THE ROLE OF IL-4 AND IL-13 IN PULMONARY TUBERCULOSIS USING GENE-DEFICIENT MICE and PROTECTIVE EFFICACY OF THE PURINE-DEFICIENT AUXOTROPH OF *MYCOBACTERIUM TUBERCULOSIS*

Najmeeyah Brown

This thesis is presented for the degree of DOCTOR OF PHILOSOPHY in the Department of Immunology at the UNIVERSITY OF CAPE TOWN

September 2002

ABSTRACT

Absence of the TH2 inducing cytokine IL-4 has been shown not to increase the TH1 response and resistance to mycobacterial infection. This study asked whether the combined absence of IL-4 and IL-13, as compared to IL-4 deficiency only, would increase resistance to an aerogenic *Mycobacterium tuberculosis* infection. By using IL-4 gene-deficient mice, it was confirmed that endogenous IL-4 does not reduce host immunity. In contrast, IL-4Rα gene-deficient mice, which lack both IL-4 and IL-13 signalling, had reduced bacterial burden in the lungs and other organs, increased survival and cellular immunity with macrophage activation. Therefore IL-4Rα gene-deficient mice have increased resistance to *Mycobacterium tuberculosis* infection. These data suggest that endogenous IL-13, unlike IL-4, has the potential to suppress the normal protective immune response to *Mycobacterium tuberculosis* infection.

In the second part of the study, it was asked whether immune-compromised mice would be able to control infection with an attenuated *Mycobacterium tuberculosis* strain. In two experiments, interferon gamma receptor gene-knockout mice were infected via aerosol with 100 wild type *Mycobacterium tuberculosis* and purine-deficient *Mycobacterium tuberculosis*, respectively. Interferon gamma receptor-knockout mice succumbed to wild-type bacilli earlier than to purine-deficient mutants. Wild type mice, whether infected with wild type or purine-deficient *Mycobacterium tuberculosis*, survived. This indicates that the purine-deficient mutant had a slightly reduced virulence when compared to the wild type mycobacterium.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 TUBERCULOSIS AND WORLD HEALTH STATISTICS

Tuberculosis (TB) is a global problem. One third of the world population is infected with *Mycobacterium tuberculosis* (MTB), but only a small percentage develops active TB (7, 12, 33). Of the people infected with TB, 1.86 billion are considered to have latent TB (120). The immune response is generally successful in containing infection but not in eliminating the pathogen (7, 12, 33). If, at some stage, there are changes in the immune system, latent MTB can reactivate and cause active TB. This explains why the human immunodeficiency virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS) epidemic has caused resurgence in the number of TB cases (Figure 1).

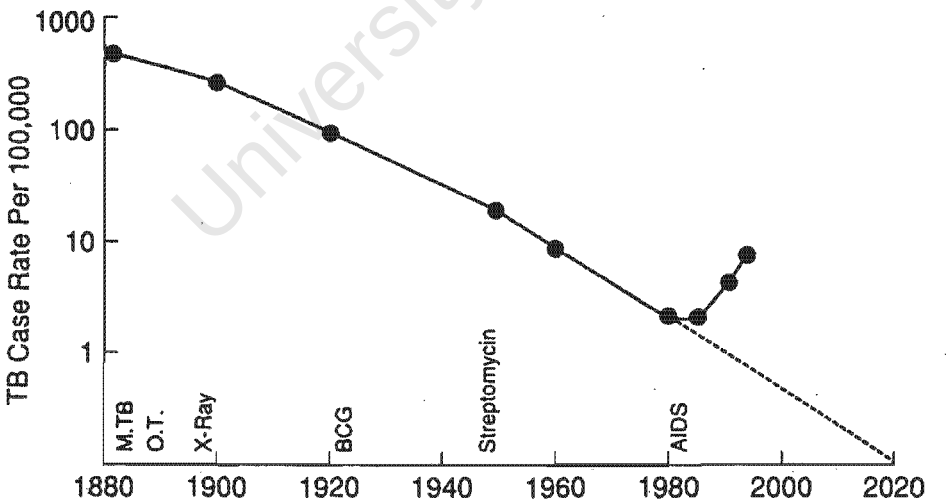


Figure 1. Incidence of pulmonary tuberculosis in the USA during the past century. The dotted line represents the predicted rate of decline (18).

TB is the single biggest killer of people living with HIV/AIDS (109, 120). It has been estimated that at the end of 2000, 36.1 million people were living with HIV/AIDS and 70% of these individuals were living in sub-Saharan Africa. About a third of those infected with HIV/AIDS were co-infected with MTB. Up to 70% of patients found to be positive for MTB were HIV-positive and 50% of these patients died of TB. Infection with HIV is therefore the greatest risk factor for reactivation of latent TB, and TB is the commonest cause of HIV-related deaths.

TB can be treated using a 4-drug combination therapy of isoniazid, rifampicin, pyrazinamide and ethambutol, taken over a period of 6-9 months (39). This therapy is most effective when given under DOTS (direct observed therapy system) – the delivery of standardised chemotherapy directly observed by a health care provider or other responsible individual. The WHO (World Health Organisation) launched DOTS in 1994, and by 2000, 21 countries were implementing it (Figure 2) (120). DOTS programs identified 2 million new TB cases and successfully treated 80% of all registered smear-positive patients. The DOTS program has its limitations, however. Lack of qualified staff and management skills, shortage of equipment, absence of collaboration between TB and HIV programs, decentralisation of health services, misdiagnosis and poor drug supply have all lead to the emergence of multiple drug resistant (MDR) MTB strains (39, 120). At least 4% of TB cases are MDR.

Figure 2. Regional profile for Africa: notification, detection and DOTS coverage, 2000 (120).

Country/Territory	Country Information									WHO TB control strategy (DOTS)								Other Strategy (non-DOTS)					
		Notifications				Estimated cases				%	Notifications						% of	%	Notifications				% of
	Pop	all cases		New ss+		number		CDR	DOTS	of	all cases		New ss+		DDR		pulm	of	all cases		New ss+		pulm
	thousands	number	rate	number	rate	all cases	New ss+	%	category	pop	number	rate	number	rate	%		cases ss+	pop	number	rate	number	rate	cases ss+
	a	b	b/a	c	c/a	d	e	c/e	f	g	h	h/(g*a)	i	i/(g*a)	i/e		j	k	l	l/(k*a)	m	m/(k*a)	n
Algeria	30 291	18 294	60	8 050	27	13 909	6 259	129	4	100	18 294	60	8 050	27	129		80	0					
Angola	13 134	16 062	122	9 053	69	36 083	15 860	57	1	0								100	16 062	122	9 053	69	63
Benin	6 272	2 706	43	2 286	36	16 266	7 167	32	4	100	2 706	43	2 286	36	32		95	0					
Botswana	1 541	9 292	603	3 091	201	11 661	4 597	67	4	100	9 292	603	3 091	201	67		39	0					
Burkina Faso	11 535	2 310	20	1 560	14	37 319	16 013	10	4	100	2 310	20	1 560	14	10		88	0					
Burundi	6 356					25 820	10 808		0														
Cameroon	14 876	5 251	35	3 960	27	50 685	21 594	18	3	46	4 754	32	3 522	24	16		86	54	497	3	438	3	94
Cape Verde	427					777	341		0														
Central African Republic	3 717					16 544	6 857		0														
Chad	7 885					21 571	9 487		0														
Comoros	706	120	17	87	12	447	201	43	4	100	120	17	87	12	43		86	0					
Congo	3 018	9 239	306	4 244	141	10 206	4 377	97	4	100	9 239	306	4 244	141	97		68	0					
Côte d'Ivoire	16 013	12 943	81	8 497	53	62 353	26 163	32	3	60	12 943	81	8 497	53	32		85	40					
DR Congo	50 948	60 627	119	36 123	71	162 901	70 443	51	3	70	60 627	119	36 123	71	51		82	30					
Equatorial Guinea	457					1 117	500		0														
Eritrea	3 659	6 652	182	590	16	10 559	4 636	13	3	80	6 652	182	590	16	13		10	20					
Ethiopia	62 908	91 101	145	30 510	48	249 457	104 734	29	3	85	91 101	145	30 510	48	29		50	15					
Gabon	1 230					3 606	1 569		0														
Gambia	1 303					3 436	1 520		0														
Ghana	19 306	10 933	57	7 316	38	55 147	24 094	30	3	87	10 325	53	7 042	36	29		76	13	608	3	274	1	54
Guinea	8 154	5 440	67	3 920	48	22 046	9 785	40	4	100	5 440	67	3 920	48	40		90	0					
Guinea-Bissau	1 199	1 273	106	526	44	3 251	1 432	37	4	95	1 273	106	526	44	37		47	5					
Kenya	30 669	64 159	209	28 773	94	148 579	61 556	47	4	100	58 067	189	26 162	85	43		54	0	6 092	20	2 611	9	55
Lesotho	2 035	9 746	479	3 041	149	11 768	4 742	64	4	100	9 746	479	3 041	149	64		52	0					
Liberia	2 913					8 012	3 521		0														
Madagascar	15 970					40 642	18 264		0														
Malawi	11 308	23 606	209	8 265	73	50 539	20 793	40	4	100	23 606	209	8 265	73	40		48	0					
Mali	11 351	4 216	37	2 527	22	30 318	13 403	19	4	95	3 845	34	2 221	20	17		74	5	371	3	306	3	94
Mauritania	2 665	3 067	115	1 583	59	6 028	2 699	59	1	0								100	3 067	115	1 583	59	70
Mauritius	1 161	160	14	115	10	803	357	32	4	100	160	14	115	10	32		89	0					
Mozambique	18 292	21 158	116	13 257	72	79 144	32 878	40	4	100	21 158	116	13 257	72	40		77	0					
Namibia	1 757	10 474	596	3 911	223	9 147	3 723	105	4	100	10 474	596	3 911	223	105		46	0					
Niger	10 832	4 292	40	2 693	25	27 701	12 315	22	3	70	4 292	40	2 693	25	22		80	30					
Nigeria	113 862	25 821	23	17 423	15	347 385	150 301	12	3	47	25 821	23	17 423	15	12		72	53					
Rwanda	7 609	6 093	80	3 681	48	30 793	12 896	29	4	100	6 093	80	3 681	48	29		81	0					
Sao Tome and Principe	138	97	70	30	22	183	82	37	1	0								100	97	70	30	22	35
Senegal	9 421					24 617	10 906		0														
Seychelles	80	20	25	11	14	34	15	73	4	100	20	25	11	14	73		61	0					
Sierra Leone	4 405	3 760	85	2 472	56	12 225	5 365	46	3	50	3 760	85	2 472	56	46		75	50					
South Africa	43 309	111 269	257	77 391	179	227 941	92 683	84	3	77	87 836	203	62 399	144	67		84	23	23 433	54	14 992	35	77
Swaziland	925	5 877	635	1 823	197	5 551	2 229	82	1	0								100	5 877	635	1 823	197	36
Togo	4 527	1 409	31	984	22	14 368	6 181	16	4	100	1 409	31	984	22	16		92	0					
Uganda	23 300	30 372	130	17 246	74	81 780	34 717	50	4	100	30 372	130	17 246	74	50		66	0					
UR Tanzania	35 119	54 442	155	24 049	68	126 103	53 589	45	4	100	54 442	155	24 049	68	45		58	0					
Zambia	10 421	49 806	478	12 927	124	55 152	22 425	58	1	0								100	49 806	478	12 927	124	34
Zimbabwe	12 627	51 918	411	15 455	122	73 701	29 603	52	4	100	51 918	411	15 455	122	52		36	0					

