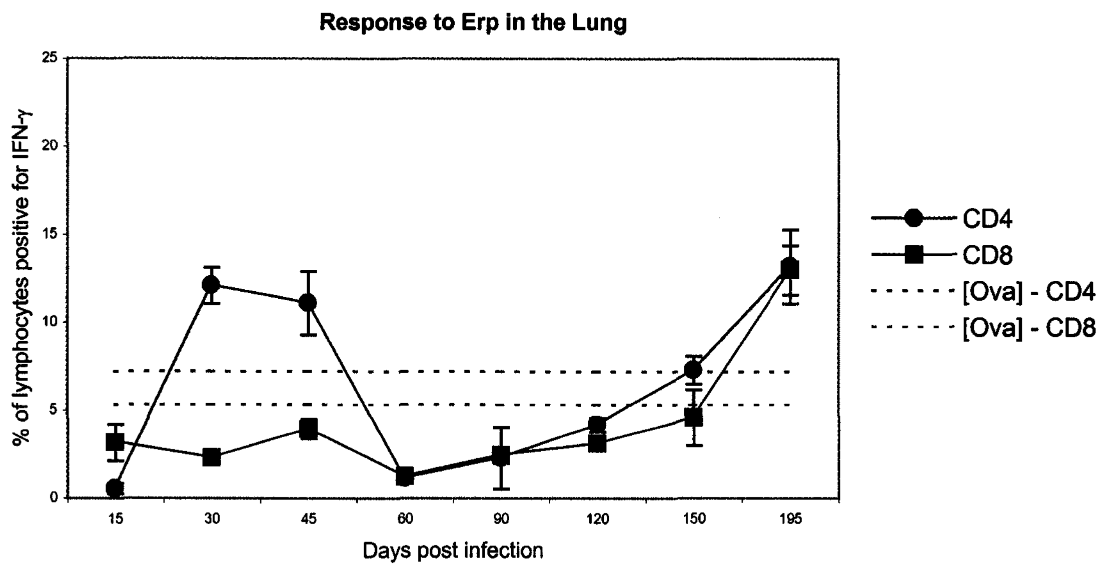

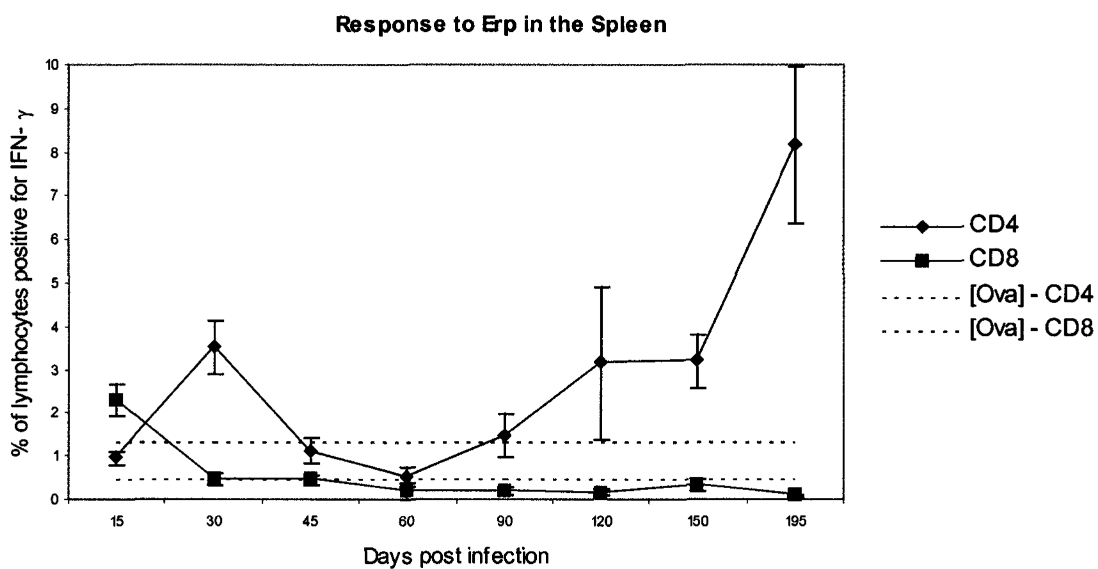

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on Erp-pulsed dendritic cells.

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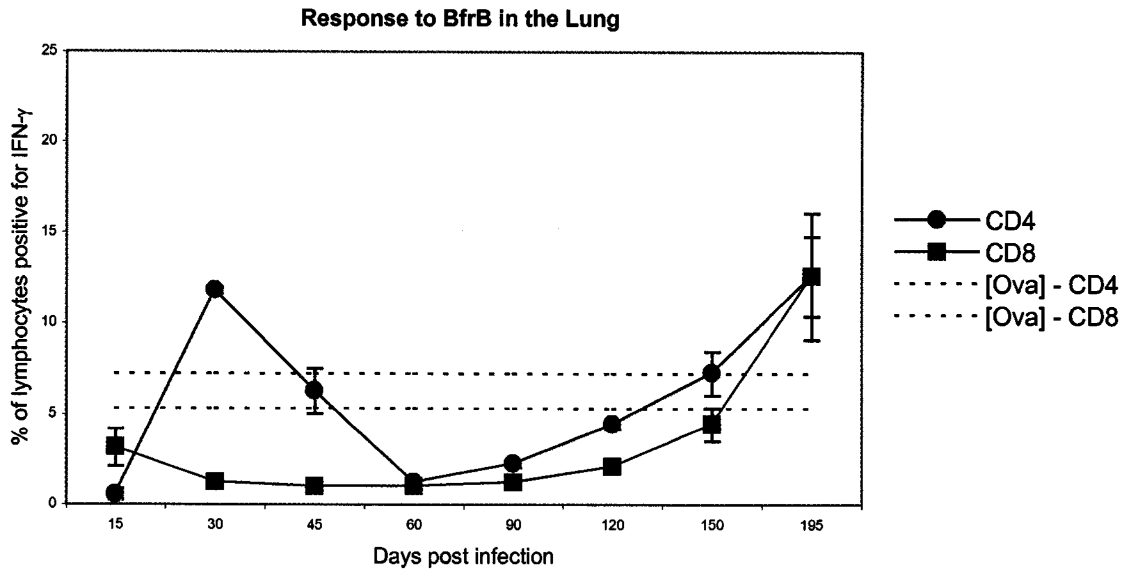

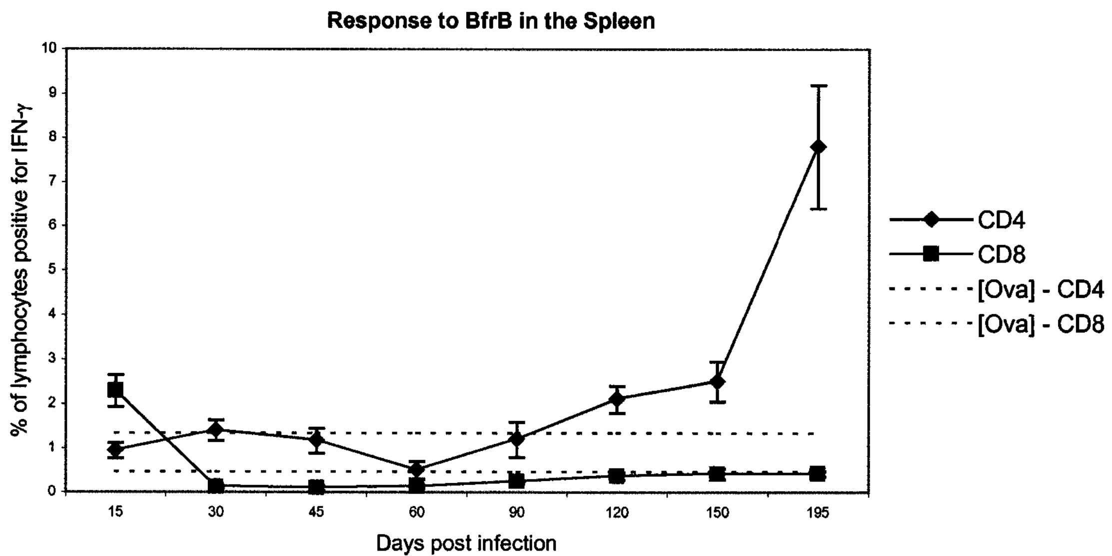

Figure 4.2 (cont.) Percentage of IFN-γ positive $CD4^+$ and $CD8^+$ lung and spleen cells after overlay on BfrB-pulsed dendritic cells.

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Table 4.4 **CD4 and CD8 lymphocyte response to *M. tuberculosis* proteins during the early and late stages of infection.**

	Early Response (30-45 dpi)				Late Response (120-195 dpi)			
	Lung		Spleen		Lung		Spleen	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
CFP	++	+	+	-	+	+	+++	-
Ag85A	+	-	-	-	+	+	+	-
ESAT-6	+	-	-	+	+	+	++	-
MPT-32	-	-	-	-	-	-	-	-
Fba	-	-	-	-	+	+	++	+*
Icl	+	-	+	-	-	-	-	-
Rv1738	+	+	+	-	+	+	++	-
Rv1813c	+	-**	+	+	+	++	++	-
Acr	++	+	++	-	-	-	+	-
Acg	-	-	-	-	+	+	+	-
Rv2624c	+	-	+	-	+	+	++	-
Rv2626c	-	-	-	-	-	-	-	-
Ppa	-	-	-	-	-	-	-	-
Erp	+	-	+	-	+	+	++	-
BfrB	+	-	-	-	+	+	++	-

- No significant response over ovalbumin. (If error bar crosses baseline it is scored as negative.)

+ < 3x value over ovalbumin, ++ 3-5x value over ovalbumin, +++ >5x value over ovalbumin.

* A slight response to Fba was observed by CD8+ cells in the spleen at day 120, but the response did not continue through days 150 or 195.

** CD8+ data was not collected at day 45.

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4.3.2 BCA Assay of cytokine concentrations.

Using cytokine-specific capture beads in a soluble fluorometric sandwich ELISA assay coupled with flow cytometry, CBA test allows the measurement of cytokine concentrations within a solution. In order to better understand the complex connections and interactions between host immune machinery and pathogen components during infection, the concentrations of key cytokines (IL12p70, TNF-α, IFN-γ, MCP-1, IL10, and IL-6) in the supernatants of the overlays were measured by FACS analysis after 24 h of stimulation as described previously. While all cytokines noted are produced mainly by macrophages and dendritic cells except for IFN-γ, which is produced primarily by lymphocytes, the evaluation of these molecules will provide a better understanding of the complex interplay between effector cells as it is directed by activated lymphocytes. As demonstrated in Figure 4.3, cytokine production to each antigen tested differed from each other and at different time points of infection.

In many cases, the cytokines, IL-12p70, TNF-α, and IFN-γ were found to be expressed in approximately the same pattern through each time point, but in a pattern specific to the individual antigen. For example, CFP treatment induced a peak in expression of IFN-γ, IL-12p70, and TNF-α at 60, 90, and 90 days, respectively, followed by a dip in cytokine production at 120 days post infection. This reduction was followed by increased levels of these cytokines during the remainder of the experiment. This pattern of correlated expression is also observed when Ag85A was used. However the timepoints of peak cytokine levels are unique to this protein with the highest levels of all three pro-inflammatory molecules occurring at 45 days followed by a trough of basal

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level expression occurring from 60-120 days with a subsequent resurgence of expression at later times. ESAT-6, Fba, Rv1738, Rv1813c, Acr, Rv2624c, Erp, and BfrB are all seen to fall into this trend to some degree. Proteins MPT-32, Icl, Acg, Rv2626c, and Ppa, where levels of IL-12p70, TNF-α, and IFN-γ were rarely seen to increase above the amount generated in response to ovalbumin, did not exhibit this biphasic pattern, nor did it appear that coordinated cytokine expression occurred.

In this assay, the levels of MCP-1 and IL12p70 appeared low in relative abundance compared to other cytokines and were often present in quantities similar to negative controls, even when other inflammatory cytokines were seen to peak significantly. This could be due to several factors: intrinsic limitations of the assay to accurately determine biologically relevant increases in cytokine levels, the antigens do not stimulate DC to produce IL-12, susceptibility of these cytokines to breakdown during freezing, or in the case of IL12p70, dissociation of the p40 and p35 subunits before analysis could occur.

In this experiment, the strongest IL-6 production was observed in response to those antigens that produced a dominant pro-inflammatory response in both cytokine (Figure 4.3) and T-cell activation (Figure 4.2) assays. Expression of this cytokine can be categorized according to its production during early (days 30-45), middle (days 60-90), or late (days 120-195) stages of infection. Proteins CFP, Rv1738, Rv2624c, Erp, and BfrB elicit expression during all three stages, while Ag85A stimulates IL-6 production only during the early time points. Moreover, ESAT-6, and Acg stimulated expression only during the late stage of infection, while Icl and Acr yielded production during early and late stages, but not during the middle stage of infection with Acr exhibiting strong

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biphasic peaks at 45 and 150 days post infection. Fba and Rv1813c demonstrated significant levels of this cytokine during the middle and late phases of infection preferentially, however early data for Rv1813c was not available due to sample loss.

In response to the need for balance in the immune response and to avoid excessive tissue damage and pathology from excessive inflammation, the host produces cytokines such as IL-10 that counteract Th1 mechanisms of activated cells [17]. IL-10 has been implicated in at least some of the inhibition observed in IL-12 levels during *M. tuberculosis* infection [18], but other factors are clearly also in play [19]. IL-10 concentrations increased significantly from days 30- to 45-post infection in response to almost all proteins tested. Proteins that failed to induce this pattern of increased IL-10 expression early were those which failed to manifest indications of an inflammatory response either in the CBA assay or intracellular staining for IFN-γ production, including MPT-32, Rv2626c, and Ppa. The only exception to this, with levels of IL-10 remained statistically equivalent to baseline early in infection, is seen in response to Fba where immune responses in general were not seen to occur until after 60 days post infection determined by CBA analysis (Figure 4.3) or by intracellular IFN-γ staining (Figure 4.2).

In general, this early spike in IL-10 production was followed by decreased levels over the remainder of the experiment (Figure 4.3). In the case of CFP, Rv1738, and Rv2624c IL-10 concentrations remained above baseline for every time point, while Ag85A, Acr, Fba, Icl, Erp, and BfrB-induced expression dropped below baseline during 60 and/or 90 days followed again by significant levels at 120-195 days post infection. ESAT-6 and Rv1813c produced baseline levels of IL-10 at middle time points with statistical levels at later times; early time points were not available.