Figure 2. Regional profile for Africa, cont'd: treatment outcomes for cases registered in 1999 – WHO TB control strategy (DOTS) and other (non-DOTS) control strategies (120).

Country/Territory	New smear-positive cases - DOTS									Retreatment cases - DOTS									New smear-positive cases - non-DOTS								
	Regist-ered	% cured	% compl-eted	% died	% failed	% default	% trans-ferred	% not eval	% success	Regist-ered	% cured	% compl-eted	% died	% failed	% default	% trans-ferred	% not eval	% success	Regist-ered	% cured	% compl-eted	% died	% failed	% default	% trans-ferred	% not eval	% success
	a	c	d	e	f	g	h	b	c+d	i	k	l	m	n	o	p	j	k+l	q	s	t	u	v	w	x	r	s+t
Algeria	7 622	87		3	1	5	5	0	87	810	85		0	2	8	5	0	85									
Angola																											
Benin	2 192	57	20	6	2	15	1	0	77	200	45	24	8	4	21	0	0	68									
Botswana	2 746	25	46	7	0	10	11	0	71	383	21	51	9	0	12	7	0	72									
Burkina Faso	1 411	51	9	14	3	14	7	1	61	75	56	5	12	11	12	4	0	61									
Burundi																											
Cameroon	2 003	67	8	11	2	11	2	0	75	243	62	5	11	8	13	2	0	67									
Cape Verde																											
Central African Republic																											
Chad																											
Comoros	115	93	0	2	2	3	0	0	93	8	100	0	0	0	0	0	0	100									
Congo	2 222	49	12	1	0	23	2	13	61	98	43	16	2	1	38	0	0	59									
Côte d'Ivoire	6 946	53	10	4	3	16	15	0	63	691	57	9	5	7	16	6	0	66									
DR Congo	34 923	59	10	5	1	7	9	9	69	2 608	60	7	7	2	7	11	6	67									
Equatorial Guinea																											
Eritrea	465	40	4	8	2	7	6	33	44	84	60	10	6	6	7	12	0	69									
Ethiopia	15 980	60	16	7	1	10	4	3	76	846	59	15	9	3	10	4	0	74	4 531	28	39	4	1	18	5	4	68
Gabon																											
Gambia																											
Ghana	5 605	49	6	8	2	17	6	12	55										1 272	25	6	4	3	11	1	50	32
Guinea	3 563	65	9	8	2	9	7	0	74	239	58	8	5	5	13	10	0	66									
Guinea-Bissau	616	19	16	7	0	20	8	30	35	65	11	14	8	0	29	8	31	25									
Kenya	24 670	64	14	6	0	9	6	0	78	1 545	62	11	11	1	10	5	0	73	2 747	82	6	5	2	4	1	0	88
Lesotho	2 831	48	21	13	1	7	6	3	69	374	50	11	19	1	6	3	8	61									
Liberia																											
Madagascar																											
Malawi	8 185	69	3	21	1	4	3	0	71	668	64	6	23	1	3	3	0	71									
Mali	2 690	50	18	3	1	26	2	0	68										309	61	15	3	2	18	2	0	76
Mauritania																											
Mauritius	122	87		4	4	5		0	87	3	67			33			0	67									
Mozambique	11 791	69	2	11	1	12	3	1	71	1 404	69	2	12	2	11	4	0	71									
Namibia	3 791	37	12	5	1	12	6	27	50	589	39	16	14	6	12	9	5	54									
Niger	1 368	37	24	12	2	17	9	0	60	1 941	27	31	12	2	18	9	0	58									
Nigeria	14 868	60	15	6	3	13	3	0	75	1 639	61	13	8	5	9	4	0	74									
Rwanda	4 652	56	11	6	1	4	6	16	67	325	39	9	10	2	6	5	31	48									
Sao Tome and Principe																			97	62	20	7	4	5	0	2	81
Senegal																											
Seychelles	21	86	5	0	0	5	5	0	90										1	100		0	0	0	0	0	100
Sierra Leone	1 904	66	9	2	16	7	1	0	75																		
South Africa	63 304	52	8	7	1	13	17	2	60	21 125	40	7	8	4	20	20	1	47	18 191	33	16	6	1	19	23	1	49
Swaziland																											
Togo	893	74	1	10	2	12	1	0	76																		
Uganda	14 250	30	31	8	0	16	5	9	61	1 111	28	20	11	1	11	5	24	48									
UR Tanzania	23 994	71	6	10	0	7	6	0	78	1 737	69	5	13	1	6	5	1	74									
Zambia																			11 645	50	19	7	3	9	5	7	69
Zimbabwe	12 791	59	14	10	0	7	11	0	73	943	50	17	16	1	8	9	0	66									

1.2 THE BCG VACCINE

Although vaccination is considered the most successful prophylactic measure against infectious diseases, most vaccines currently in use are directed against viruses and extracellular bacteria (46). BCG (Bacillus Calmette-Guerin), one of only two vaccines directed against intracellular bacteria, is an attenuated derivative of the virulent *Mycobacterium bovis* (*M bovis*) strain (46, 106).

BCG is the most widely used vaccine to date. It is safe enough to be given at birth, can be given as an oral vaccine, is cost effective, is heat stable, can persist in an innocuous form for many years and has potent adjuvant properties (115). Although BCG vaccination is considered safe, disseminated BCG does occur (46). This is mainly due to the individual having some kind of immune-deficiency. Patients suffering from Mendelian susceptibility to mycobacterial disease have an impaired IFNγ-mediated immune response due to a defect in their IFNγ receptor gene or IL-12 gene and these individuals may experience disseminated BCG (26). The BCG vaccine is also not fully effective against the adult form of TB. It displays a higher protective efficacy against miliary TB and tuberculous meningitis. Infection with HIV is the greatest risk factor for development of active TB. Complications with BCG vaccination have been reported in adults who are HIV-positive (115).

The efficacy of BCG vaccination has yielded varying results. One explanation was that there are two types of cell-mediated responses that occur after mycobacterial infection (9, 115. In the Koch phenomenon, animals develop necrosis at the site of vaccination a few days after MTB infection. In the listeria-type response, T cells appear within days after

infection and no necrosis occurs. It is believed that prior exposure to mycobacteria that produce the listeria-type response may enhance the protective effects of subsequent BCG vaccination. Infection with mycobacteria that cause the Koch response inhibits development of protective immunity after BCG vaccination.

The efficacy of BCG vaccination may also depend on whether disease is due to reactivation or re-infection (46, 115). This hypothesis is supported by trials which indicate that in areas where re-infection is high, the efficacy of BCG vaccination may be lower than in areas where reactivation predominates.

1.3 DEVELOPMENT OF A RECOMBINANT BCG VACCINE

The major difficulty in the development of a recombinant BCG (rBCG) vaccine was the problem of introducing foreign DNA into the mycobacterium through its thick waxy cell wall. This problem was overcome by the use of mycobacteriophages, which could effectively introduce the foreign DNA (Figure 3) (114, 115). The rBCG vaccine was therefore a BCG vector modified to express antigens of interest such as MTB antigens (111, 112).

Considerable efforts have been made to identify specific mycobacterial antigens and virulence genes involved in the immune response to TB and to incorporate these into a rBCG vaccine (83, 112) (Figure 4). Subunit vaccines and adjuvants are highly specific and have a low risk of side effects but their immunostimulatory and protective efficacy is generally weak and short-lived (106). Another method of vaccine employment is the application of naked plasmid DNA encoding the gene of interest directly into the skin (118). The mycobacterial protein Ag85A has been shown to induce a protective immune response in mice comparable or only slightly lower than BCG vaccination (47).

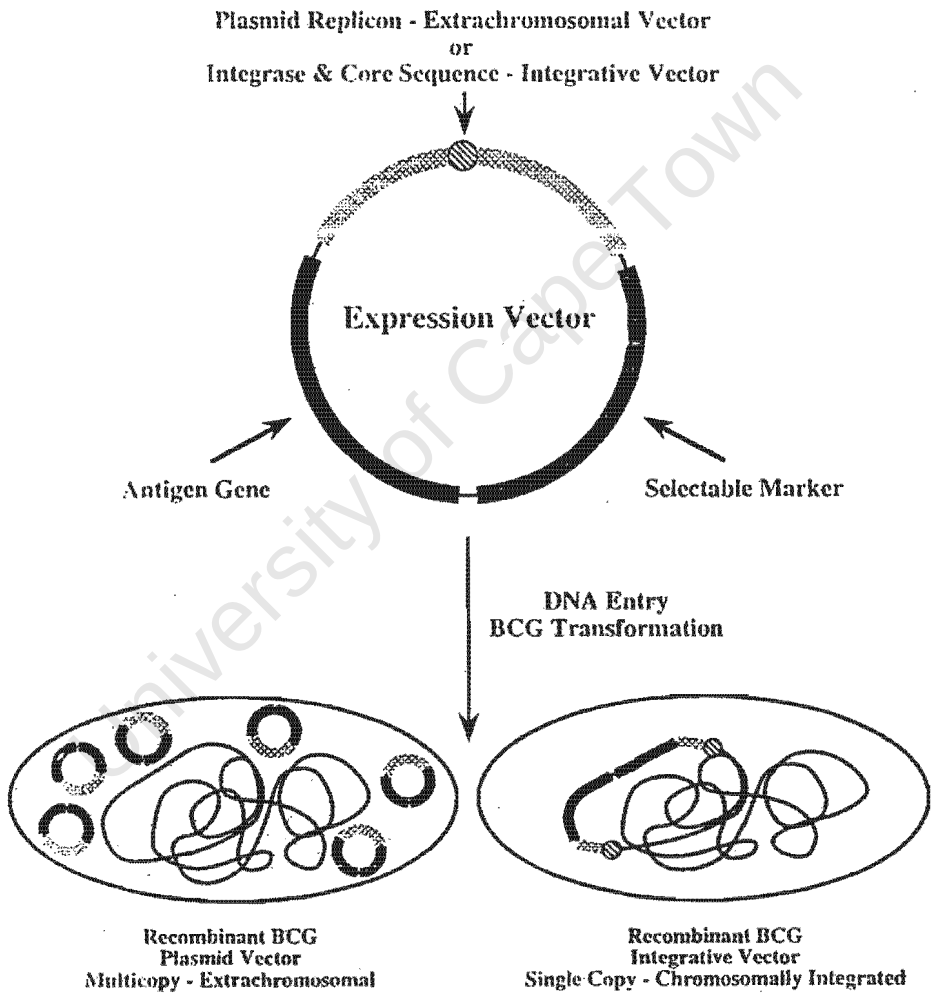


Figure 3. Mycobacterial transformation to construct rBCG vaccine (115).

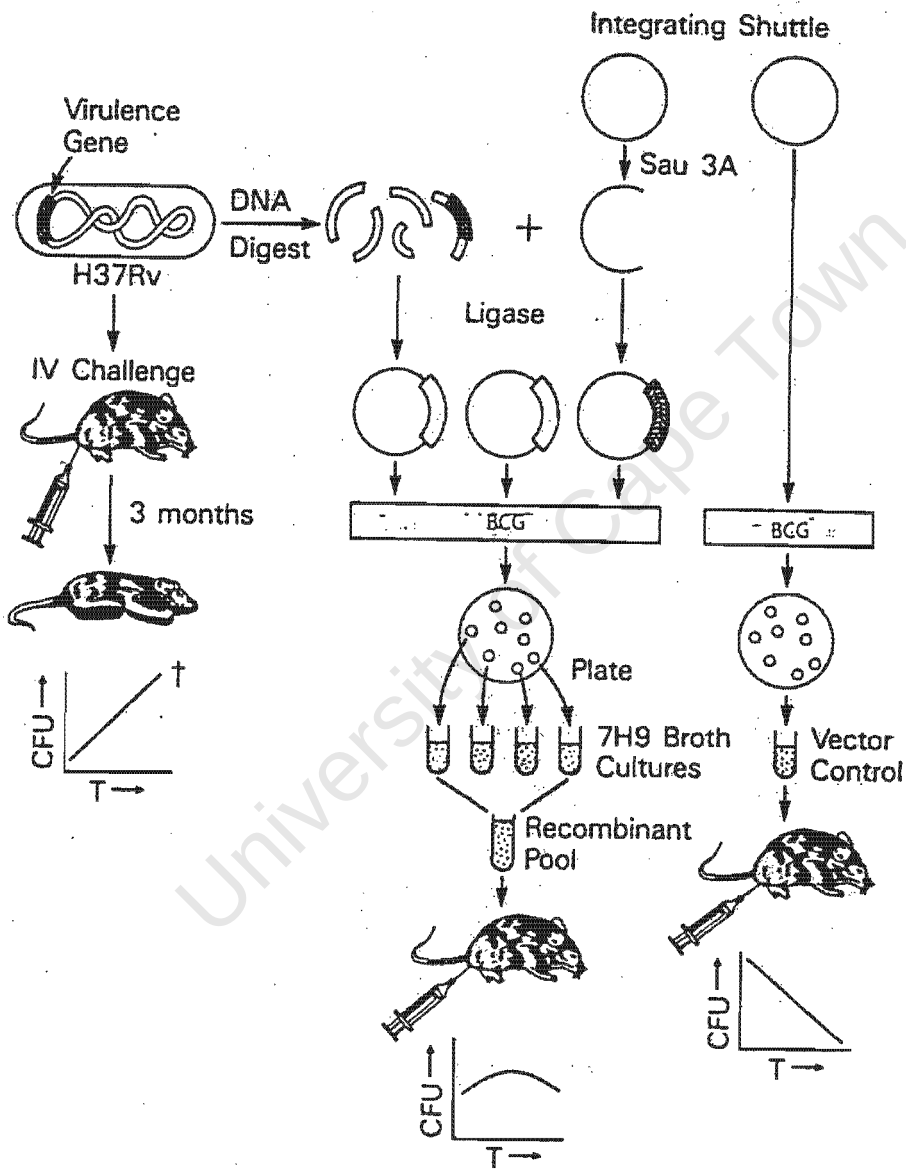


Figure 4. Cloning procedure used to transfer putative virulence genes from MTB to BCG by the use of an integrating shuttle vector(modified from 18).

1.4 MYCOBACTERIA

1.4.1 *Mycobacterium tuberculosis*

MTB is a slow-growing acid-fast bacillus (AFB) transmitted mainly via the respiratory route. A notable feature of pathogenic mycobacteria is their extremely slow growth (81). The estimated doubling time is 17 hours and this is due to the single rRNA operon. Mycobacteria are phylogenetically more closely related to Gram positive bacteria and they stain weakly positive for the Gram stain. Their most distinctive feature is the mycobacterial cell envelope consisting of peptidoglycan and glycolipids such as lipoarabinomannan, mycolic acids and complex lipids. (71). This lipid layer is responsible for their resistance to certain drugs and also their acid-fast characteristic. During the Gram stain, heat is used to infuse the lipid envelope with a dye. Organisms which do not posses this lipid layer, and are therefore not mycobacteria, lose the dye when acid is added. Mycobacteria are not affected by the acid wash, retain the dye and are therefore called acid-fast.

1.4.2 Auxotrophic mutants of mycobacteria

An avenue of exploration in vaccine development is alteration of the pathogen's ability to cause an immune response in its host. The possibility of using live mutant strains of mycobacteria as vaccines have also been explored (4, 45, 49). An advantage of using attenuated live vaccines over subunit vaccines are that they are believed to provide a greater complement of antigens since they produce most of the antigens normally expressed *in vivo* (7). Live attenuated MTB strains should express antigens which are lacking in the BCG vaccine and which may be important in the immune response to TB.

Attenuated auxotrophic mutants of bacterial pathogens have already shown potential as live vaccine candidates because they were attenuated *in vivo* and still remained immunogenic (21, 110).

BCG auxotrophs have been tested on severe immune-deficient mice that survived infection with these mutants although they succumbed to the wild type (WT) BCG infection (4, 11, 41, 45, 46). It was therefore proposed that attenuated BCG strains could present potentially safe and useful vaccines against TB and specifically that they could be useful for people infected with HIV.

1.5 GENERAL PATHOLOGY OF TUBERCULOSIS

The first point of entry and major organ of disease during MTB infection is the lung (12). After being inhaled, the pathogen is engulfed by alveolar macrophages which transport it to the draining lymph nodes. Infected macrophages produce chemokines that cause extravasation of additional phagocytes and lymphocytes. T cells activated in the draining lymph nodes, and natural killer (NK) cells, are recruited to the site of infection (12, 33). Here, the T cells and NK cells interact with macrophages to produce interferon gamma (IFNγ), which in turn activates the macrophage to become bactericidal (32). Activated macrophages secrete tumour necrosis factor (TNF), which initiates granuloma formation (54, 55, 98). A productive granuloma with high cellular turnover develops to confine the bacteria and restrict their growth (Figure 5) (12, 33).