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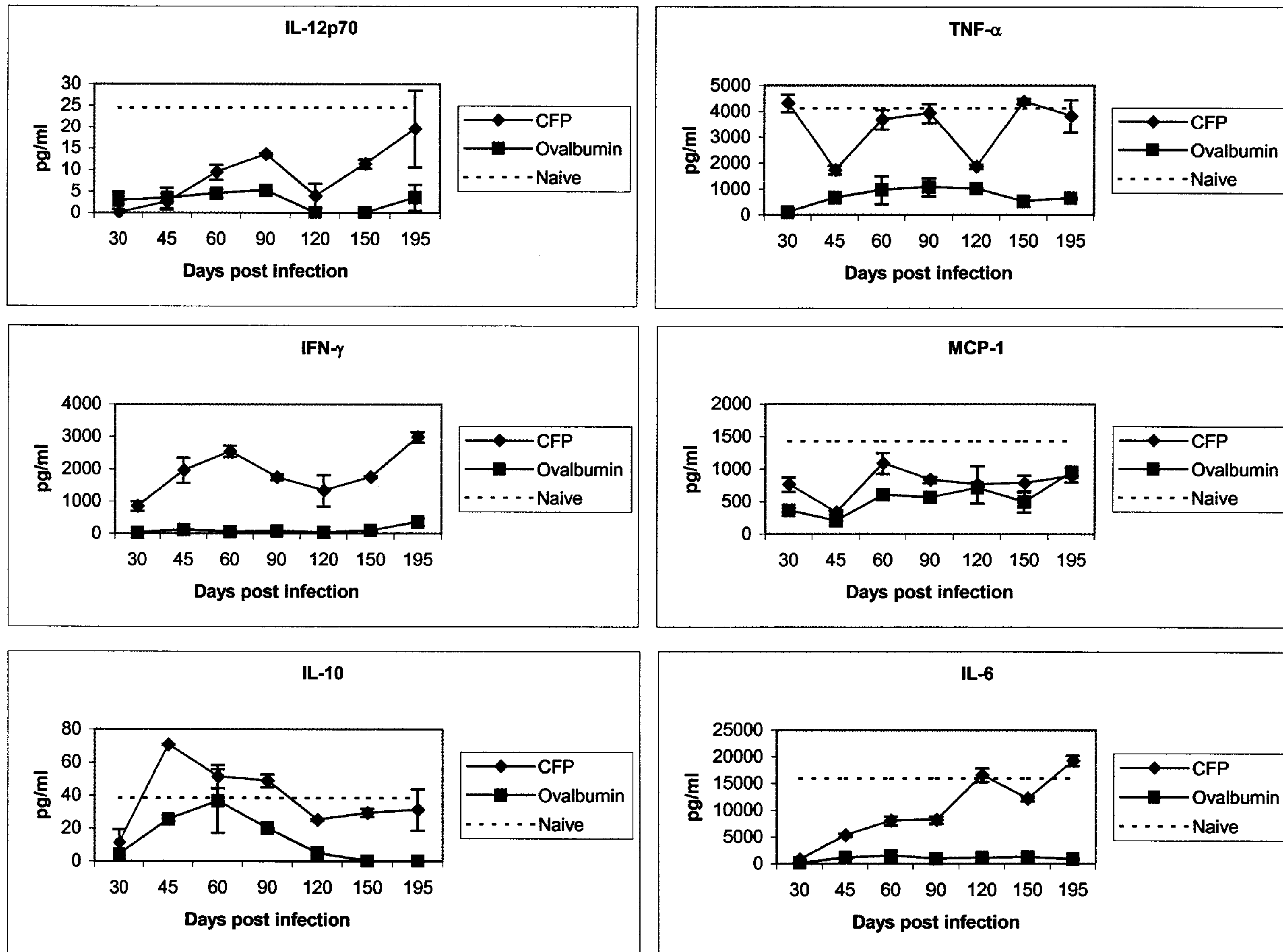

Figure 4.3 CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from CFP- and ovalbumin-pulsed dendritic cells. All values are an average of four mice from each time point +/- SEM. Naïve values are an average of 8 mice.

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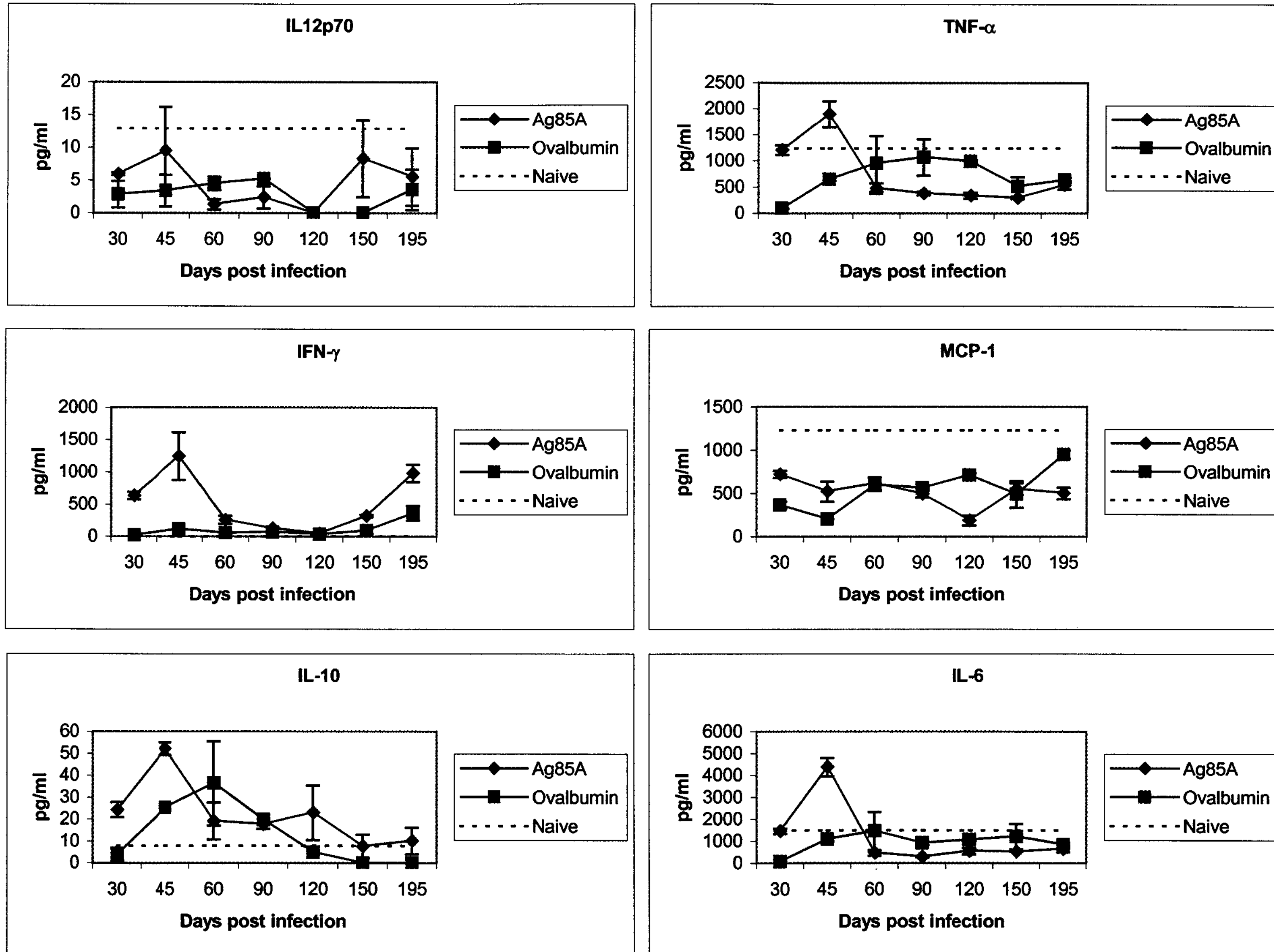

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Ag85A- and ovalbumin-pulsed dendritic cells.

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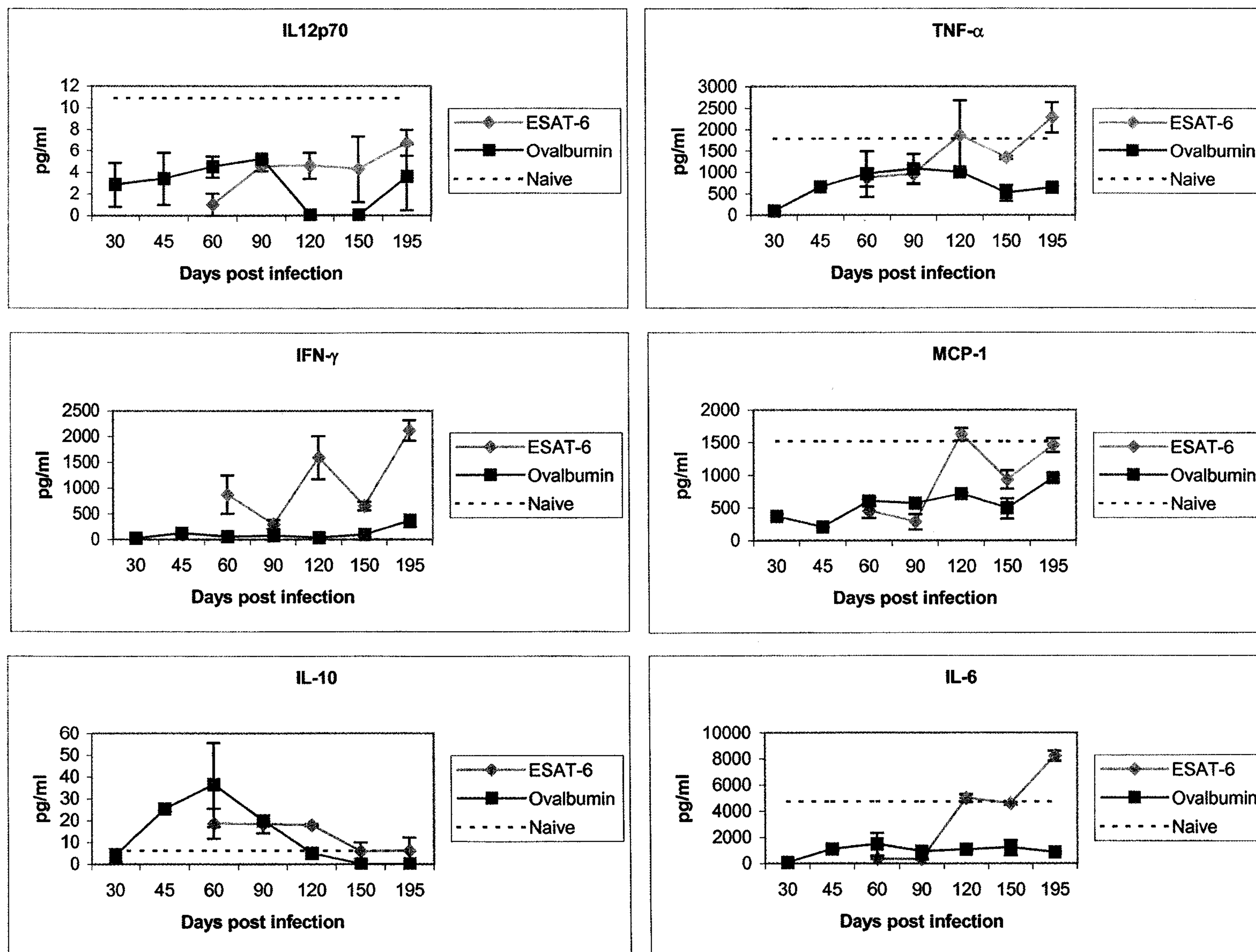

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from ESAT-6- and ovalbumin-pulsed dendritic cells.

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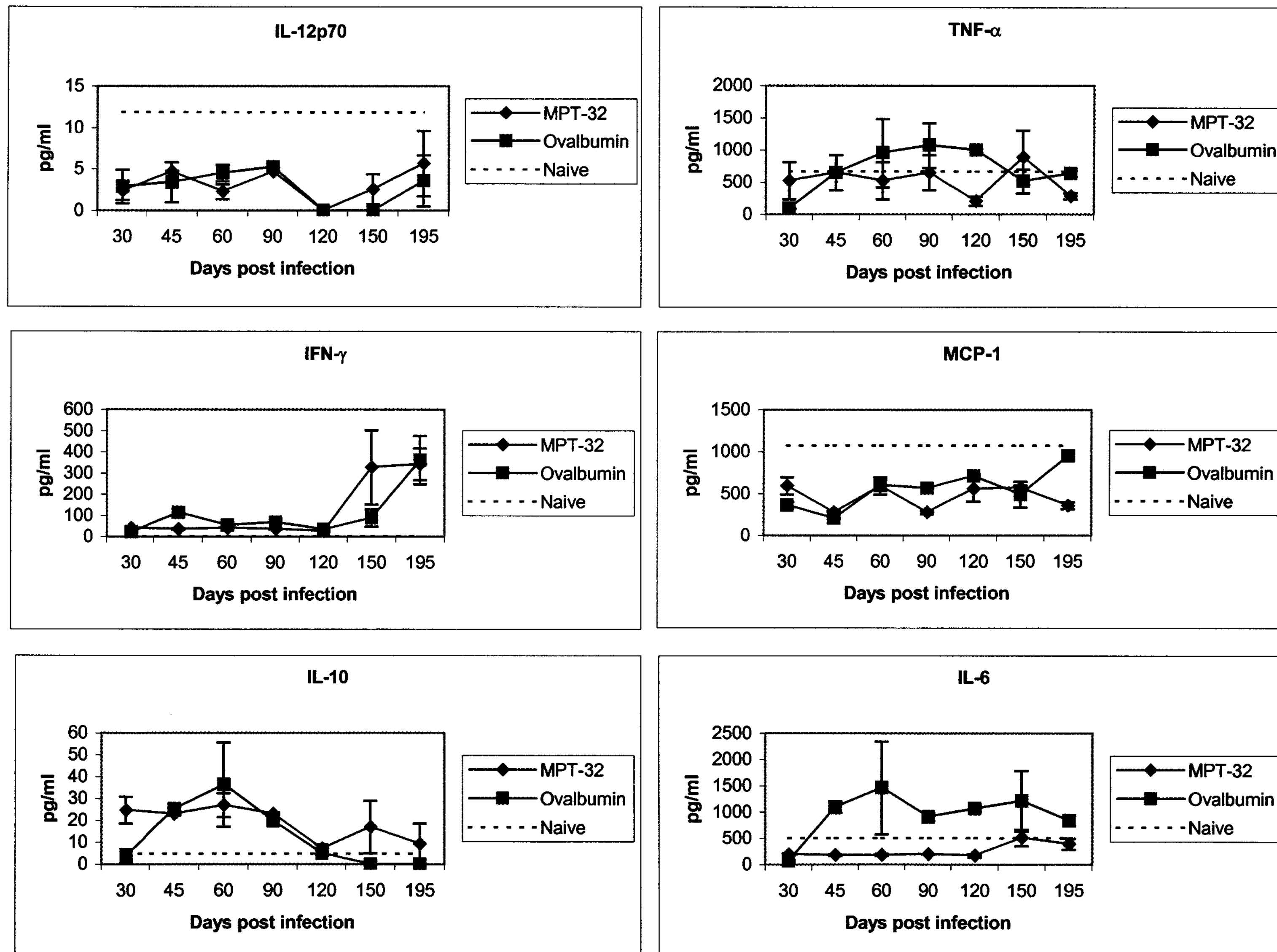

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from MPT-32- and ovalbumin-pulsed dendritic cells.

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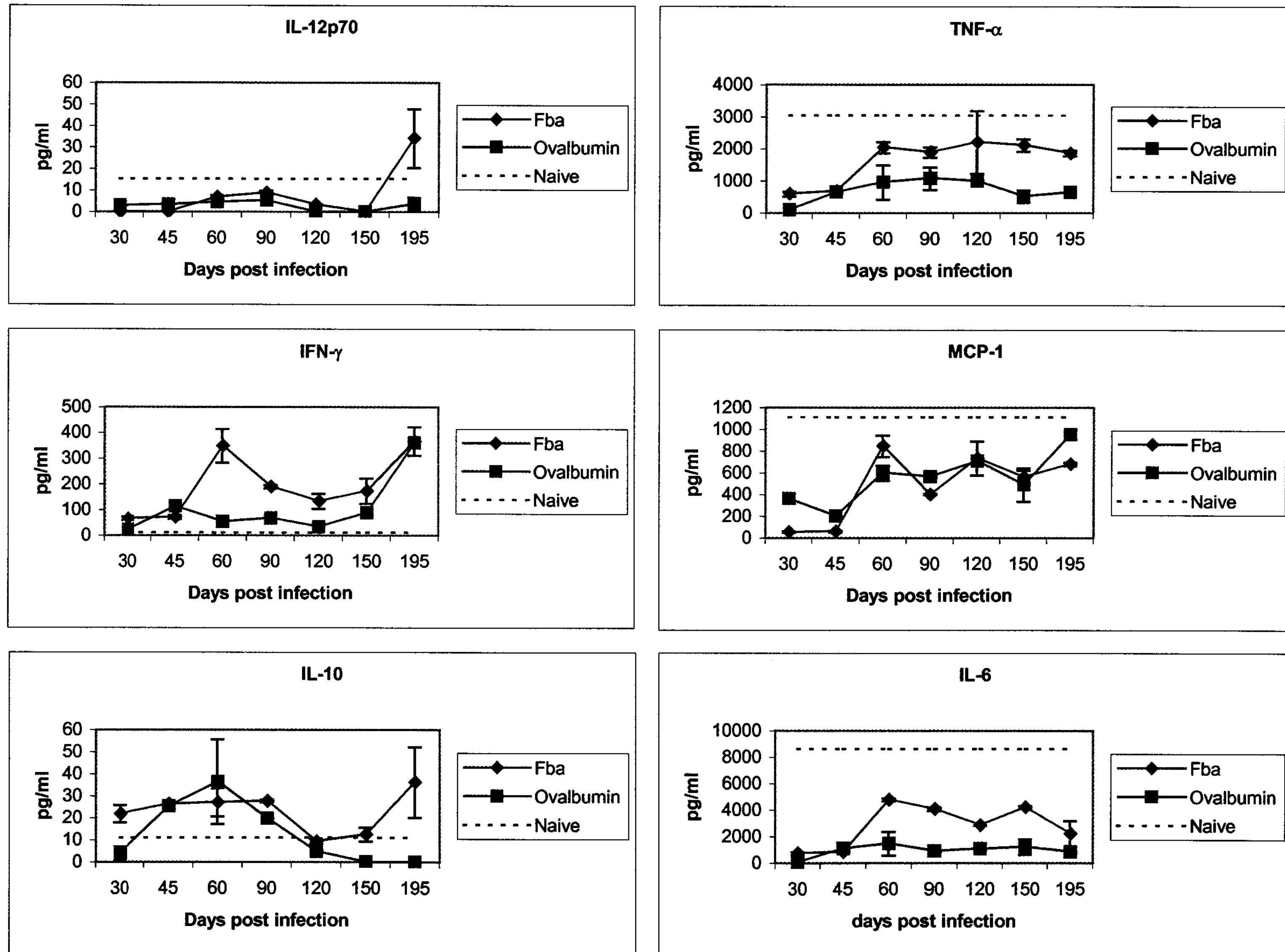

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Fba- and ovalbumin-pulsed dendritic cells.

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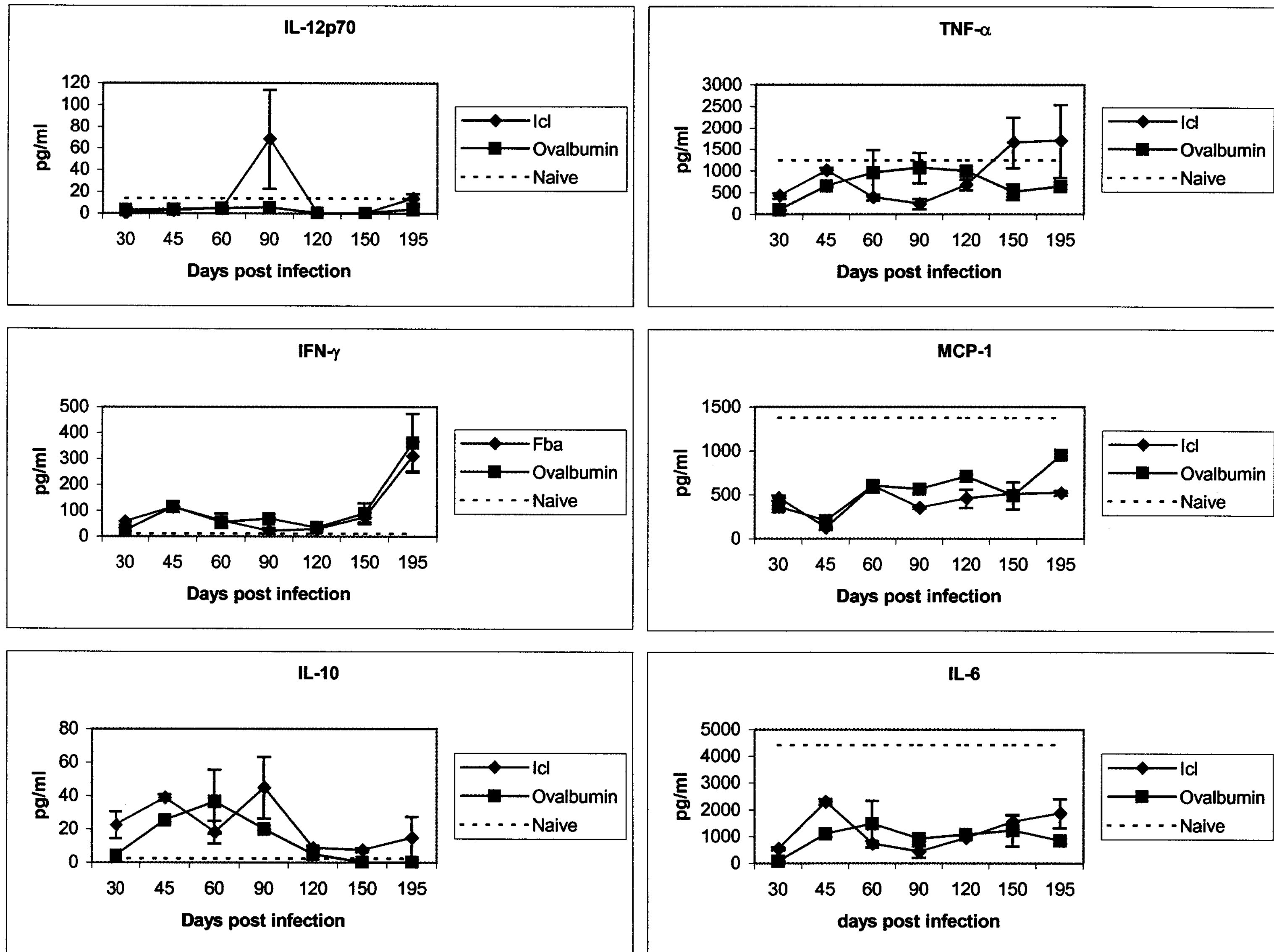

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Icl- and ovalbumin-pulsed dendritic cells.

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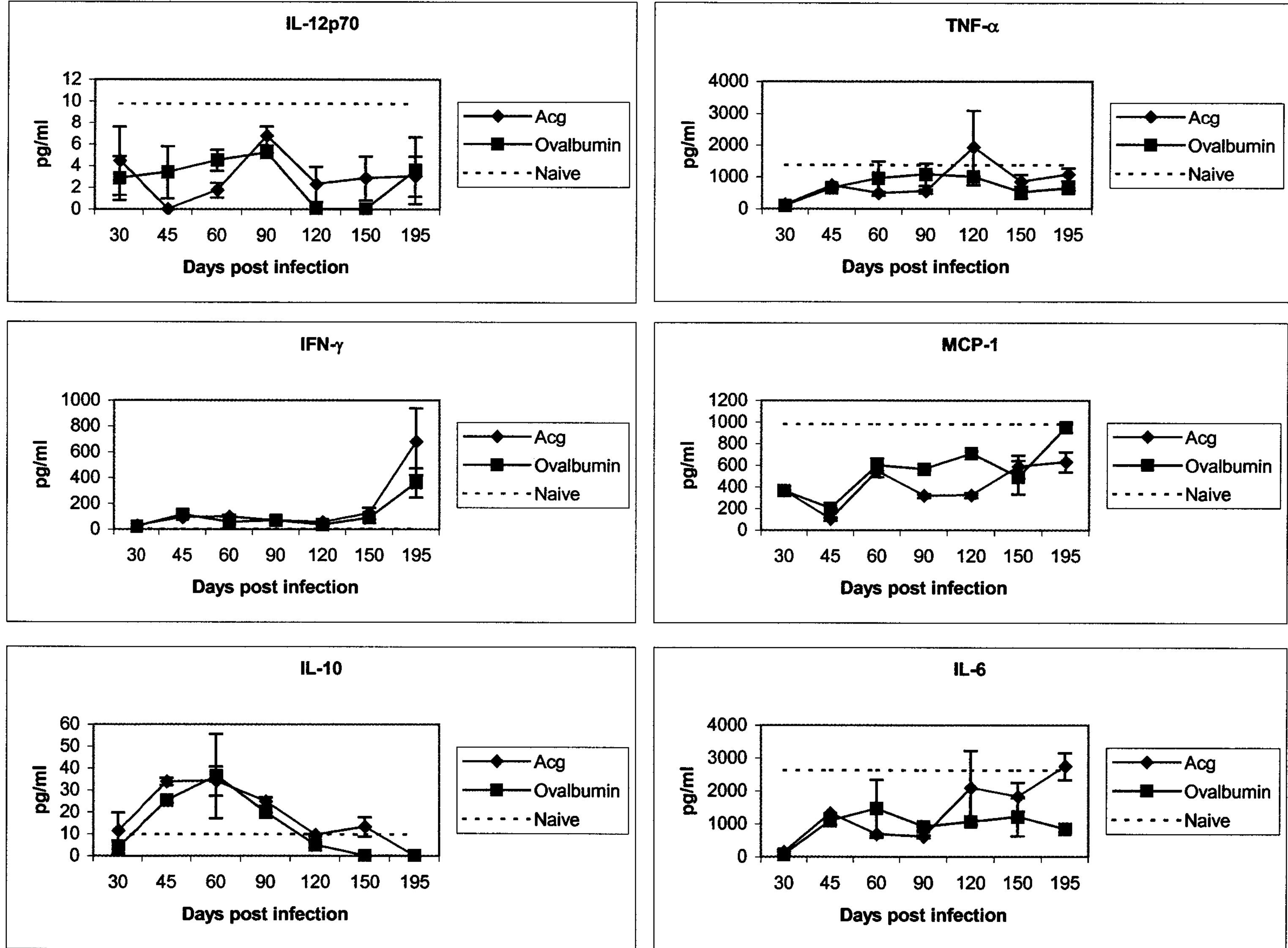

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Acg- and ovalbumin-pulsed dendritic cells.

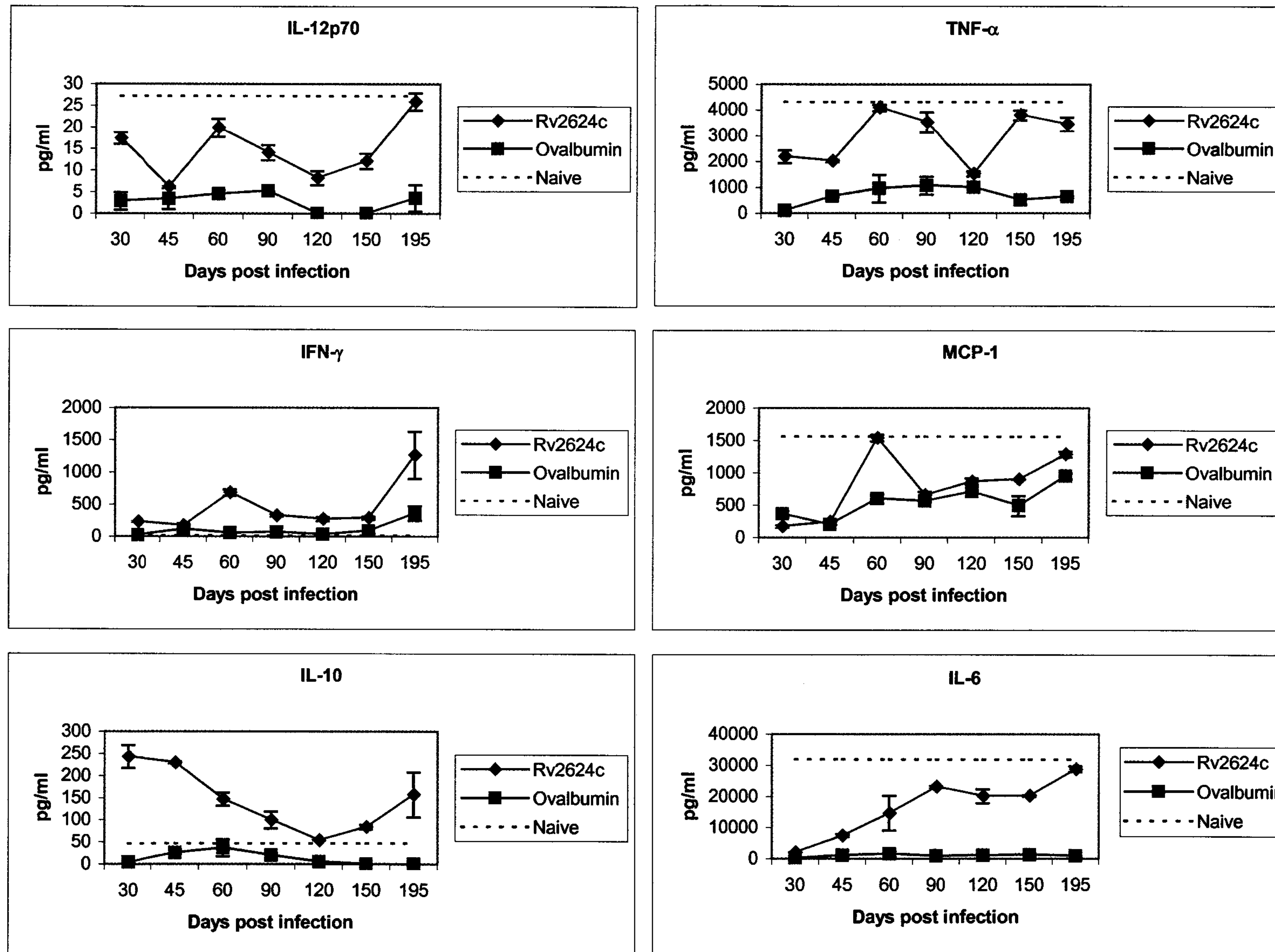

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Rv2624c- and ovalbumin-pulsed dendritic cells.

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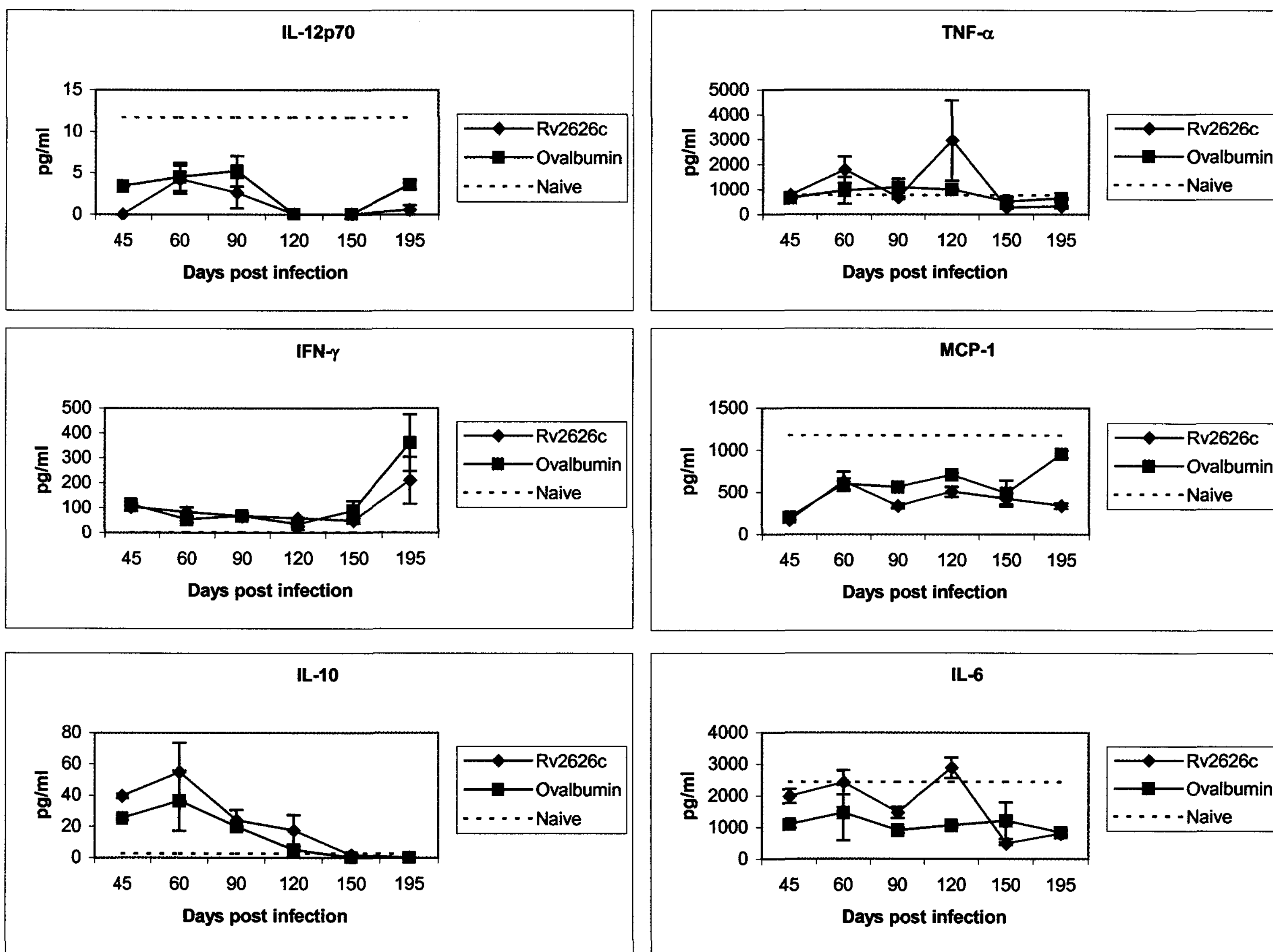

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Rv2626c- and ovalbumin-pulsed dendritic cells.