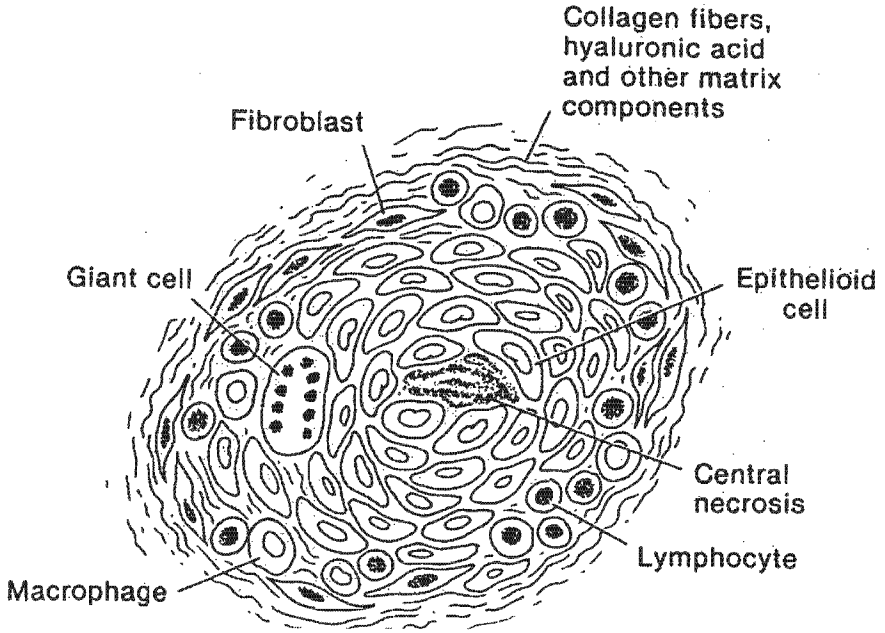


Figure 5. General architecture of a tuberculous granuloma with a core of macrophages and epithelioid cells surrounded by lymphocytes (121).

Although granulomas inhibit bacterial replication, they often are unable to completely eliminate the bacteria so cytolytic T cells move through the granuloma searching for residual live bacteria (12, 33, 84). They lyse infected host cells and lysis of such cells releases bacteria and allows them to be taken up by more efficient phagocytes. This also promotes discharge of toxic macrophage products to further inhibit mycobacterial growth. Later, granulomas are encapsulated by fibrotic walls and the centre of the granuloma becomes necrotic. Encapsulation contributes to microbial containment and lowers partial oxygen pressure providing unfavourable growth conditions for MTB. When uncontrolled cell destruction by cytolytic T cells, NK cells and macrophages promote granuloma liquefaction, they encourage rupture of the bronchoalveolar and vascular systems and dissemination of the mycobacteria into these systems.

The co-ordinated interplay between macrophage activation and target cell lysis appears to be required for protection (12). Mycobacteria can reside in a host for years without

causing disease and this indicates that the immune system generally fails to completely eliminate MTB and must rely on mycobacterial containment and growth inhibition.

1.6 MECHANISMS OF PROTECTION

Anti-tuberculosis immunity involves a critical interplay of macrophages, lymphocytes and cytokines (7, 12, 33).

1.6.1 MACROPHAGES

Macrophages play multiple roles in the containment of MTB infection. They are the innate cells that initially recognise the presence of inhaled MTB. After phagocytosis of MTB, alveolar macrophages process the antigens for presentation to the adaptive immune system. Macrophages also possess antibacterial activities that are largely responsible for restriction of MTB growth (7, 12, 13, 32, 33, 61, 62, 104, 105). The antimycobacterial activities include production of reactive oxygen and nitrogen intermediates and the creation of an acidic phagolysosomal environment.

1.6.2 T CELLS

Different stages of experimental infection involve different T cell subsets (Figure 6) (84, 85, 117). Presentation of mycobacterial antigens to $CD4^+$ T cells occurs via the MHC Class II molecule, and the MHC Class I molecule which is used to sensitise $CD8^+$ T cells (36, 59, 60, 113) (Figure 7).

During haematogenous spread of the infection, bacteria in the blood are picked up within a short time by macrophages in the spleen and liver (7, 33). Some of these bacteria can be detected in lungs. In an aerosol infection, many bacteria are cleared by lung alveolar

macrophages and carried to draining lymph nodes. Some may erode directly into alveolar endothelial cells and within 10-15 days, small granulomas develop around these alveolar cells. $CD8^+$ cytolytic cells destroy the infected cell, releasing the bacteria for ingestion and destruction by surrounding macrophages (85, 108). If there are no $CD8^+$ T cells, bacilli survive and grow, causing abscess formation and necrotic destruction. Some macrophages are damaged by high bacterial loads and others may be refractive to cytokine activation. Under these circumstances, mycobacterial proteins may leak into the cytoplasm and become part of the MHC Class I processing pathway (67). $CD8^+$ T cells are therefore essential for the detection and resolution of these bacteria (84).

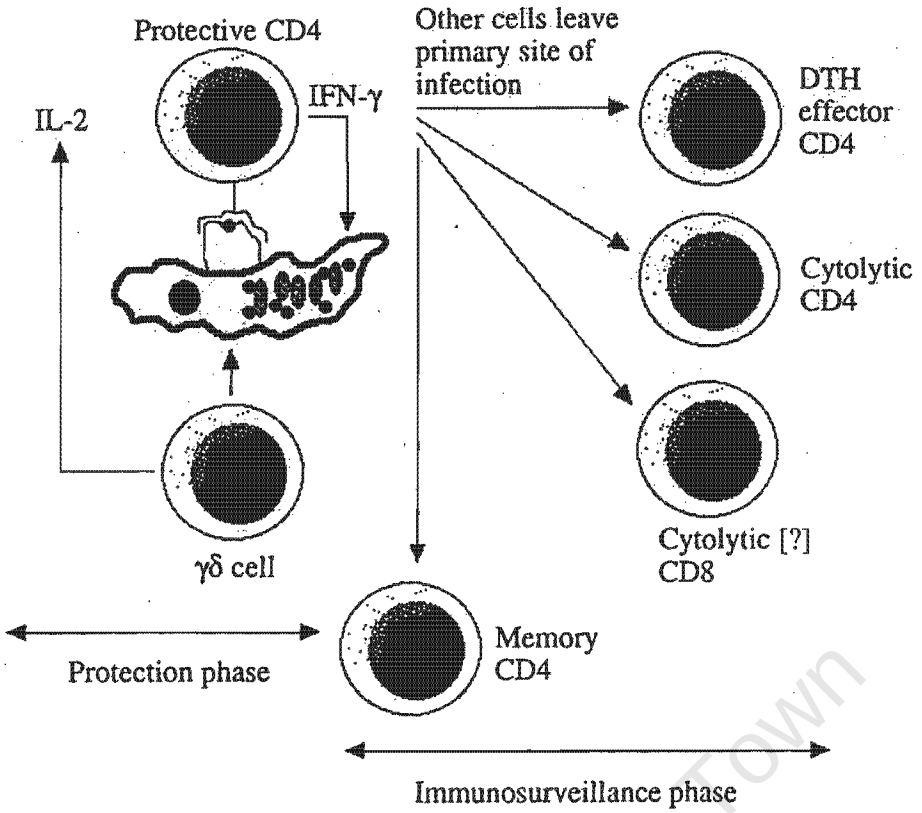


Figure 6. Hypothetical model for T cell generation during the course of tuberculosis in the mouse. In the early protective phase, CD4 T cells recognise secreted/export proteins presented on MHC II molecules and secrete IFNγ for macrophage activation and recruitment. γδ T cells accumulate and secrete IL-2. As the infection progresses, some sensitised T cells leave the site of infection and play a part in immunosurveillance. DTH T cells circulate and can rapidly initiate a granuloma on recognition of mycobacterial antigens. Cytolytic T cells release mycobacteria from heavily infected macrophages. CD4 memory T cells rapidly mediate resistance to subsequent mycobacterial infection (87).

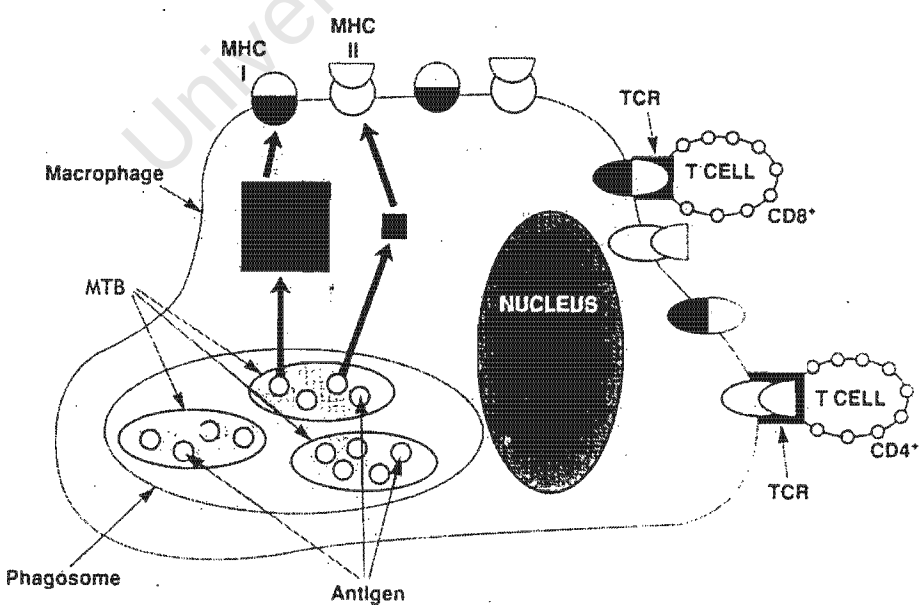


Figure 7. Relationship of MTB inside the macrophage and its relationship to the antigen presentation pathway (114).