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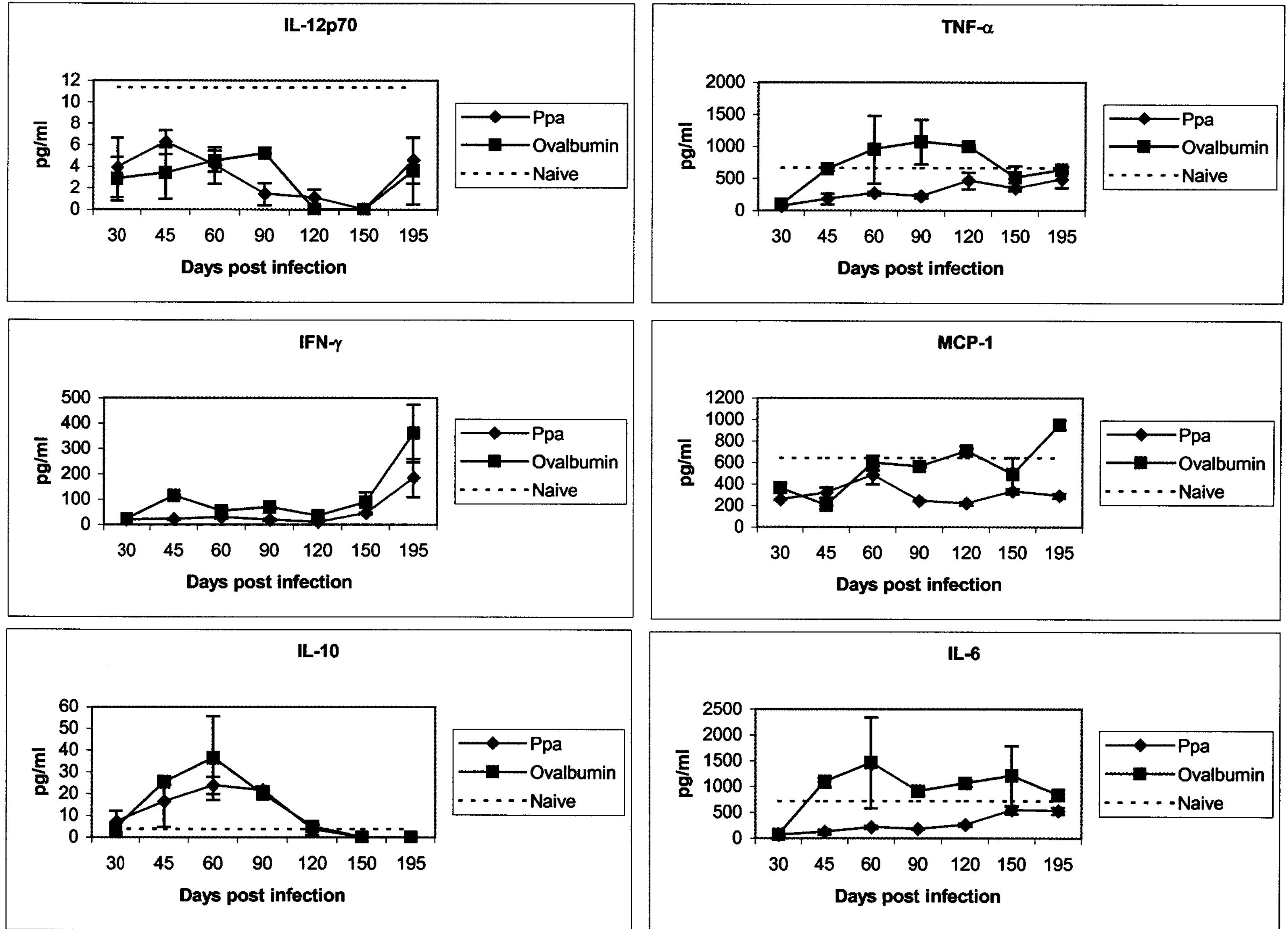

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Ppa- and ovalbumin-pulsed dendritic cells.

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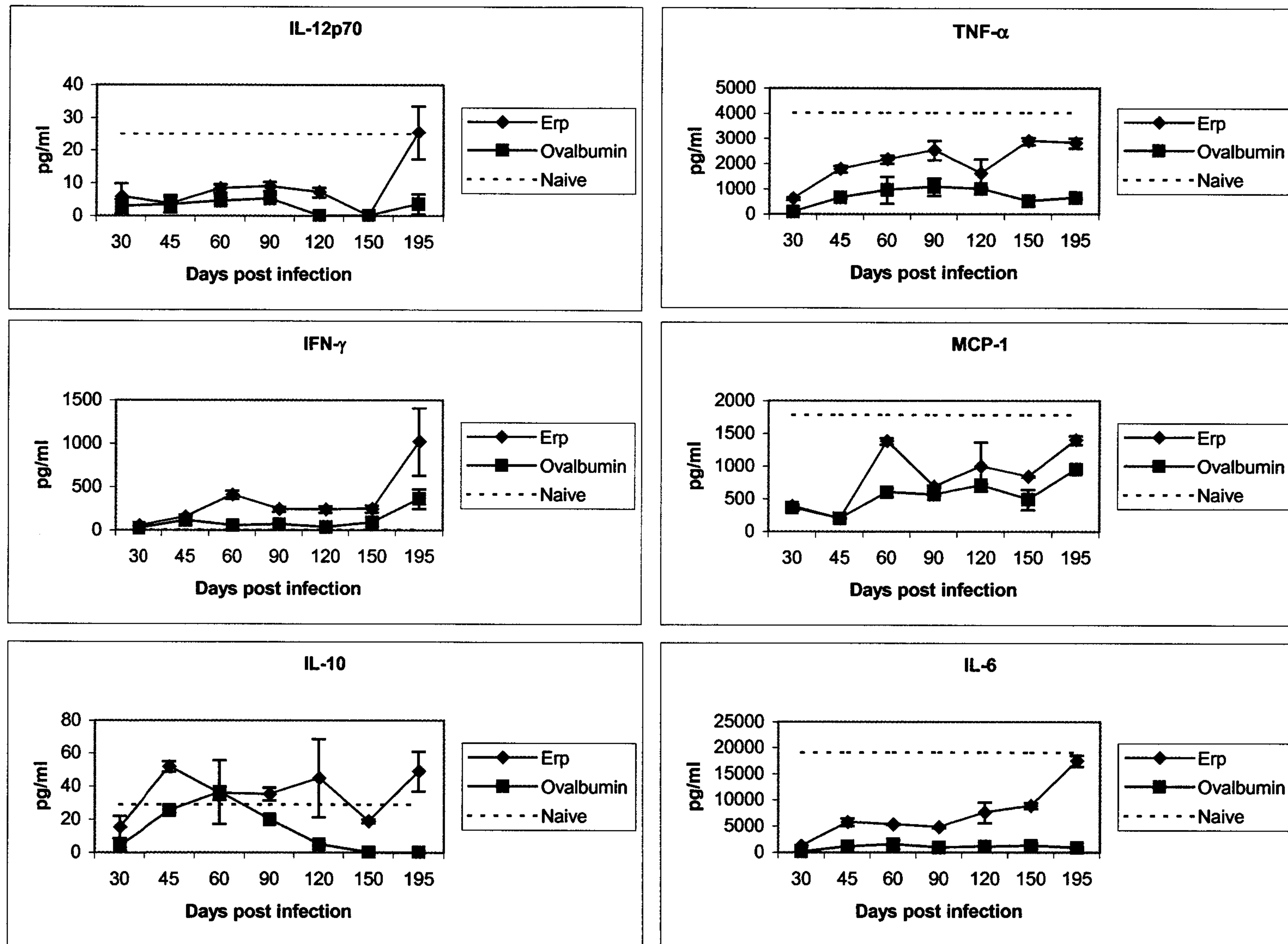

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Erp- and ovalbumin-pulsed dendritic cells.

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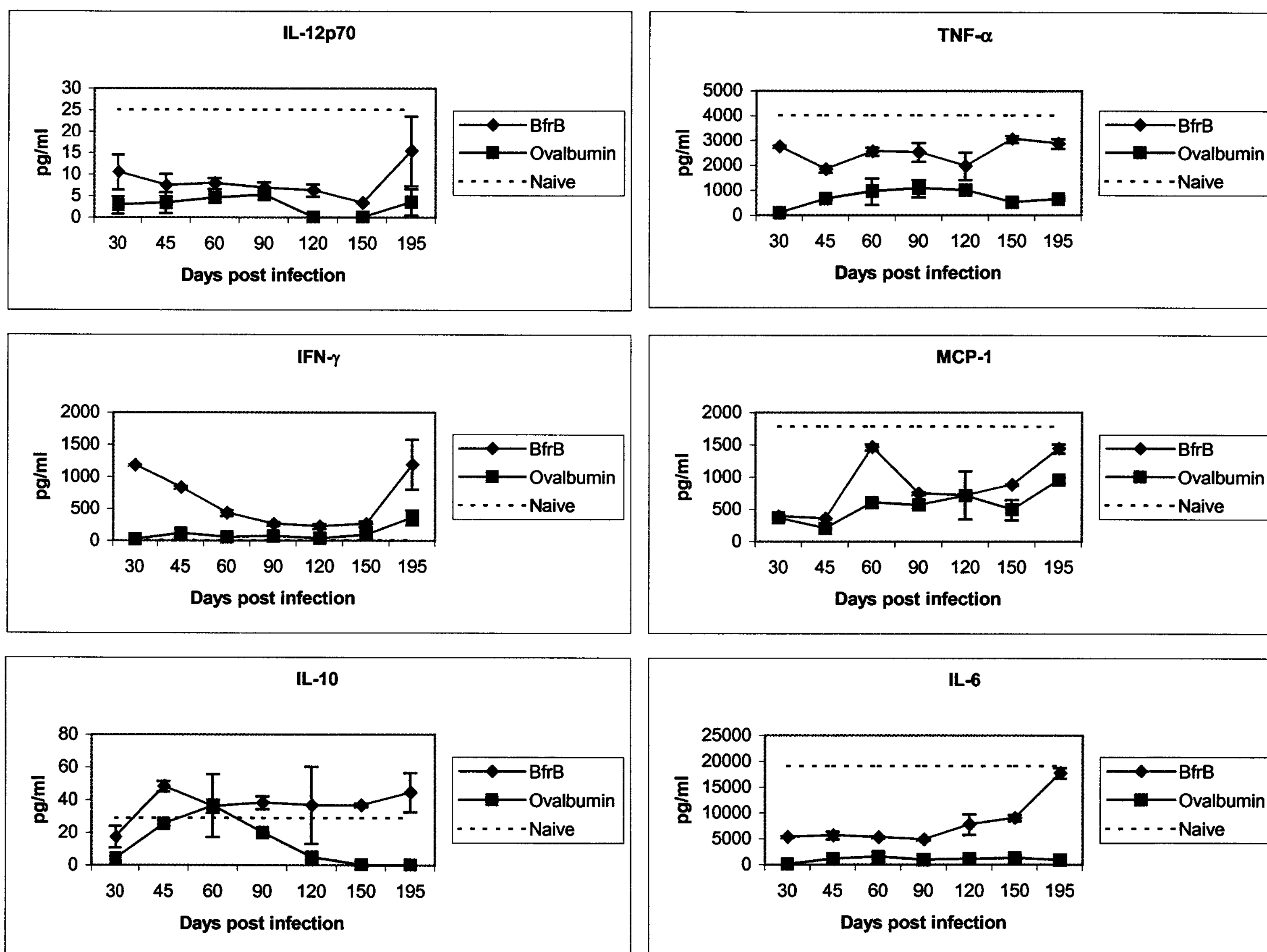

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from BfrB- and ovalbumin-pulsed dendritic cells.

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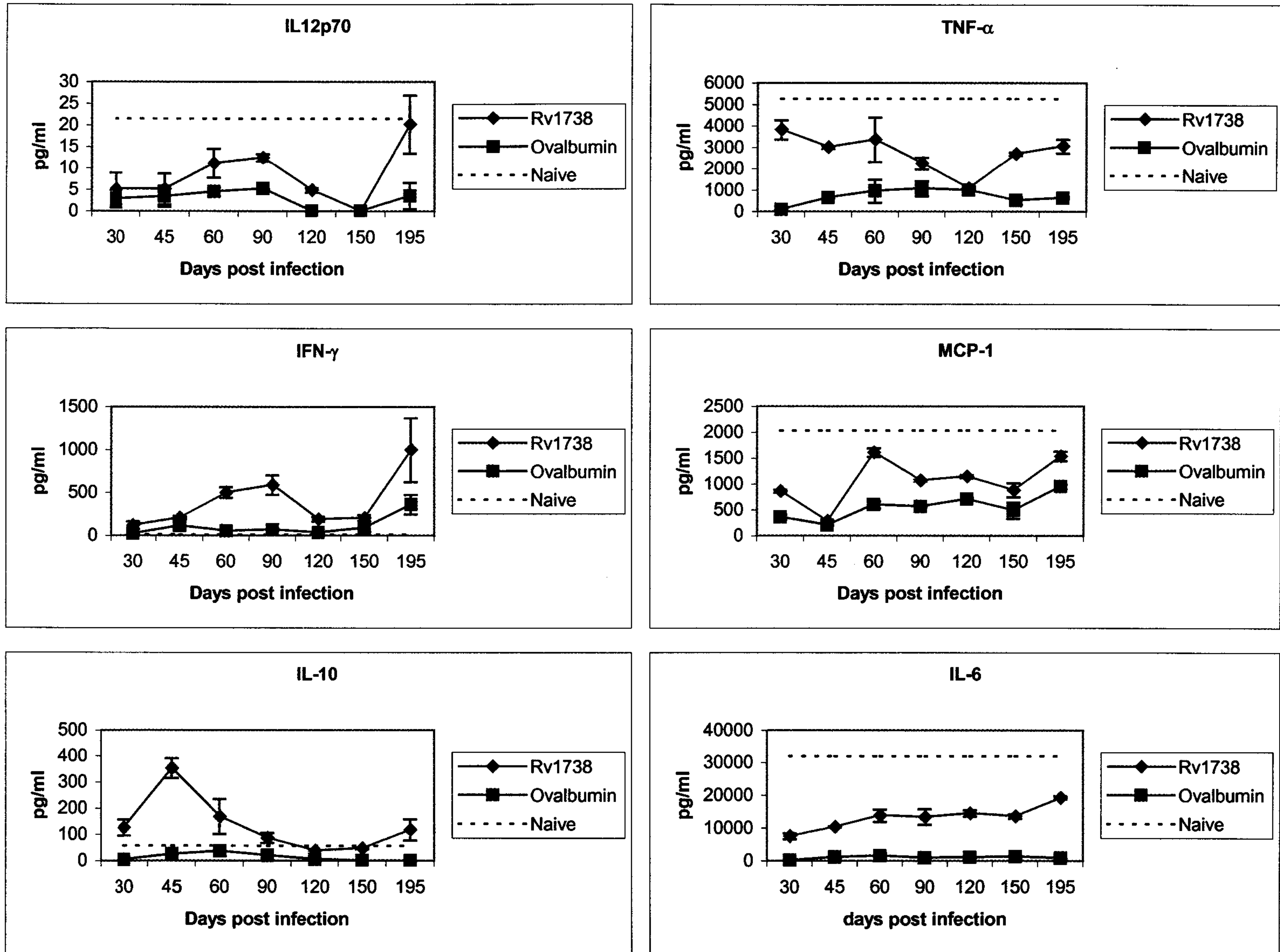

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Rv1738- and ovalbumin-pulsed dendritic cells.

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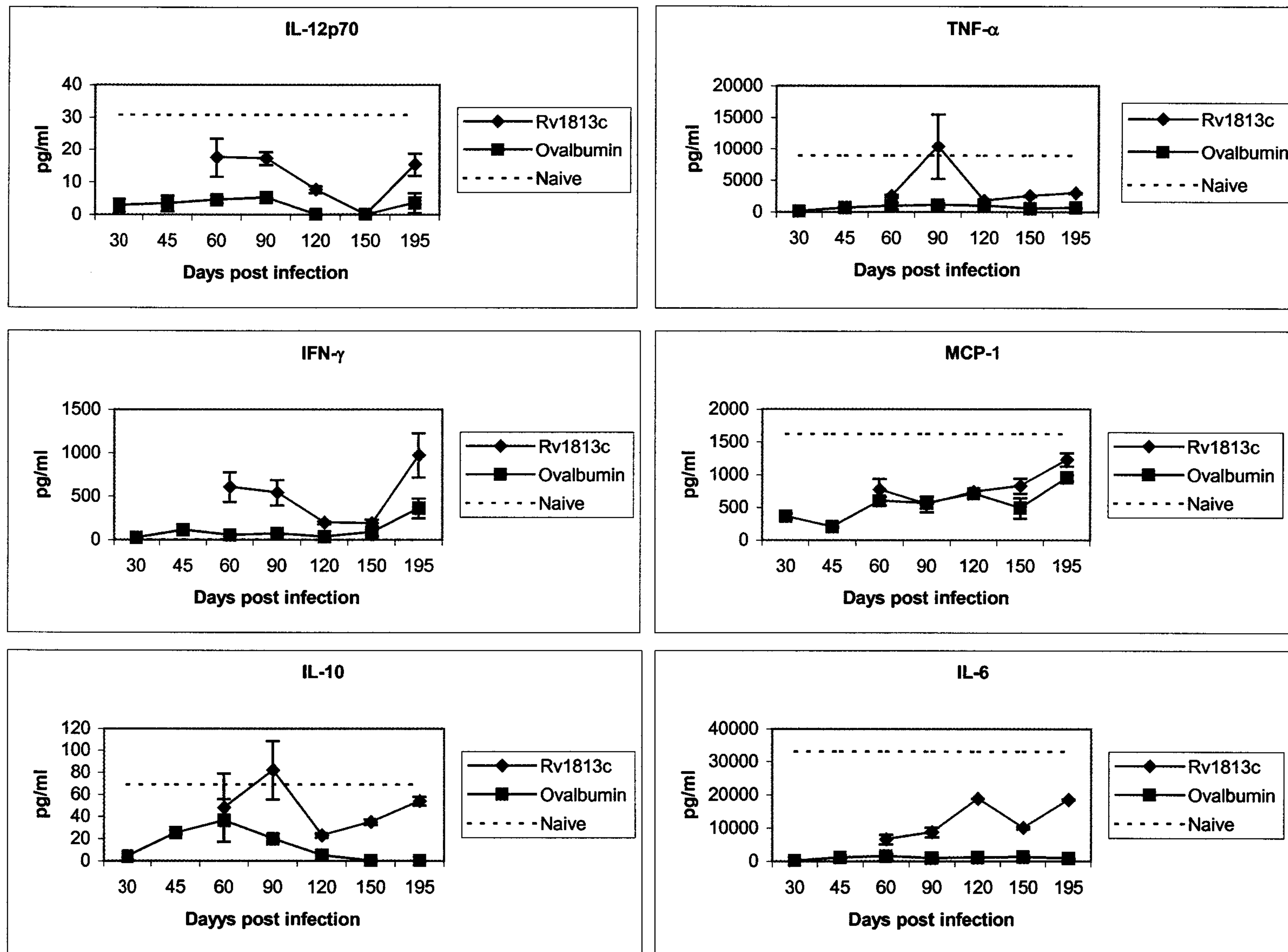

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Rv1813c- and ovalbumin-pulsed dendritic cells.

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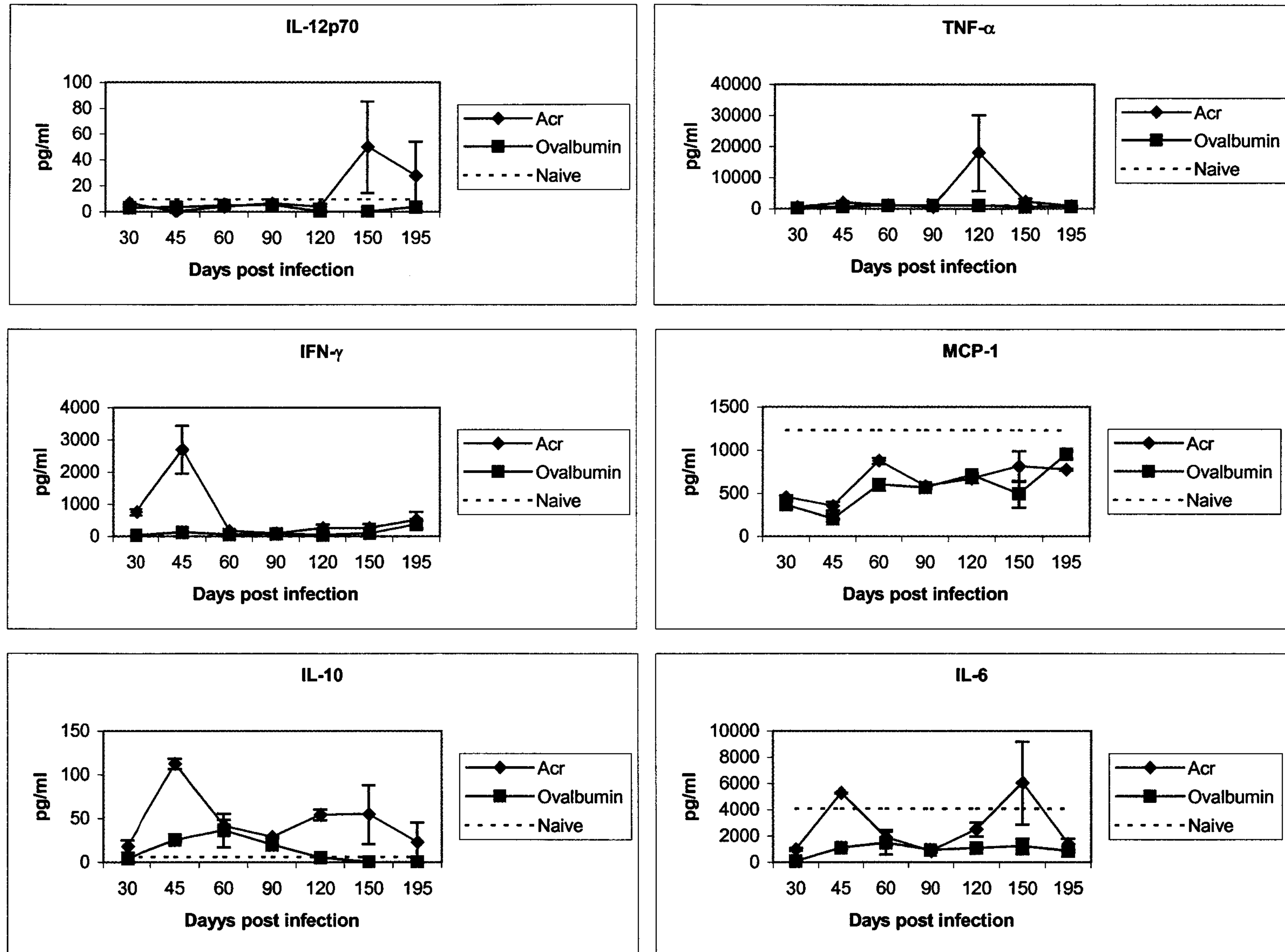

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from Acr- and ovalbumin-pulsed dendritic cells.

4.3.3 Cytokine levels of naïve T-cells overlays in response to various M. tuberculosis *proteins*

Employing the same overlay strategy using lymphocytes from uninfected C57BL/6 mice demonstrates the stimulatory nature of some of the proteins used, independent of *M. tuberculosis* infection (Figure 4.4). CFP and proteins Rv1738, Rv1813c, Rv2624c, Erp, and BfrB induced high levels of IL-12, TNF-α, IL-6 and IL-10, with the latter two cytokines exhibiting the strongest increase. IFN-γ also displayed modestly, though not statistically significant, increased levels in response to CFP, Fba, Rv1738, and Rv2624c. Comparing these data from naïve mice with data from infected mice leads to an intriguing question about the ability of *M. tuberculosis* infection to inhibit the production of various cytokines produced in response to antigenic stimulation. In multiple cases where antigens were capable of inducing cytokine production significantly above baseline throughout infection, the concentration was still well below the levels produced by the same antigen in the naïve mice (i.e. see Figure 4.3 proteins Rv1738 and Rv2624, especially IL-6 values). This decreased cytokine expression in response to infection, which was independent of the protein used for stimulation, was observed for every cytokine except IFN-γ where baseline values exceeded naïve values throughout the experiment.

4.3.4 A priming vaccination of BCG followed by boosting injections of HPP affords protection from subsequent challenge greater than BCG or HPP alone, which increases during chronic infection.

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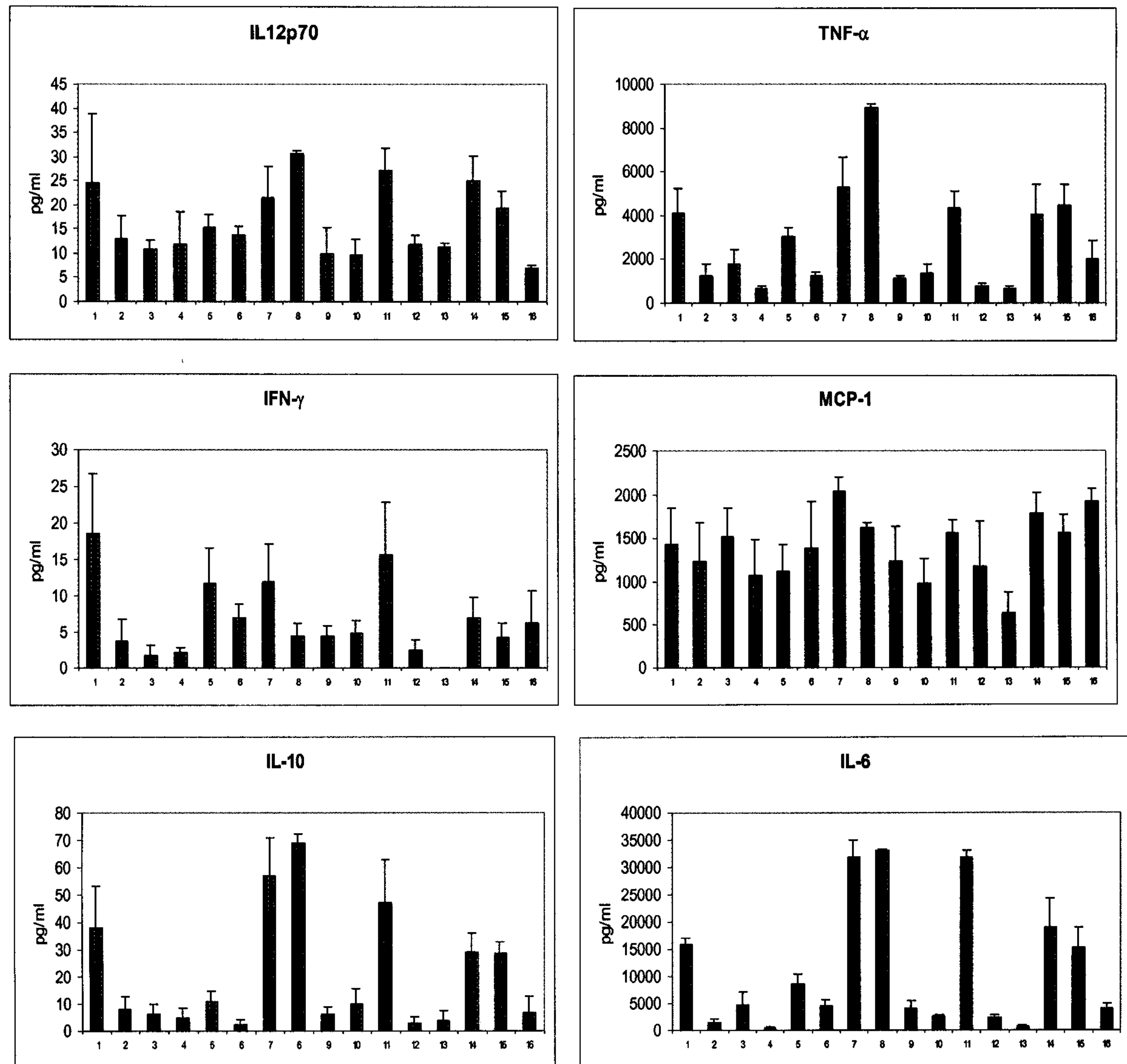

Figure 4.4 Cytokine production by lymphocytes from naive mice in response to *M. tuberculosis* proteins. Lymphocytes from the lungs of non-infected C57BL/6 mice were overlaid onto dendritic cells that had been incubated for 24 h with *M. tuberculosis* proteins (10μg/ml). Supernatants were collected after 24 h and analyzed by CBA assay. Proteins used : 1) CFP, 2) Ag85A, 3) ESAT-6, 4) MPT-32, 5) Fba, 6) Icl, 7) Rv1738, 8) Rv1813c, 9) Acr, 10) Acg, 11) Rv2624c, 12) Rv2626c, 13) Ppa, 14) Erp, 15) BfrB, 16) Ovalbumin. Results are the average of 8 mice +/- SEM.

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Eleven proteins produced in greater abundance under hypoxic conditions were pooled and administered to C57BL/6 mice as put forth in Table 4.3. The mice were subsequently challenged by low dose aerosol with *M. tuberculosis*-H37Rv at 72 days after the first vaccination. The hypoxia-induced protein pool (HPP) consisted of equal quantities of Fba, Icl, Rv1738, Rv1813c, Acr, Acg, Rv2624c, Rv2626c, Ppa, Erp, and BfrB [20-22]. In this experiment, a single injection of 10^6 CFU of BCG-Pasteur given SC gave 1.2 and 1.0 logs protection at 30 days post infection (pi), 0.8 and 1.3 logs protection at 60 days pi in the lungs and spleens, respectively ($p<0.05$) (Figure 4.5). At day 90 pi 0.8 logs protection was realized in the lungs of BCG vaccinated mice. When the HPP and adjuvant (MPL-SE and DDA) was administered three times over the course of six weeks, no significant protection was observed over the course of the experiment. Interestingly, when BCG and HPP were combined in a prime-boost strategy with a single BCG injection at day 1 and three injections of HPP and adjuvant at days 1, 21, and 42, protection was seen to be 1.8 and 1.1 logs at 30 days pi, and 1.9 and 0.4 logs at 60 days pi in the lungs and spleens, respectively ($p<0.05$). At 90 days pi protection was observed at 1.8 logs in the lung. Moreover, the increase in protection using the prime-boost method was statistically significant over the single BCG injection ($p<0.05$). Histology was not performed on the organs of these mice.