1.6.3 CYTOKINE RESPONSE

Infection with MTB induces an early type-1 (TH1) T cell response associated with production of IL-12 and IFNγ (15, 86, 88, 89, 108). Within 24 hours after infection, host macrophages begin to secrete proinflammatory cytokines (IL-1, IL-6, M-CSF, GM-CSF, TNF, IL-10) (89). Secretion of TNF is usually induced by lipoarabinomannan, a component of the mycobacterial cell wall, and this activates host macrophages to inhibit the mycobacterial growth and also promotes a granulomatous response at the site of infection (14, 55). IFNγ together with TNF are the key activating agents that trigger macrophages to become antimycobacterial (19, 25, 29, 31, 32, 98, 101). Deficient TNF results in poor granuloma formation and dissemination of infection (55, 98). Administration of recombinant IL-12 may confer resistance to tuberculosis in mice. (44). Events triggered by IL-12 help identify NK cells as critical cellular components in defence against MTB. Since NK cells can produce IFNγ in response to IL-12, NK cells can rapidly activate macrophages to express microbiocidal functions during the early non-immune phase of TB, before the expansion and differentiation of T cells (56).

Forty days post-infection, TH2-like IL-4-secreting $CD4^+$ T cells appear. Both TH1- and TH2-like responses therefore occur in mice and humans infected with MTB (57, 117). The early TH1-like response mediates protective immunity by release of IFNγ and other cytokines while the later emerging TH2-like response drives helper T cells to facilitate antibody production to mycobacteria (19). It has been suggested that very low and very high antigen doses promote a TH2 response, while moderate antigen levels predispose naïve cells to become TH1 cells (86, 94, 100). Low and high doses of high affinity antigen yield TH1 cells, while moderate doses of high affinity antigen yield TH2 cells.

Three cytokines are central to the initial stages of development of TH1 and TH2 cells (104). IL-4 is considered dominant over IL-12 (57, 78, 107). It up-regulates expression of its own receptor, inhibits secretion of IL-12 and down-regulates expression of IL-12β2 subunit (58, 91). It induces its own expression and induces TH1 to TH2 switch, possibly through activation of its own receptor on TH1 cells (58). IL-12, in contrast, cannot block IL-4 production and cannot induce a switch from TH2 to TH1 (78). This may be due to the fact that TH2 cells are constantly making IL-4 and IL-4 down-regulates IL-12Rβ2 subunit, making these cells unresponsive to IL-12. IFNγ inhibits IL-4R expression and interferes with IL-4 and IL-5 production (90, 99). Given the dominant position of IL-4 over IL-12, timing and amounts of IL-4 dictate the pathway of T cell development (90, 100, 107). Unlike IL-12, which has a source immediately adjacent to the activated T cell, IL-4 is made by a variety of cells distant from the site of antigen activation (97). IFNγ and IL-4 are also made by antigen-naïve T cells. If enough endogenous IL-4 is made to overcome early endogenous IFNγ inhibition and APC-derived (antigen-presenting cell) IL-12 stimulation, IL-4 dominance will drive cells to a TH2 phenotype (58, 107). The key is the relative timing and ratios of IL-4 to IFNγ and IL-12 (107).

1.7 IL-4 AND IL-13 IN INFECTIOUS DISEASES

1.7.1 FUNCTIONS OF IL-4

IL-4 acts on B cells to induce proliferation in the presence of anti-IgM, stimulates IgG1 and IgE synthesis and allows the expression and release of low affinity IgE receptors (58, 91). It also inhibits IgM, IgG2a, IgG2b, and IgG3. On T cells, it inhibits IL-2 induced cytotoxic T cell activity. IL-4 stimulates mast cell growth and haematopoetic progenitor

cell growth. It induces macrophage aggregation and cytotoxicity and also induces MHC Class II expression (91). It blocks IL-1, IL-6, IL-8 and TNF production by macrophages but stimulates G-CSF and GM-CSF production by monocytes. IL-4 is made by activated TH2 $CD4^+$ cells and has receptors on T cells, B cells, monocytes, fibroblasts, epithelial cells, and endothelial cells (91).

1.7.2 FUNCTIONS OF IL-13

IL-13 is a TH2 cytokine related to IL-4 and they both belong to the same α-helix protein family (72, 123). Human genes for IL-4 and IL-13 are found on chromosome 5 and the mouse genes are found on chromosome 11. IL-4 and IL-13 production is restricted to activated T cells, especially TH2 cells, mast cells, basophils, dendritic cells and NK cells (68, 69, 124). IL-4 and IL-13 act on a variety of cell types, e.g. NK cells, fibroblasts, eosinophils, airway smooth muscle cells, endothelial cells. The effects of these cytokines include up-regulation of MHC Class II and CD80/86 on macrophages and monocytes, up-regulation of members of the integrin superfamily that may contribute to enhanced extravasation, mobility and trafficking of macrophages and monocytes (68, 73). There are also important immunosuppressive effects and anti-inflammatory activities on macrophages and other cells (5, 8). These include inhibition of pro-inflammatory cytokines and chemokines, inhibition of nitric oxide production, antibody-dependant cell-mediated cytotoxicity and FcγR surface expression. Both cytokines have a role in asthma (99). Although IL-4 and IL-13 have many shared activities, each cytokine also has its own individual functions.

1.7.3 DIFFERENCES BETWEEN IL-4 AND IL-13

IL-4, but not IL-13, promotes TH2 cell differentiation which is characterised by the secretion of IL-4, IL-5, IL-9, IL-10 and IL-13 (70, 122, 124). IL-4 is important for the generation of the humoral immune response and immunoglobulin switch of B cells, leading to the secretion of IgG1 (human IgG4) and IgE (95). In mice, no direct effect of IL-13 has been demonstrated on TH2 differentiation or on B cell switch and this is probably due to the absence of IL-13 receptors on the surface of these cells (123). Human B cells, but not T cells, are responsive to IL-13 (68, 95). Activated TH0, TH1-like and TH2-like $CD8^+$ cells make IL-13.

1.7.4 IL-4 AND IL-13 RECEPTORS

1.7.4a The IL-4 receptor

The overlapping biological functions of IL-4 and IL-13 are due to the shared receptor component (1, 10, 43, 82). The type 1 IL-4R is a heterodimeric complex consisting of the IL-4Rα chain and the common γ chain (γc) (102). The γc chain was found not to be the common component of the IL-4R and IL-13R since effects from both cytokines could be detected in the absence of the γc chain (1, 66). Monoclonal antibodies against IL-4Rα chain blocked responses to both IL-4 and IL-13 and therefore the IL-4Rα chain had to be the common component of the IL-4R and IL-13R (43). The type 2 IL-4R comprises an IL-4Rα chain and IL-13Rα1 chain (52). It is a high affinity IL-4 receptor with a low affinity for IL-13 since the IL-13Rα1 chain binds to IL-13 with low affinity (77). Soluble IL-4R is predominantly secreted by mature lymphoid cells and may act as an inhibitor at high concentrations (17). At low concentrations it acts as a carrier for IL-4. In all the receptors,

signal transduction via the IL-4Rα chain leads to activation of Jak-1 independent pathways, which involves tyrosine phosphorylation of STAT6 (signal transducer and activation of transcription) or IRS 2 (insulin response element) (76). IL4Rα$^{-/-}$ and STAT6$^{-/-}$ mice have no IL-4- or IL-13-mediated macrophage functions therefore IL-4Rα had to be an important component for both receptors and STAT6 signalling (76).

1.7.4b The IL-13 receptor

IL-13Rα1, IL-13Rα2, IL-4Rα and γc chain form the 4 types of IL-13R complexes (Figure 8) (82). In the Type I IL-13 receptor, IL-13 binds to two 70kDa isomers (IL-13Rα and α') and IL-13Rα' also binds to IL-4. IL-13Rα corresponds to IL-13Rα1 and IL-13Rα' corresponds to IL-13Rα2. IL-13 will have greater binding affinity than IL-4. The Type II IL-13R may be composed of heterodimeric proteins of 70kDa (IL-13Rα') and 140kDa (IL-4Rα). Both IL-4 and IL-13 bind to both proteins. The Type III IL-13R may be composed of the 70kDa protein (IL-13Rα'), the 140kDa protein (IL-4Rα) and the 64kDa protein (γc). IL-13 will bind weakly to IL-4Rα and to IL-13Rα' but will not bind to γc. IL-4 binds to both IL-4Rα and to γc. In this case both IL-4 and IL-13 will compete for the binding of both IL-4R and IL-13R. The fourth model is similar to the Type III IL-13R but here IL-13 binds weakly. The type of IL-13R complex expressed depends on the type of cell and which combination of receptor components are present (37, 40, 82). Different cells therefore display different binding properties for IL-13 and IL-4. IL-13Rα1 is for signalling and binds IL-13 with low affinity (77). IL-13Rα2 has greater affinity for IL-13 than IL-13Rα1 but it is non-signalling (40, 93). IL-13Rα1 has been found on human and mouse B cells but not on human or mouse T cells (37, 40, 93). It is also found on mouse germinal centre B cells and follicular dendritic cells (91). Soluble IL-13Rα1 has been

found in the supernatant of activated human T cells and soluble IL-13Rα2 was found in mouse serum (98, 122). A soluble IL-13Rα2/Fc fusion protein blocked mitogenic responses suggesting that IL-13Rα2 could serve as dominant negative inhibitor or decoy receptor (16).