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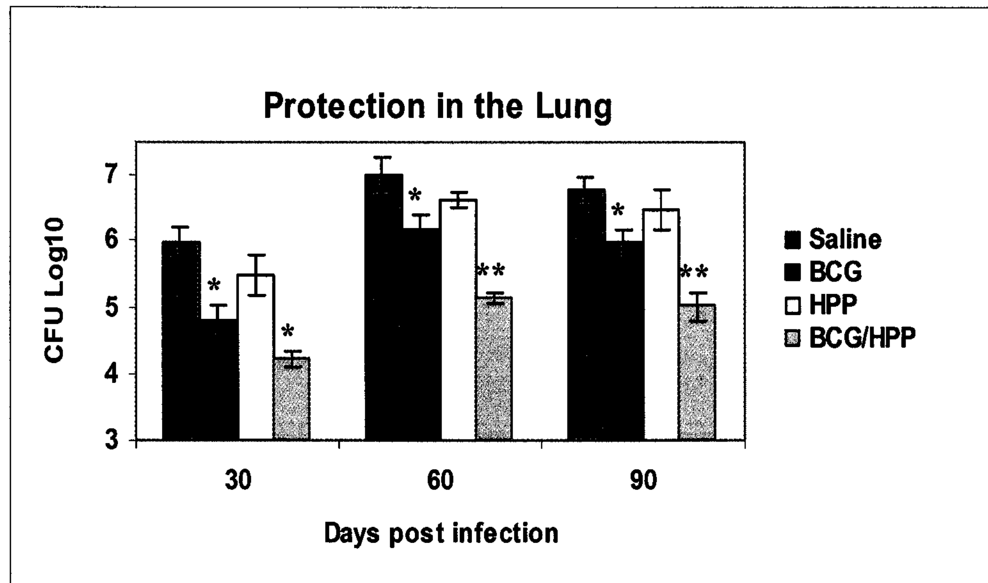

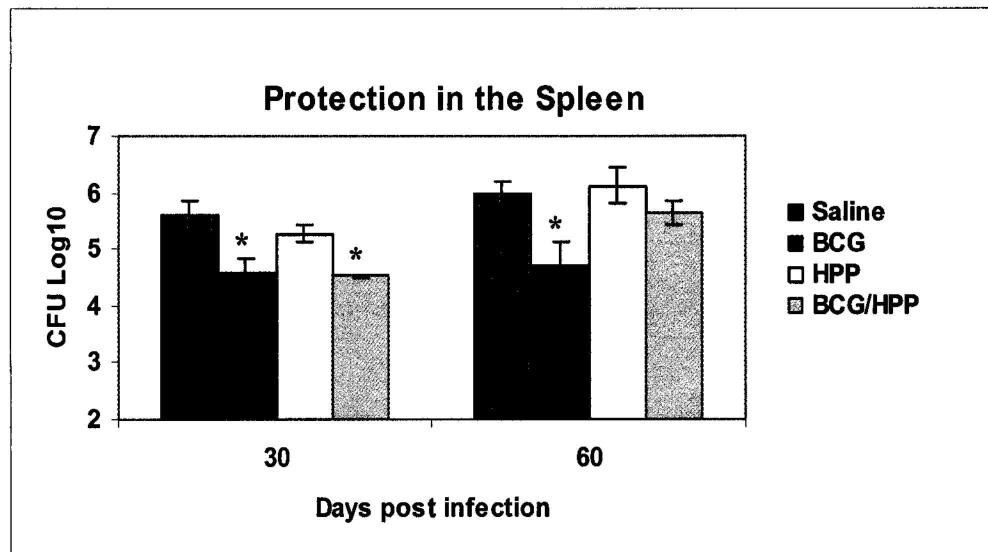

Figure 4.5 Subcutaneous (SC) vaccination with BCG boosted with a pool of hypoxia-induced proteins (HPP) give nearly two logs of protection in the lung more than the saline control. C57BL/6 mice were vaccinated three times, three weeks apart by subcutaneous (SC) injection with Saline, 10^6 BCG-Pasteur, 11μg HPP (1 μg each of Fba, Icl, Rv1738, Rv1813c, Acr, Acg, Rv2624c, Rv2626c, Ppa, Erp, and BfrB), or 10^6 BCG-Pasteur once and 11μg HPP three times as put forth in Table 4.3. HPP was prepared in MPL-SE and DDA adjuvant. Viable counts of whole lungs and spleen from infected mice at days 30, 60, and 90 were used to determine vaccine protective efficacy. Day 90 spleen data is not available due to plate contamination.

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4.4 Discussion

In light of the enormous number of persons latently infected with *M. tuberculosis*, characterizing the dynamics between the pathogen and the host during the acute, chronic and reactivation stages of infection is of critical importance to the control of tuberculosis. To date, most research in this area has focused on the time preceding and immediately following the initiation of acquired immunity. However, as the pathology of infection in the mouse lung is clearly progressive [23], it is reasonable to speculate that changing conditions within the host will necessitate adaptation by the bacillus reflected in a dynamic protein profile expressed by the bacterium throughout the entire infection. Therefore, I hypothesized that the activation of antigen-specific immune cells and the *omnium gatherum* of cytokines and chemokines at the site of infection would reflect these dynamics in response to the changing pathology in the host, metabolic state of the bacillus, and microbial components accessible to the host. Combining proteomic and immunological tools to address this question, I have demonstrated that the interaction between host and pathogen is highly responsive and antigen-specific while still exhibiting general and global characteristics determined by the stage of the infection.

4.4.1 Lymphocytic activation assay

To investigate the immuno-stimulatory potential of various recombinant *M. tuberculosis* proteins, I used in vitro-cultured bone marrow-derived dendritic cells (BMDC) pulsed individually with protein and overlaid them with *M. tuberculosis* immune lymphocytes at consecutive time points throughout infection. In these studies, I

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demonstrated that the lymphocytic immune response is antigen specific and changes over the course of a long-term resistant mouse infection model and cytokines are produced in a manner specific to the antigen and time point of infection, suggesting that distinct bacterial products are produced and available to the host antigen processing and presentation machinery at different stages of the infection. Using IFN-γ production as a marker for activation, $CD4^+$ and $CD8^+$ T lymphocytes were analyzed for their ability to recognize discrete proteins. The observed trend of initial $CD4^+$ lymphocyte activation early in infection in this model alludes to a healthy, robust immune response to an actively growing, invasive organism confirming earlier work describing the importance of this lymphocytic subset to initially control infection [1-3]. This early activation was observed to various degrees in response to some proteins while others failed to induce any sign of lymphocytic stimulation, further evidence of a dynamic inflammatory response directed by multiple bacterial products. Except in a few cases, $CD8^+$ lymphocytes were only observed to become activated after 150 days of infection, supporting the theory that these cells are more essential for prevention of reactivation than for initial control of infection [9, 24].

While CFP, Ag85A, ESAT-6, Icl, Rv1738, Acr, Rv2624c, Erp, and BfrB were capable of inducing significant activation of $CD4^+$ lymphocytes at days 30-45 pi in this assay, the stimulatory effect of Ag85A, ESAT-6, and BfrB were exclusive to the lung, but were not observed in the spleen, irrespective of the magnitude of activation, possibly due to different mechanisms of cellular trafficking and lymphocytic activation where some antigens are engulfed and removed to the lymph node to prime the lymphocytic response while other antigens are presented to lymphocytes locally in the lung. As the

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disease progresses into the reactivation stage of infection, however, those antigens which are recognized by lung lymphocytes are also seen to stimulate splenocytes strongly as well, perhaps due in part to systemic infection resulting in high populations of circulating activated lymphocytes.

4.4.2 Cytokine analysis of T cell overlays

To more fully determine the kinetics of the immune response to these proteins the supernatants from these overlays were quantified for their concentrations of IL-12(p70), TNF-α, IFN-γ, MCP-1, IL-10, and IL-6. Similar to observations of intracellular IFN-γ production, soluble TNF-α, IFN-γ, and IL-12 were also largely seen to reflect a general pattern of two-stage activation separated by a period of relative quiescence, with activation peaks separated by 60 or more days. While this trend is seen in response to many proteins (CFP, Ag85A, ESAT-6, Fba, Icl, Rv1738, Rv1813c, Acr, Rv2624c, Erp, and BfrB) in at least one of these cytokines and often in all three, the peaks of concentration were specific to the protein and to the cytokine measured. For example, in response to CFP, IL-12p70 and TNF-α are seen to peak at day 90 post infection, while IFN-γ levels peak at day 60, followed by secondary peaks at 195 days for IL-12 and IFN-γ and 150 days for TNF-α. On the other hand, in response to Ag85A, IL-12p70, IFN-γ and TNF-α are all observed to peak at day 45 pi, but only IL-12 and IFN–γ are seen to exhibit secondary peaks at days 150 and 195, respectively.

The other divergence to the pattern exhibited in the lymphocyte activation assay is the observation that the first peak in inflammatory cytokine concentration occurs at days 60-90 pi, precisely when the $CD4^+$ and $CD8^+$ lymphocytes are seen to be non-reactive to

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antigen in the previous assay. Several possible explanations could account for this difference. First, through fine tuning of the immune response and elimination of ineffective activated lymphocytes, the few responding T cells during the chronic phase of infection could be more potent responders than the large percentage of activated cells initially observed at days 30-45 pi. Second, the lymphocytic activation assay data is based on the percentage of IFN-γ-producing cells rather than total numbers, which were not determined, therefore it is conceivable that greater cell numbers during the chronic stage could reduce the actual percentage of activated versus non-activated lymphocytes. This argument would seem to contradict research showing the numbers of $CD4^+$ and $CD8^+$ T cells peak in the lungs at day 28 pi then decrease over time [25]. However, since IL-2 was added to the overlay, those cells that are obtained from 60 and 90 day infections, for example, could proliferate *in vitro* more readily than those obtained from earlier time points, thus providing greater cell numbers. Third, since this overlay was performed with total lung leukocytes instead of purified lymphocytes, the measured IFN-γ concentration could have derived from non-$CD4^+$/$CD8^+$ cells, such as NK or γδ T cells, which have been shown to produce large amounts of IFN-γ in response to *M. tuberculosis* infection, and are present in the lung during infection [26-28].

One trend of interest becomes apparent when comparing the levels of the inhibitory IL-10 cytokine and the pleiotropic IL-6 molecule. In large part these cytokines display an inverse relationship of concentration in the supernatants of this assay. IL-10 is largely seen to peak early (day 45 for CFP, Ag85A, Rv1738, Acg, Rv2624c, Erp, and BfrB) followed by an often gradual, but distinct reduction over the remainder of the experiment. In the case of Fba, Rv1738, Rv1813c, Rv2624c, and Erp increasing levels of

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IL-10 are seen at 195 days post infection, but these are modest. Alternatively, IL-6 levels are observed on average to increase over the course of infection, often to very significant concentrations by 195 days post infection as in the example of CFP, ESAT-6, Rv1738, and Rv2624c at 486, 208, 487, and 726 times the level induced by ovalbumin, respectively. High levels of IL-10 initially could be responsible for the observed down-regulation of inflammatory processes and the establishment of a chronic infection while increasing levels of IL-6 over time could lead to decreased TNF-α production resulting in subsequent necrotic pathogenesis in the lung [29]. This necrosis would lead to loss of containment of infected macrophages and release of bacilli followed by recrudescence of active disease.

Because of the inhibitory functions of IL-10, especially with regard to lymphocyte proliferation and IFN-γ production, an inverse correlation between levels of pro-inflammatory cytokines and IL-10 was expected [29-32]. In this assay, small changes in IL-10 concentration did not always result in coordinately inverse changes in either IFN-γ or TNF-α, however, a general trend of high IL-10 concentrations did result in lower IFN-γ production with the inverse relation also being true. For example, CFP, ESAT-6, Ag85A, and BfrB levels of IL-10 were relatively low (5-70 pg/ml) while IFN-γ levels were relatively higher (600-3000 pg/ml) over the course of infection. Conversely, Rv1738 and Rv2624c induce higher levels of IL-10 (100-370 pg/ml) with a coinciding decrease in IFN-γ production (100-600 pg/ml) at certain points during infection. This trend confirms a correlated interplay between IFN-γ and IL-10 during the course of *M. tuberculosis* infection in which overwhelming pathogenesis at the site of infection is precluded by inhibitory control of inflammatory Th1 processes.

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4.4.3 Possible explanations for observations

An interesting observation from this study was the appearance of a biphasic lymphocytic activation pattern demonstrated in response to several antigens: initial and late-stage activation of $CD4^+$ and, more commonly during the late stage, $CD8^+$ lymphocytes separated by a 30-90 day period of relative inactivity. Even those proteins that yielded responses only during early or late time points, exclusively, failed to induce significant percentages of activated cells during the chronic portion of the infection (days 60-90 pi). While it could be argued that this recurrent pattern is simply a product of the proteins chosen for analysis where these proteins are simply not produced by the pathogen, the universal nature of this change to non-reactivity argues against a shift in the transcriptional set as the sole determinant. Moreover, the proteins analyzed were chosen for the very fact that greater production would occur under hypoxic and stressed conditions: those conditions believed to be encountered by the bacilli during the granulomatous or chronic stage of the infection. Furthermore, it is difficult to envision that CFP, which contains over 200 individual, identified proteins would not induce a response in the T-cell population if the bacilli were metabolically active and the host lymphocyte population was responsive to stimulation. Explanations for this biphasic activation motif, therefore, consist of several remaining possibilities: (1) bacterial metabolic shutdown during chronic infection in response to the onslaught of acquired immune processes and formation of the granuloma, (2) a shift in the immune response of the host away from an IFN-γ-mediated inflammatory response after initial cessation of bacterial growth, or (3) physical separation of the pathogen from the host immune cells

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during the chronic stage of infection due to granulomatous sequestration precluding an immune response.

Much of the research over the last decades dealing with bacterial physiology and host interaction has been based on the premise that immune pressures exerted on the pathogen result in adaptive metabolic shutdown or transcriptional shifts within the bacillus [20, 33-41]. Both *in vitro* and *in vivo* studies have demonstrated the adaptive capacity of *M. tuberculosis* to thrive in the face of environmental stresses. Most convincing is work demonstrating the essential character of individual genes and entire transcriptional sets (regulated by alternative sigma factors) during sub-optimal *in vitro* growth as well as survival within an animal [42-47]. Moreover, tubercle bacteria are extremely hardy and capable of surviving extended periods (even decades) in severe conditions with little or no nutrients or oxygen. The hypothesis, therefore, that the observed drop in lymphocytic activation could be due to bacterial metabolic dormancy with an ensuing absence of bacterial products to stimulate an immune response could be valid in this respect. In this regard, the renewal of IFN-γ production by $CD4^+$ and $CD8^+$ cells observed during the late stage of infection would therefore be due to resumption of bacterial growth and metabolic activity. The trigger for this reactivation could be general decreased immune competence of the host because of aging, exhaustion caused by the chronic infection, or an as yet unidentified signal. If the IFN-γ concentration levels from the CBA analysis can be extrapolated to the environment within the host then another impetus for reactivation could be the overall decrease in IFN-γ observed immediately preceding reactivation.

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On the other hand, work by Gennaro, et al. [25], looking at the antigen composition of multiplying and non-replicating bacilli shows that the production of some antigens (such as Ag85A) do, in fact, peak early followed by a sharp drop in production by day 30 pi, whereas other antigens (such as ESAT-6) display continual production throughout infection [25]. Others have also documented the constituative production of ESAT-6 through chronic infection [48]. Another argument against this theory is that cytokine levels throughout infection are constantly above baseline for at least one antigen tested and a period of global inactivity at any time-point is not observed, indicating a constant stimulation of immune effectors by some facet of the infection. This constant recognition of active or dormant infection is alluded to by the fact that removal of $CD4^+$ or $CD8^+$ T cells immediately results in reactivation of disease [49-51]. It is more likely that the observed production of ESAT-6 but not Ag85A after the acute phase is reflected in the lymphocytic activation observed in response to ESAT-6 but not Ag85A in this experiment during reactivation (day 195 pi), but does not explain the contraction of the T cell response during the chronic phase.

The second possibility is a global down-regulation in the inflammatory response or a switch from Th1 to Th2-dominated mechanisms. It is well established that control of acute infection requires a Th1 response, including IL-12 induction of IFN-γ resulting in macrophage activation and bactericidal RNI production [12]. A Th2 cytokine response, however, has also been observed in human tuberculosis patients and has been linked to the extent of pathology and reactivation [52-55]. In mice it was shown that the dose of infection determines the Th1 or Th2 characteristics of infection [56]. In this experiment, the administration of a low dose aerosol infection and use of C57BL/6 mice would

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accordingly yield an almost exclusive Th1 phenotypic immune response during the acute phase of infection. Several labs have further demonstrated that this initial Th1 response is followed by a shift to a Th2 cytokine profile during the chronic phase [57, 58]. This shift is characterized in cattle by a constant production of IFN-γ followed by a delayed production of anti-inflammatory IL-4 [58]. In my work IL-4 concentrations were not analyzed but the analogous contraction of Th1-type lymphocytic activity was observed during chronic infection. Interestingly, LeClerc et al. established that this shift is not necessarily antigen driven since the Th1 and the lagging Th2 responses were both directed toward the same epitope on the same antigen, but was rather a reflection of the strength of antigen presentation [59]. In other infectious diseases such as HIV, leishmaniasis, and schistosomiasis distinct, unambiguous changes from a Th1 to a Th2 response leads to active disease. Alternatively, failed attempts to isolate true Th2 cells from the site of infection [12, 60], as well as studies showing lower IL-4 concentrations in infected versus non-infected individuals [61-63] argue against a definitive, clear-cut switch from a Th1 to a Th2-dominated immune response. Rather, research indicates that relative intensities of pro- and anti-inflammatory cytokine concentrations are a more likely determinant of the outcome of disease [24, 64].

Other than the inhibitory action of IL-10 already discussed, other host factors implicated in regulation of the inflammatory response are the selective inhibition of CIITA transcription in macrophages by *M. tuberculosis*-induced IL-6 production [65], TNF-α controlled type 1 immune activation resulting in suppression of T cell proliferation [29], and CD25 (IL-2Rα) T cell inhibition of inflammatory processes. Bacterial factors possessing anti-inflammatory capabilities include the 19 kDa lipoprotein

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[66], LAM [67, 68], and crude cellular extracts [69] just to name a few. It is clear that both pathogen and host-derived mechanisms of directing inflammatory operations are employed to restrict Th1 immunity, but the precise contribution of these processes to the lymphocytic inactivation observed in this assay, or in the establishment of latency in human disease is not entirely understood.

The third possibility of a physical sequestration of the pathogen forestalling antigen presentation to antigen-specific T cells finds corroboration in the histology of the lung during disease. It is well recognized that the pathology of tuberculosis in the lungs of *M. tuberculosis*-infected C57BL/6 mice is progressive, resulting in the eventual death of the animal due to cachexia and acutely diminished respiratory capacity [23], and the progressive character of this pathology can be described as stage-specific.

Stage I: within 7 days of aerosol infection, interstitial edema is apparent as molecular signals promoting extravasation allow the diffusion of exudate into the infected tissue and initial lymphocytic infiltration from the lung vasculature can be observed. At 30 days pi, epithelioid macrophages are apparent with pronounced perivascular and peribronchial cuffing visible around the site of infection. This first stage of pathology is generally considered to be the beginning of the granulomatous phase of tuberculosis in the mouse. In contrast to human pathogenesis of disease, however, distinct central regions of infected macrophages surrounded and delineated by lymphocytic borders is not observed.

Stage II: by 50 days post infection, foamy macrophages, extensive lymphocytic cuffing and interstitial fibrosis are prominent with alveolar spaces being filled with immune cells. Over the succeeding 2-3 months the granulomas continue to enlarge while

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tight lymphocytic aggregates move throughout the granulomatous foci. During this time, the granulomas continue to increase in size with the infected macrophages observable as distinct centrally located entities.

Stage III: as the granulomas increase in size they begin to coalesce into large areas of multifocal necrosis and much of the architecture of the lung and granuloma begins to breakdown. During the end of the infection, these areas of disease often comprise more than 50% of the volume of the lung eventually culminating in the death of the animal due to extremely diminished lung capacity.

Correlating the kinetics data with the pathology of the lung, one can envision a situation where, during stage I, the pathogen is growing exponentially in the lung until the granuloma formation begins to restrict bacterial growth. Upon formation of the granuloma and the ensuing spatial separation of the infected macrophage from effective immune cells during stage II, the disease enters a homeostatic phase where the host restricts inflammation in the lung and the pathogen proliferation decreases, perhaps due to nutrient and oxygen restriction. The period of lymphocytic inactivity observed in this experiment, from days 60-90 and beyond, correlate to the time where the granulomas in the mouse lung appear to by structurally sound and distinct [23]. Due to the slow progression of pathogenesis, however, and the breakdown and coalescing of granulomas in stage III of infection, the bacilli are no longer contained and increased inflammation and necrosis ensues resulting in the eventual death of the animal. At the time indicated by the lung pathology when this loss of containment occurs, this work demonstrates a marked increase in inflammatory activation within the lung as well as the spleen. This

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activation is distinguished by augmented percentages of activated $CD4^+$ and $CD8^+$ lymphocytes as well as enhanced production of inflammatory cytokines.

In the final analysis, however, the most likely explanation for the biphasic activation patterns observed in the kinetics data is a combination of multiple possibilities. It is clear that transcriptional patterns in the pathogen are altered upon ingestion by the host macrophage and that a multitude of gene products are induced by stressed conditions, including ROI and RNI production by the host [70-73]. It is also clear that the pathogen is exposed to many antibacterial forces in response to innate as well as acquired immunity, which further force the pathogen to adapt and decrease metabolic processes [74-76]. We know that host-mediated changes occur in the immune response, most likely in attempts to regulate destructive inflammatory processes, especially within the lung. And this down-regulation of inflammatory signals without concurrent increases in bacilli numbers is due most likely to the sequestering power of the granuloma. Finally, *M. tuberculosis* produces many components known to regulate the immune response and suppress inflammatory processes and signaling mechanisms [66, 68, 77-81]. These products are both ubiquitous on the surface of the organism (as in the case of LAM) and actively produced and exported (as in the case of the 19 kDa lipoprotein). The resulting homeostasis between the host and the parasite is a careful balance of forces where the advantage can be gained by either side if any of these opposing forces are removed as has been verified by numerous gene deletion, knock-out, cell depletion, and antibody-mediated neutralization studies.

Another intriguing finding from the CBA assay is in the observation that *M. tuberculosis* proteins were less capable of inducing expression of TNF-α, IL-12, MCP-1,

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IL-10, and IL-6 in this experiment when overlays of infected lymphocytes were performed versus naïve lymphocytes, suggesting a global down regulation of immune activity caused by infection with *M. tuberculosis*. It must be pointed out that these cytokines are primarily derived from the macrophage/monocyte lineage and not lymphocytes. In this assay dendritic cells from naïve mice were used as APCs for each time-point and were overlayed with macrophage-depleted lymphocytes. Therefore, the decrease or increase of these cytokines over time results solely from the stimulatory or inhibitory effects of the infection-derived T cell upon the naïve dendritic cell, not from any antigen:DC kinetics. Interestingly, however, several of these proteins (CFP, Rv1738, Rv1813c, Rv2624c, and to a lesser extent Erp and BfrB) were seen to induce high levels of cytokines TNF-α, IL-6 and IL-10 in the absence of infection. This could be explained by the presence of pathogen-associated molecular patterns (PAMPs) within the antigen which would signal through toll-like receptors or other unknown receptors similar to other observed *M. tuberculosis* antigens [82].

4.4.4 Vaccine studies

In further analysis, to determine whether these hypoxia-induced proteins are capable of conferring protection as a vaccine mice were immunized with recombinant, purified hypoxia-induced proteins or BCG boosted with these same proteins. Results were compared with the efficacy of BCG alone. Significant levels of protection (1.8 logs above saline and 0.6 logs above BCG alone) were observed at 30 days after challenge when BCG was boosted with a pool of eleven hypoxia-induced proteins. The kinetics data suggest that several of these proteins are, in fact, produced early in infection and the

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protection afforded early substantiate that assessment. This level of protection, however, increased to 1.1 and 1.0 logs above BCG in the lungs at 60 and 90 days after challenge, respectively, attesting to the new or increased production of proteins in the hypoxia pool during the chronic disease state, thus conferring even greater protection when these antigens are produced. While the idea that BCG boosted with various additional protective antigenic targets has yielded success in the past [83-85], this is the first research to demonstrate this level of protection by a prime-boost vaccine and gives reason for hope that these and other mycobacterial products produced during the chronic state of infection are capable of producing immunoprotection in man.

4.4.5 Final thoughts

This research has demonstrated the dynamic and antigen-specific nature of the immune response to *M. tuberculosis* infection over the course of a long-term infection. While many questions have been raised concerning the metabolic capacity and viability of the pathogen, the capacity of immune effectors and the effects of cytokine hierarchies especially during chronic disease, it is clear that a determination of potential vaccine candidate antigens can be achieved based upon the assays employed in this work. Moreover, one of the most significant conclusions of this research is that individual proteins can induce a stage-specific immune response that can be used as a marker for reactivation disease. And finally, this work demonstrates the powerful potential of coupling the tried and proven protective efficacy of a standard BCG vaccination with further treatments using additional antigenic proteins targeting the latent state of disease.

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CHAPTER 5: Concluding Remarks

In the first portion of this research *secA2* was demonstrated to function in the secretion of certain virulence factors essential for pathogenesis during the early stages of infection. The translocation mechanisms of these proteins, however, have not been elucidated. The SecA2-dependent exported (S_2DE) proteins identified in this research possess no canonical signal sequence by which they are marked for translocation across the membrane, whereas some, but not all, of the S_2DE proteins observed in other organisms possess a classic signal sequence. It is interesting that to date, the only other prokaryotes that have been identified to possess a non-redundant *secA* paralogue in their genome are gram-positive bacteria, which do not possess a SecB chaperone as part of their Sec-dependent translocation machinery. One possible function of SecA2, therefore, could be the recognition, binding and shuttling of nascent proteins to the membrane-bound SecA1 homodimer where it could serve to enable translocation in the absence of signal sequence recognition and binding. The lack of any cleavable or internal sequence motif among S_2DE proteins, however, weakens the argument for direct contact between SecA2 and the protein destined for transport due to the lack of a defined protein subset slated for export. However, this could rather indicate that SecA2 serves in the establishment of translocation machinery or components within the membrane either supplementary to or apart from the classical Sec apparatus that functions in the export of specific proteins. In order to determine SecA2 protein binding and more definitively

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determine functionality, cell fractions could be run through resin columns bound with SecA2 and the retentate could be analyzed by PAGE to identify SecA2-binding proteins. Additionally, to determine how SodA and KatG are recognized for transport, PhoA reporter protein fusions with diminishing portions of the exported proteins could be utilized to ascertain regions critical for transport. Specifically, portions of these genes could by cloned upstream of a truncated *phoA* gene lacking the promoter and export signal. Once the PhoA protein reaches the exterior of the cytoplasmic membrane it becomes enzymatically active thus providing a method of detection for exported proteins [1].

To date, the proteins observed to be influenced by SecA2 in mycobacteria as well as *S. gordonii, S. pneumoniae, L. monocytogenes,* and *S. aureus* are intimately connected with the pathogenic fitness of the organism, yet *secA2* mutations are not observed to effect bacterial viability outside of a mammalian host. This suggests an intriguing evolutionary parallel, where the acquisition of *secA2,* and by inference the pathway in which SecA2 operates, coincides with the time when the organism acquired pathogenic competence. In fact, this event could have facilitated the potential for colonization within a human host. Furthermore, the sequence identity between SecA2 proteins found in different bacterial species points to a common ancestral source from which this gene was acquired. Another possibility, suggested by its high homology to *secA1* genes across all species in which it is found, is that *secA2* evolved from *secA1* in order to facilitate mammalian colonization. Determining an evolutionary timeline using homology and SNP analysis of the genomic sequence of *secA2* as well as the location of the gene within the entire genome could be performed in order to confirm one or both of these

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possibilities. In further evidence of this idea, *M. leprae*, which has undergone massive genome decay, and can only be cultivated within a select few mammalian hosts, possesses a fully functional SecA2 gene, attesting to its essentiality for virulence.

In the next portion of my research a model of *in vivo* environmental characteristics was developed by decreasing pO_2 pressure and changing the carbon source available to *M. tuberculosis* from the three-carbon glycerol to 40-carbon dipalmitoyl-phosphatidylcholine. Based on empirical evidence a reduced O_2 environment is encountered by the bacilli upon phagocytosis, and circumstantial evidence alludes to an increasingly hypoxic environment within the granuloma. The research performed in this work utilizes the sensitive tools of 2DE and MS/MS to identify proteins that are found exterior to the bacillus during growth in these conditions. While other research has focused largely on the gene transcription patterns during stress conditions, transcription of genes does not necessarily represent the bacterial products encountered by the immune mechanisms of the host, especially if the resultant proteins are produced in low quantity or are retained within the pathogen. As such, this work more accurately identifies possible immunoreactive proteins produced by the organism. To further characterize the bacterial products presented to the host during infection, the 2DE gels of CFP from these *in vivo*-mimicking conditions could be analyzed by Western blot against human or animal sera. Since T cell help is required for a humoral response, this approach could provide a new set of antigens for further evaluation of T cell reactivity.

In order to determine the degree of oxygenation of the granuloma and at what stage of infection, if any, the bacilli experience hypoxic conditions research in tumor characterization can be exploited [2]. Based on the increasing longitudinal relaxation rate

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of perfluorocarbons (PFCs) with increasing pO_2 it is possible to employ ^{19}F magnetic resonance imagery (^{19}F-MRI) to determine spatial gradients of oxygen concentration within a tissue. As such, the i.v. injection of PFC into an infected animal followed by ^{19}F-MRI would allow the direct determination of the degree of hypoxia experienced by the pathogen. This procedure could be repeated without damage to the animal at various stages of infection to correlate *in vitro* gene expression and protein production data with a real time infection. An additional method of testing the pO_2 within the granuloma is to expand the method employed by James, et al. in which they used an oxygen-sensitive, chemical probe to measure phagosomal pO_2 within activated macrophages. These probes, nitroxide radicals that react with oxygen in a gradient fashion, could be infused into the lungs dissected from infected mice and would be incorporated into the cells of the organ. In order to ascertain oxygen concentrations, the granulomas would be dissected away from healthy lung tissue and both samples, kept under a nitrogen blanket, would be measured by electron paramagnetic resonance spectrometry, as described [3].