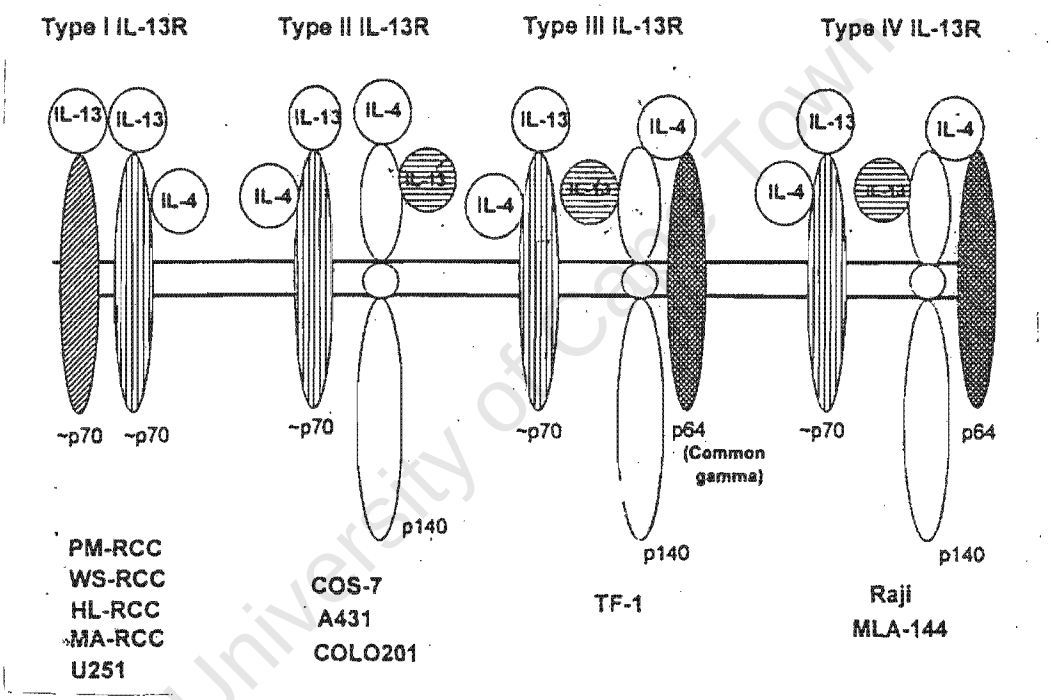


Figure 8. Proposed models of IL-13R structure. Examples of each model are shown below the schematic diagrams (82).

1.7.5 IL-4 AND IL-13 AND INFECTIOUS AGENTS

1.7.5a *Leishmania major* infection

The protozoan flagellate *Leishmania major* (*L major*), transmitted by sand flies, causes human leishmaniasis. During cutaneous *L major* infection, leishmaniasis-resistant mouse strains develop dominant TH1 responses with high IFNγ and low IL-4 production resulting in a protective cellular immune response (75). Leishmaniasis-susceptible strains have a dominant TH2 response with high IL-4 and low IFNγ production (74). IL-4 inhibition by IL-4 itself is a key component determining polarisation to TH2 cell phenotype and susceptibility to *L major* infection. Several studies have revealed that IL-$4^{-/-}$, IL-$13^{-/-}$, IL-$4^{-/-}$/IL-$13^{-/-}$ and IL-4Rα$^{-/-}$ mice are resistant to *L major* therefore IL-4 and IL-13 have major influences on disease outcome (65, 75). There are, however, IL-4Rα-independent mechanisms for TH2 development, as demonstrated by IL-4Rα$^{-/-}$ and IL-$4^{-/-}$ mice being able to produce IL-4 (74, 79). IL-13R is also responsible for protection from chronic *L major* infection as IL-4Rα$^{-/-}$ mice but not IL-$4^{-/-}$ mice, undergo disease progression. This data suggest that there are both IL-4- and IL-13-dependent and independent factors which determine disease progression (75).

1.7.5b Nematode infection

IL-4 plays a various roles in resistance to nematode infection (10). Abrogation or neutralisation of IL-4 activity or infection of IL-$4^{-/-}$ mice with *Heligosomoides polygyrus*, *Trichinella spiralis*, *Trichus muris* hinders parasite expulsion from intestine (2, 3, 10).

Nippostrongylus brasiliansis (*N. brasiliansis*) expulsion is independent of IL-4 and relies more on IL-13 for protection (5, 69, 70, 119). Mice deficient in IL-4 and IL-4Rα were

infected with *N. brasiliansis* (5, 779. *N brasiliansis* is a strong inducer of TH2 responses in mice. TH2 cell differentiation was more severely affected in IL-4Rα$^{-/-}$ than IL-4$^{-/-}$ mice. IL-4$^{-/-}$ mice were still responsive to IL-13 therefore TH2 cell development seemed to be regulated by IL-4 and IL-13. STAT6$^{-/-}$, IL-13$^{-/-}$ and IL-4/IL-13 double knockout mice had impaired TH2 development (69, 70, 119). IL-13 could not directly promote TH2 polarisation and neither IL-4 nor IL-13 addition could reverse the IL-13-deficient phenotype.

1.7.5 c *Schistosoma mansoni* infection

IL-13 plays an important role in pulmonary granuloma formation and fibrosis in Schistosome infection (30). IL-4Rα$^{-/-}$ and IL-4/IL-13$^{-/-}$ have no granulomatous inflammation or fibrosis and had reduced TH2 responses (16, 51, 96). Blocking IL-13 mediated functions with antibody completely eliminated Schistosome-induced granuloma formation in IL-4$^{-/-}$ mice (16).

1.7.5d Mycobacterial infection

Cell-mediated immunity plays a key role in host resistance to MTB infection (7, 33). The cell-mediated immune response is regulated by a balance between TH1 cytokines (IFNγ, IL-12, TNF) and TH2 cytokines (IL-4, IL-10, IL-13). Studies in mice deficient in TH1 cytokines show that these cytokines are essential to control mycobacterial infections (6, 19, 20, 25, 34, 35, 42). Besides the dominant TH1 response, mycobacterial infections also induce TH2 cytokines such as IL-4 and IL-10, which are known to down-regulate the TH1 response but have been demonstrated to play a minor role in mycobacterial infections (28, 33, 38, 42, 50, 79, 116).

1.8 ANIMAL MODELS OF TUBERCULOSIS

For the study of TB, the guinea pig and mouse models are the most popular animal models used. Each model has its specific advantages, which will depend on the aim of each project.

1.8.1 TB AND THE GUINEA PIG

Since the guinea pig is sensitive to a low dose pulmonary infection, it therefore depicts the natural cause of the infection in humans with active TB (87). It is mainly used for studies of pathogenesis and for the evaluation of vaccines. Vaccine efficacy and therefore resistance to mycobacterial infection is evaluated with respect to increased survival, reduction in size and severity of pulmonary TB and lowered bacterial loads in the guinea pig (87).

1.8.2 THE MOUSE MODEL OF TB

The mouse is the most common animal model used in TB research due to its breeding capabilities and low-cost maintenance (63, 87). Most human genes have a related mouse version, making it possible to gain insights into human disease using gene-deficient mouse models. By using low-dose mouse models of infection, it is possible to define the immune response that occurs in individuals who become infected but who do not develop active TB.

Although the guinea pig model is gaining popularity, there is a large supply of immunological tools that support using the mouse model of infection eg. variety of gene-knock-out mice, availability of mouse antibodies, molecular mouse probes. It can be said that a large amount of information gained from mouse models may not pertain directly to

human infection but it does provide some understanding about the immune response in general.

Immunology has been revolutionised by the availability of gene-deficient mice (Figure 9) (27). Before the advent of knockout mice, most work was tested in *in vitro* systems. It is now well known that the *in vitro* and *in vivo* systems operate differently and that results obtained using *in vitro* techniques may not always mimic what happens during the natural course of infection in the host. The immune system and immune response is the result of the interaction of a variety of factors and therefore the use of animal models may give a better understanding of this interaction. The use of cytokine-knockout mice furthers this understanding by giving some idea of the role of particular cytokines in disease. Many cytokine-knockouts changed the way cytokines were previously viewed and some knockouts were lethal indicating far broader functions than anticipated (27).

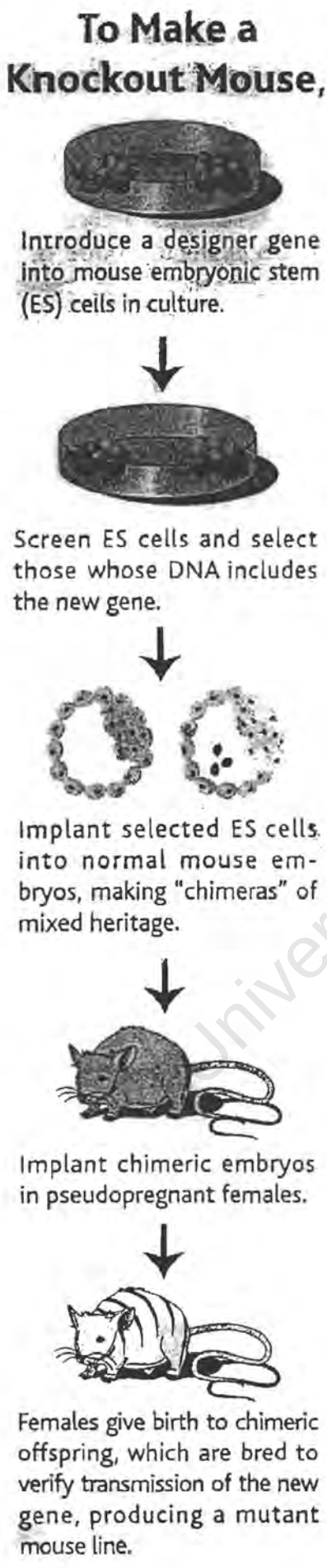


Figure 9. Generation of gene-deficient mice (63).