The hypoxic response in *M. tuberculosis* is regulated by the two-component system DosR/DosS or DevR/DevS which signals the transcription of multiple genes, including α-crystallin, in response to low oxygen concentrations. Counterintuitively, mutation of the DevR gene results in hypervirulence of the organism, including rapid acute-stage growth in immunocompetent mice and uncontrolled growth in SCID mice [4]. This led to rapid death of the host, suggesting that the hypoxia response of the pathogen slows bacterial growth in order to allow the organism to survive without causing the death of the host. Perhaps this would allow a balance between the host and the bacilli in which greater opportunity for the spread of infection could occur. Another

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perspective is that uncontrolled growth of the pathogen would result in an overwhelming immune response, thus allowing clearance of the pathogen because of continuous antibacterial pressures by host immunity. Either way, it is an intriguing aspect of bacterial pathogenesis when a parasite can regulate its own growth to balance bacterial clearance by the host with the instinctual desire for self-propagation, and speaks to the effectiveness to which this organism has become one of the most successful pathogens known to man. An intriguing development in this area is the finding that increasing NO concentrations, encountered by the pathogen within the activated macrophage, induce the same profile of genes as those induced by hypoxia, suggesting that acquired immunity and activation of infected macrophages rather than hypoxia are responsible for the dormancy program observed in so many hypoxia models [5].

Recent efforts by Timm, *et al.* to confirm the hypothesis that the adaptation of *M. tuberculosis* to the intracellular environment involves the differential transcription of stress-response genes led to the confirmation that the bacilli exhibit similar transcriptional activity during host infection as they do during the shift into stationary phase *in vitro* [6]. This research demonstrated, based on the transcription of several reporter genes, that conditions of nutrient deprivation and oxygen limitation are present during the shift from acute to chronic stages of infection. Also, fatty acid catabolism is induced during later stages of infection in the mouse. Two of the reporter genes utilized in this research, *icl* and *acr,* were also used in the research presented in this work. The transcription patterns of these genes observed over the first nine weeks of infection parallel the lymphocytic reactivity manifested in this work. Specifically, both the transcription levels and lymphocytic stimulation was seen to increase from two to four

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weeks returning to lower levels at nine weeks post challenge. While many of the other reporter genes used by Timm were not employed in this work, it does confirm that mimicking *in vivo* conditions by limiting nutrient and oxygen availability, and by inducing fatty acid catabolism is a useful model of infection. Additionally, based on the trend, the transcription patterns observed argue that the decrease in lymphocytic activation after nine weeks (roughly 60 days) may in fact be due to a metabolic shift of the bacilli and not inhibition of the immune response. However, gene transcription was not analyzed after nine weeks, therefore care should be taken when inferring bacterial metabolic activity past the initiation of the chronic stage of infection.

In the culminating section of this work, the kinetics of the acquired immune response were evaluated by determining the reactivity of immune cells isolated from the lungs and spleens of infected mice during different stages of disease to exogenous antigen. The character of the immune response was analyzed based on the IFN-γ production of CD4 and CD8 T cells, and cytokine concentrations in the lymphocyte overlay supernatants. During the initial, acute phase of infection strong CD4 responsiveness was observed followed by significant, though not as strong CD8 activity in response to individual antigens. Several known T cell antigens used in this work, however, did not demonstrate any stimulatory potential. This is most likely due to the lack of proper post-translational modifications present on the native protein. If MPT-32 and Rv2626c were isolated in their native state from mycobacteria the lost reactivity would likely be restored as has been documented for GroEL [7]. While no post-translational modifications were characterized for Rv2626c, glycosylation of MPT-32 is well defined [8, 9]. Perhaps other weak (Icl, Fba) or non-stimulatory (Ppa) proteins used

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in this work would demonstrate greater immune potential in their native state as well. But the ultimate goal for this research must be remembered: to formulate a subunit vaccine that is protective against acute, chronic and reactivation disease that can be administered cheaply and efficiently to billions of people. In this respect, the rapid production of recombinant *M. tuberculosis* proteins is the only economically feasible option for large-scale vaccine production. As such, because of the cost and difficulty of purifying proteins from *M. tuberculosis* even strong native antigens should be dropped from consideration if they fail to elicit the same response when produced as recombinants.

Following the strong acute-phase response, a lull in lymphocytic activation occurred in which even the strongest initial stimulators failed to induce IFN-γ production in infection-derived T cells. The duration of this inactivity was 60-90 days, following which a renewed antigenic immune response was observed by some, but not all, of the early stimulatory antigens. Additionally, a couple of antigens (Fba and Acg) that failed to induce a response early were able to do so late in infection. This attests to the fact that the immune response is changing over time, either due to transcriptional changes within the pathogen or plasticity of the host immune response responding preferentially to certain antigens at different times of infection.

The ability of *M. tuberculosis* to establish long lasting and often sub clinical disease states has been a well recognized hallmark of this disease since its discovery. As such, two of the critical questions concerning tuberculosis host-pathogen interaction are regarding the metabolic state of *M. tuberculosis* bacilli and the fitness of the immune response toward this organism during the chronic or latent stage of infection. The

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lymphocytic inactivity during the chronic stage of infection observed in this research could correlate with the phenomenon of latency in human disease and will, therefore, be treated the same in the remainder of this work.

In 1907, Hans Much describes the appearance of coccoid granules in the lesions of tuberculosis patients. Interestingly, Much found these non acid-fast staining granules in tuberculous abscesses where classical bacilli could not be isolated. While it was demonstrated that infection of naïve animals with these granules did not lead to typical tuberculosis disease, the injection of tissue from animals where the granules, but not acid-fast bacilli, were observed lead to the formation of classical lesions with tubercle bacilli [10]. In 1987 Khomenko observed ultra-fine coccoid forms in guinea pig and human lung lesions by electron microscopy [11]. These coccoid forms were seen to revert to typical rod-shaped tubercle bacilli upon passage and inoculation of naïve animals with these coccoid forms resulted in granulomatous inflammation typical of tuberculosis. If these coccoid forms can be compared to spore forms seen in other gram positive bacteria, then it is reasonable to suggest that metabolic processes are halted to a large extent resulting in little to no antigen production by the infection. The characteristic drop in T cell activity in this case, therefore, could be due to the lack of stimulatory antigen. Even in the absence of granular forms, metabolically quiescent or sluggish bacilli could account for decreased inflammatory processes. Since the C57BL/6 chronic stage is characterized by stable numbers of CFU in the lungs, one possible experiment to evaluate the level of proliferation of bacteria at each stage of infection is to pulse-feed mice BrdU, which would incorporate into the RNA of all cells. Lung homogenate could then be analyzed by flow cytometry with gating of the bacterial cells so that rounds of replication

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could be determined. However, metabolic activity does not necessarily equate to proliferation, therefore other readouts for bacterial activity would need to be employed. For instance, staining of lung homogenate with the fluorescent redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride) followed by flow cytometric analysis would determine respiratory activity of the bacteria.

On the other side of the argument is the possibility that some component of the host immune response is truncated, resulting in the lack of a full inflammatory response capable of clearing the organism. Mycobacteria produce molecules known to inhibit or regulate host immune processes. One of these products is the lipoprotein LprG. Recent research has shown that prolonged LprG signaling through TLR2 inhibits MHC class II expression and antigen presentation by infected macrophages in both mice and humans. A mutation in the *lprG* operon does not inhibit growth *in vitro* but results in marked attenuation of virulence in mice, which increases as the infection progresses, highlighting the pathogen's inability to establish a chronic infection. In order to determine the ability of macrophages and dendritic cells from different stages of infection to induce activation of lymphocytes the following experiments could be performed. Macrophages and dendritic cells could be purified from the mediastinal lymph nodes and lungs during acute, chronic, and reactivation stages of disease, pulsed with CFP then overlaid with lymphocytes isolated from an acute infection. The lymphocytes could then be analyzed as to their production of activation molecules such as IFN-γ. This would determine whether or not the APCs themselves are competent during the time when this research observed decreased lymphocytic activity.

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Alternatively, truncated lymphocytic competence even in the presence of fully competent APC mechanisms could account for inactivity during chronic infection. In this research, lymphocytes from the lungs of a 60-day infection were determined to respond less to non-specific stimulation by ConA than did naïve lymphocytes, suggesting a deficit in activation ability during chronic infection. However, the levels of stimulation observed in the naïve T cells were far lower than expected casting doubt on the usefulness of this measure of lymphocytic fitness. To further evaluate this possibility T cell clones could be transferred into *M. tuberculosis*-infected mice. Periodic stimulation with antigen would ensure a sufficiently high population of clones. At days 30 and 90 post infection the lymphocytes could be removed and stimulated *ex vivo* whereupon T cell reactivity could be assessed.

The cytokine levels at different stages of the infection reveal a similar picture to the lymphocytic activation data in regards to the pro-inflammatory cytokines, but often exhibit a more gradual and directed shift over the entire course of infection. The levels of Il-10 were often observed to peak early (around day 45) then continuously decrease over time. Alternatively, the levels of IL-6 could be seen to generally increase as disease progressed. The inverse correlation between these two cytokines, coupled with the early and late stage activation of pro-inflammatory lymphocytic processes, could potentially provide a powerful diagnostic tool by which clinicians could monitor tuberculosis patients to determine the stage or character of disease. Furthermore, in the realm of diagnostics, perhaps single antigens such as those observed in this work to elicit a response during late stages of disease could provide a useful marker for the advent of reactivation, thus allowing preemptive chemotherapeutic treatment.

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Finally, as a critical evaluation of the proteins identified and analyzed in this work, mice were vaccinated with a pool of all evaluated hypoxia-induced proteins (HPP) either with adjuvant alone or boosting a primary BCG vaccination. Subsequent aerosol infection with H37Rv *M. tuberculosis* showed that HPP vaccination alone provided little to no protection, but in combination with BCG was capable of delivering a log of protection over BCG vaccination alone and nearly two logs of protection over non-vaccinated controls. This protection was seen to increase slightly from 30 to 60 and 90 days post infection, suggesting additional increases to the anti-tuberculosis T cell repertoire during later stages of infection, perhaps due to bacterial production of

The next step to fine tune the vaccine formulation used in this work is to determine what proteins were non-essential to elicit the amount of protection observed. In addition to a more simple vaccine recipe, perhaps greater protective efficacy could be realized due to greater concentrations of relevant epitopes or the removal of potentially inhibitory proteins. Clear candidates to drop from future formulations include MPT-32, Rv2626c, and Ppa. Additionally, weak stimulators such as Icl and Fba could also be excluded. Moreover, there are hundreds of other antigenic and stress-induced proteins produced by *M. tuberculosis* that have yet to be evaluated. Potential power exists in any one of these proteins to improve greatly on this and other vaccine formulations. In any regard, the formulation of a BCG prime-boost vaccine for tuberculosis is a smart path to pursue, because of the billions of people already vaccinated with BCG.

Further work to be performed in this regard is the evaluation of this vaccine in the guinea pig model, since guinea pig lung pathogenesis more accurately parallels a human infection. Also, the guinea pig is far more susceptible to tuberculosis than is the

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C57BL/6 mouse and is observed to die within 70-100 days following an aerosol infection. The use of this animal model, therefore, would more dramatically substantiate an increase in protective efficacy afforded by this vaccine over time as was observed in the mouse model. In the final analysis, however, factors determining a successful vaccine against tuberculosis infection should not only include measures of bacterial load and degree of pathology in the lung but also the time to death after infection in the animal model. While bacterial load and pathology could correlate to rate of transmission and quality of life in infected humans, time to death experiments might more accurately reflect the degree to which infection overwhelms the host. Therefore future vaccines should be evaluated on each of these issues.

In conclusion, it is clear that our understanding of tuberculosis and its causative agent *M. tuberculosis* has come very far over the past century, yet critical questions still must be resolved if we are to eradicate this pathogen from the human population. The old methods of fighting bacterial infections are proving increasingly ineffective. The emergence of multi-drug resistant strains, long and complicated drug regimens, and the ability of *M. tuberculosis* to become dormant and refractive to antibiotics have made chemotherapeutic treatment of an existing infection a losing battle. The array of deficiencies in the current BCG vaccine, not the least of which are the varied efficacy and lack of total protection in any study, and the many decades of unsuccessful attempts to improve upon these results attest to an extremely difficult path toward conquering this pathogen. A combination of fundamental research in tuberculosis done to this point together with future research utilizing more advanced analytical techniques available

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today in addition to our greater ability to capitalize on our understanding of this organism give hope that progress will continue to be made.

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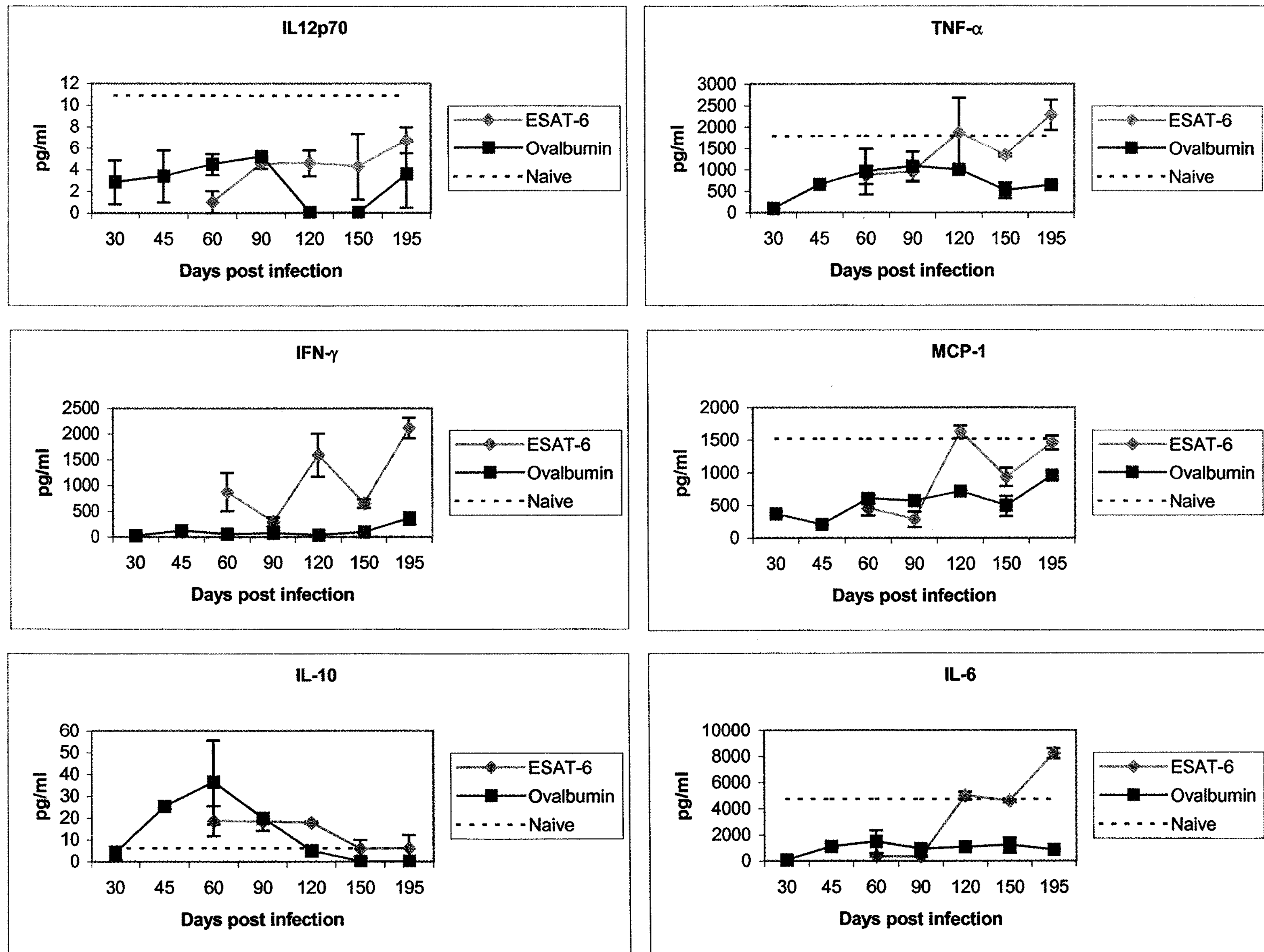

Figure 4.3 **(cont.)** CBA analysis of cytokine concentrations in the supernatants of lung lymphocyte overlays from ESAT-6- and ovalbumin-pulsed dendritic cells.