1.9 VACCINE TESTING

1.9.1 *IN VITRO* VACCINE TESTING

A number of BCG and *Mycobacterium tuberculosis* H37Rv (H37Rv) auxotrophic mutants have been tested (Table 1). Work on bone marrow-derived macrophages (BMDM) infected with WT mycobacteria showed that most of the macrophages had high intracellular bacterial counts (4, 45, 49, 111). While the H37Rv purine auxotroph persisted without the number of viable bacilli increasing, the H37Rv leucine auxotroph could not replicate inside macrophages (45, 49). The BCG purine and leucine auxotrophic mutants were gradually eliminated (4). The methionine-deficient H37Rv had growth kinetics similar to that of the WT, and proline- and tryptophan-deficient H37Rv had significantly reduced bacterial growth (108). These results demonstrated that, with the exception of the methionine mutants, disruption of these genes alters the ability of the mycobacteria to multiply within BMDM.

1.9.2 VACCINE TESTING IN GUINEA PIGS

Methionine and leucine BCG mutants were used to immunise guinea pigs and later, the guinea pigs were challenged, via aerosol, with WT H37Rv and WT BCG (11). Protection was expressed as a reduction in pathology rather than reduction in bacterial load in the lungs. Methionine BCG provided protection against H37Rv challenge. The lungs of guinea pigs vaccinated with leucine-deficient BCG displayed gross pathology. BCG vaccination conferred less protection to challenge with *Mycobacterium tuberculosis* than to challenge with BCG.

	BCG				H37Rv					
	WT	**Purine $^{-}/_{-}$**	**Leucine $^{-}/_{-}$**	**Methionine $^{-}/_{-}$**	**WT**	**Purine $^{-}/_{-}$**	**Leucine $^{-}/_{-}$**	**Methionine $^{-}/_{-}$**	**Proline $^{-}/_{-}$**	**Tryptophan $^{-}/_{-}$**
Growth in BMDM	$+^{+}/_{-}$	$^{+}/_{-}$	+	ND	+++	++	-	+++	+	+
Persistence in immune - competent mice	++	-	ND	ND	++	+	$^{+}/_{-}$	$+^{+}/_{-}$	-	-
Persistence in immune - deficient mice	+	$^{+}/_{-}$	ND	ND	+++	++	$^{+}/_{-}$	$++^{+}/_{-}$	+	-
Protects mice against iv. *M tuberculosis*	++	ND	ND	ND	ND	ND	++	ND	+++	+++
Protects mice against aerosol *M tuberculosis*	++	-	ND	ND	ND	ND	-	ND	ND	ND
Persistence in guinea pigs	++	-	-	ND	ND	+	ND	ND	ND	ND
[1]Protects gp against aerosol *M bovis* (haematogenous)	+++	ND	$+^{+}/_{-}$	++	ND	ND	ND	ND	ND	ND
[1]Protects gp against aerosol *M tuberculosis* (haematogenous)	++	-	+	++	ND	+	$^{+}/_{-}$	ND	ND	ND
[2]Protects gp against aerosol *M bovis (pulmonary)*	+++	ND	++	++	ND	ND	ND	ND	ND	ND
[2]Protects gp against aerosol *M tuberculosis (pulmonary)*	++	$^{+}/_{-}$	$^{+}/_{-}$	$^{+}/_{-}$	ND	$^{+}/_{-}$	-	ND	ND	ND

Table 1. Overview of experiments using auxotrophic mycobacteria. Degree of persistence or protection ranges from +++ (lots of growth, good protection) to – (no growth, minimal protection). [1]Used spleen CFU as a measure of haematogenous protection. [2]Used lung CFU as a measure of pulmonary protection. Abbr. BMDM – bone marrow-derived macrophages, i.v. - intravenous infection, gp - guinea pig, ND – not determined

Purine-deficient mutants (BCG and H37Rv) were also used to vaccinate guinea pigs and later the guinea pigs were challenged via aerosol with WT H37Rv (47). Purine-deficient BCG exhibited no protection in the spleen while mutant H37Rv provided more protection against an intravenous (iv) challenge than after an aerosol challenge.

1.9.3 VACCINE TESTING IN IMMUNE-COMPETENT MICE

Both BCG and H37Rv purine and leucine auxotrophs have been tested in animal models (11, 45, 49. After iv infection, the BCG purine and leucine mutants were eliminated almost immediately after infection while the H37Rv mutant numbers were dramatically reduced during the first week post-infection. Thereafter the bacilli were gradually eliminated until it became undetectable.

1.9.4 VACCINE TESTING IN IMMUNE-DEFICIENT MICE

Severe combined immune-deficient (SCID) mice have been infected with methionine-, proline- and tryptophan-deficient H37Rv and organ bacterial counts determined (111). Initially, the methionine mutants had a delay in growth but later the growth rate increased to be similar to the WT. The proline mutant also initially had a significant delay in growth but after day 20, there was a gradual loss of control in the lung. Only mice infected with the tryptophan mutant had prolonged survival and this mutant could therefore be labelled as avirulent. Since mice infected with proline and tryptophan mutants had prolonged survival, these mutants were used to immunise SCID mice. The mice were then challenged iv with WT H37Rv. Both mutants caused a reduction in tissue burden 2 months after challenge. The proline and tryptophan mutants were able to protect against challenge with virulent MTB at levels equivalent to or greater than those for BCG vaccination.

1.10 OBJECTIVES OF STUDY

It has been demonstrated that IL-4 plays a minor role in the control of BCG or MTB infection (28, 80). It has previously shown that the TH1 response is enhanced in the absence of IL-10, resulting in an accelerated elimination of BCG (50). In this study it was asked whether the combined absence of IL-4 and IL-13, as compared to IL-4 deficiency, would increase resistance to MTB infection.

Previous studies have shown the importance of IFNγ in the control of mycobacterial infections (19, 25, 34, 52, 53). In this study, IFNγR$^{-/-}$ mice were used to determine the growth kinetics of purine-deficient H37Rv. Firstly, IFNγR$^{-/-}$ mice were infected via aerosol with 100 CFU (colony forming units) purine-deficient H37Rv and WT H37Rv. Secondly, WT mice were immunised with either saline, purine-deficient H37Rv or WT H37Rv. Four weeks later, these mice were challenged with WT H37Rv via aerosol. Thus, the protective efficacy of purine-deficient BCG was determined.

2 MATERIALS AND METHODS

2.1 MICE

IL-4$^{-/-}$ mice and IL-4Rα$^{-/-}$ mice on a BALB/c background were obtained from Prof. Brombacher, Immunology Department, University of Cape Town (75, 79). IFNγR-deficient (IFNγR$^{-/-}$) mice, obtained from Prof Zinkernagel, University of Zurich, Switzerland, and their WT-129 control (WT) were used for the experiments involving mutant mycobacteria (52, 53). Mice were maintained under specific pathogen-free conditions at the University of Cape Town Animal Unit. All mice used in experiments were between 8-12 weeks old. All infected mice were housed in filter-top cages in a biohazard level 3 facility and received food, water and bedding, as needed.

2.2 GENOTYPING OF MICE

2.2.1 DNA EXTRACTION

The gene-status of all the IL-4$^{-/-}$ mice and IL-4Rα$^{-/-}$ mice (breeding stock and litters) were verified by the polymerase chain reaction (PCR) analysis of the DNA from tail biopsies. PCR machine used was a Peltier Thermal Cycler, model PTC-200 from MJ Research. A portion of the mouse tail (1cm) was removed under anaesthesia and incubated in 500µl lysis buffer overnight (O/N) at 56°C with rotation. The resulting solution was centrifuged at 10000rpm for 10 minutes. The supernatant was added to 0.5 volumes of isopropanol and the precipitated DNA was removed with a sterile hooked 150mm pasteur pipette. Pipettes were sterilised by heating the tips to form a hook. The DNA was dried at room temperature and resuspended in 500µl water. For the PCR reaction, 2µl of this DNA solution was used.

2.2.2 PCR ANALYSIS

All primers were designed by Anita Schwegmann, Department of Immunology, University of Cape Town and manufactured by the Department of Medical Biochemistry, University of Cape Town. The primer sequences and expected PCR products are outlined in Tables 2 and 3. The PCR reaction mix and thermocycling conditions are detailed in Tables 4 and 5.

Primers for PCR screening of IL-4$^{-/-}$ mice			
P1	5'	GTG AGC AGA TGA CAT GGG GC	3'
P2	5'	CTT CAA GCA TGG AGT TTT CCC	3'
Pneo	5'	GCG CAT CGC CTT CTA TCG CCT TC	3'
Primers for PCR screening of IL-4Rα$^{-/-}$ mice			
Wild-type allele			
Exon 7 forward	5'	TGA CCT ACA AGG AAC CCA GGC	3'
Exon 8 reverse	5'	CTC GGC GCA CTG ACC CAT CT	3'
Deleted allele			
IL-4R E10 reverse	5'	ACC TGT GCA TCC TGA ATG AT	3'
IL-4R intron 6-8	5'	CCC TTC CTG GCC CTG AAT TT	3'

Table 2. Primer sequences for genotyping of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice.

Expected PCR products (in base pairs)			
	+/+	+/-	-/-
IL-4 P1/ IL-4 P2	180	180	-
IL-4 P1/ IL-4 Pneo	-	1208	1208
IL-4Rα wild-type allele	800	800	-
IL-4Rα deleted allele	-	1300	1300

Table 3. Expected PCR products for genotyping of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice.

PCR reaction mix (µl)

	IL-4	IL-4Rα
DNA sample	2	2
Primer 1 (6.25µM)	2	2
Primer 2 (6.25µM)	2	2
dNTP (2.5mM)	4	4
PCR buffer (10X)	5	5
Taq (5U/µl)	0.05	0.05
Water	34.95	34.95
Total volume	50.00	50.00

Table 4. PCR reaction mix for genotyping of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice. Taq Supertherm DNA polymerase (JMR-801) used was purchased from Southern Cross Biotechnology

Thermocycling conditions

	Temperature (°C)	duration	number of cycles
IL-4	94	2 minutes	1
	94	20 seconds	
	56	20 seconds	5
	72	2.5 minutes	
	94	20 seconds	
	56	20 seconds	31
	72	2 minutes	
	72	3 minutes	
	4	hold	
IL-4Rα	94	1 minutes	1
	94	20 seconds	
	56	20 seconds	40
	72	1 minutes	
	72	3 minutes	
	4	hold	

Table 5. Thermocycling conditions for genotyping of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice.

After completion of the PCR, 50µl of the amplified product was mixed with 10µl of 6xloading dye. The mixture (30µl per well) was electrophoresed on a 1.6% agarose gel (D1 low electrosmosis, Whitehead Scientific) containing 10mg/ml ethidium bromide and using 0.5xTBE as the running buffer. A bacterial DNA marker was included in the gel to determine the band sizes (for preparation of the bacterial DNA marker, see appendix C). The gel was electrophoresed for 1 hour at 150 volts after which they were photographed under ultraviolet light.

2.3 MYCOBACTERIA

Mycobacterium tuberculosis H37Rv (H37Rv) was obtained from Prof. G Kaplan, Rockefeller University, New York. The purine mutant of *Mycobacterium bovis* BCG ($purC^{-/-}$ BCG) and its WT parent strain (WT BCG), and the purine mutant of *Mycobacterium tuberculosis* H37Rv ($purC^{-/-}$ H37Rv) and its WT parent strain (WT H37Rv) were obtained from Prof. B Gicquel, Pasteur Institute, France (48, 49). The mutant mycobacteria were generated as previously described (48, 92). A small amount of the mycobacteria was removed from a Lowenstein-Jensen slant using a sterile disposable plastic inoculating loop and inoculated into a sterile 50ml screw-capped tissue culture flask containing Middlebrook 7H9 broth supplemented with 10% OADC enrichment medium (South African State Vaccine Institute) and 1% glycerol. For the mutant strains, 20µg/ml kanamycin and 20µg/ml hypoxanthine were also added. This starter culture was grown to mid-log phase in a 37°C incubator in the presence of 5% CO_2. One aliquot was used to grow up a working stock of mycobacteria. An aliquot of the working stock was passed through a 26-gauge needle 20 times to unclump the bacteria. Ten-fold dilutions were made in Tween/saline (0.04% Tween/ 0.9% saline) and plated on Middlebrook 7H10 agar supplemented with 10% OADC and 0.5% glycerol (20µg/ml kanamycin and 20µg/ml hypoxanthine were included for the mutant strains). All

dilutions are plated in duplicate onto 90mm two-compartment bacteriological grade petri dishes (502V, Sterilab). Plates were incubated at 37°C for 2-3 weeks to determine the H37Rv concentration. Colonies were counted using a dissecting microscope (Wild, Heerbrugg). Aliquots were thawed and diluted as required.

2.4 AEROSOL INFECTION

The aerosol procedure was carried out under strict biohazard level 3 guidelines. The aerosol procedure and inoculating dose were prepared as instructed by the manufacturers of the Inhalation Exposure System (Model 099C A4224, Glas-Col) to deposit 10, $1x10^2$ CFU (colony forming units) and $3.5x10^2$ CFU, respectively, into the lungs of mice. To deliver $1x10^2$ CFU into the lungs of mice, the mycobacterial suspension prepared needs to be at $2x10^6$ CFU/ml in a total volume of 5ml sterile saline ($1x10^7$ CFU total). Therefore the suspension prepared for use needs to have 5 $\log_{10}$ CFU higher than the concentration one wants to deliver into the lungs of mice. Thus, to obtain 10, $1x10^2$ CFU and $3.5x10^2$ CFU per lung, 1 x 10^6 CFU, $1x10^7$ CFU and $3.5x10^7$ CFU respectively, were used. The infection cycle used was as follows: preheat time = 15 minutes, nebulisation time = 40 minutes, cloud decay time = 40 minutes, decontamination time = 15 minutes. The preheating cycle allows for the incinerator to reach a working temperature of 790°C. All exhaust air will pass through the incinerator before being released. During the nebulisation cycle, compressed air is passed through the bacterial suspension to form droplet nuclei. During the cloud decay cycle, air is drawn into the chamber to disperse the aerosol, and the UV lights switch on during the decontamination cycle to sterilise the top of the basket.

In 3 separate experiments, IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice were infected via the aerosol route with 10, 100 and 350 CFU respectively. Groups consisted of 4 mice per time point and the experiments were repeated two times. In one experiment, 6 $IFN\gamma R^{-/-}$ and 6 WT mice were infected with 100 CFU/lung of WT H37Rv and in another experiment, they were infected with 100 CFU $purC^{-/-}$ H37Rv, respectively.

2.5 IMMUNISATION PROCEDURE

Fifteen WT mice were anaesthetised with chloroform and infected iv via the lateral tail vein, with $1x10^6$ CFU $purC^{-/-}$ BCG. A second group of 15 mice were injected in the same way with WT BCG. A control group of 4 mice were injected with sterile saline. At 4 weeks post-infection, 4 mice from each infection group were sacrificed to determine bacterial load in the various organs. The remaining mice from each group were challenged via the aerosol route with 100 CFU per lung WT H37Rv. Ten control mice were included in the challenge and sacrificed at day 1 post-infection to determine the inoculating dose. At week 8, 4 mice from each group were sacrificed to determine whether the inoculation with $purC^{-/-}$ BCG provided protection against WT H37Rv.

2.6 DAY 1 CFU DETERMINATION

Ten control mice were sacrificed at day 1 after exposure to confirm the dosage inhaled into the lungs. Lungs were removed, weighed and homogenised, using glass tissue homogenisers (Lasec), in 2ml Tween/saline. Homogenised samples were plated onto Middlebrook 7H10 agar supplemented with 10% OADC and 0.5% glycerol, incubated at 37°C for 2-3 weeks after which the colonies were counted. For the immunisation study, liver and spleen samples were included. For the mutant strains, 20μg/ml kanamycin and 20μg/ml hypoxanthine were also added.

2.7 CLINICAL SIGNS

Mice were observed for clinical signs (posture, condition of fur) and weighed weekly, using a Mettler Toledo balance (PB 303), as reduction in body weight was considered an indicator of disease progression.

2.8 BACTERIAL BURDEN

At weeks 4, 8, 12 and 24 post-infection 3-4 mice per group were anaesthetised using diethyl ether, and sacrificed. Samples of lungs, liver and spleen were removed, weighed and homogenised in 2ml Tween/saline and plated onto Middlebrook 7H10 agar supplemented with 10% OADC and 0.5% glycerol. For the mutant strains, 20μg/ml kanamycin and 20μg/ml hypoxanthine were also added. Samples were plated in duplicate at various dilutions. Colonies were counted after 2-3 weeks incubation at 37°C.

2.9 HISTOLOGY

Tissues were fixed O/N in 20-30 volumes of 4% phosphate-buffered formalin pH 7.4. Fixed tissues were dehydrated O/N by an automated tissue processor (TP-1020, Leica) before paraffin wax embedding (embedding station, EG 1140H, Leica). The following cycles were used: 1 x 70% ethanol for 2 hours, 2 x 96% ethanol for 2 hours, 4 x 100% ethanol for 2 hours and 2 x xylene for 2 hours. Samples were embedded in paraffin wax (Histosec pastilles, Merck) and left to solidify. Once set, 2μm thick sections were cut on a rotary microtome (RM-2125, Leica). Sections were fixed onto glass slides O/N at 65°C, and stored at room temperature. Before staining, slides were dewaxed at 65°C O/N.

2.9.1 HAEMATOXYLIN AND EOSIN STAIN (HE)

After dewaxing paraffin wax-embedded sections O/N at 65°C, sections were rehydrated for 1 x 3 minutes in xylene, 2 x 1 minute in xylene, 2 x 1 minute in 100% ethanol, 2 x 1 minute in

96% ethanol, 2 x 1 minute in 70% ethanol and finally 1 minute in running tap water (22). Sections were left in haematoxylin for 8 minutes, then washed in running water for 1 minute. Excess stain was removed by rinsing in 1% acid alcohol for 10 seconds. Blue colour was regained in water for 30 minutes. Sections were then placed in 1% eosin for 2 minutes, rinsed in running water and dehydrated through alcohols. Sections were cleared in xylene (Merck) and mounted in Entellen (Merck).

2.9.2 ZIEHL-NEELSON STAIN (ZN)

Paraffin wax-embedded sections were dewaxed and rehydrated as before. Slides were flooded with 6% carbol fuchsin (Merck) solution and flamed until the steam rises (23). Sections were left to cool for 5 minutes before the process was repeated. The carbol fuchsin should then be fixed to the sections. After the slides have cooled, they were rinsed in running water. Excess carbol fuchsin was removed by rinsing sections in 1% acid alcohol, followed by running water. Sections were then left to decolourise in 25% H_2SO_4 for 20 minutes and washed in running water for at least 10 minutes to remove excess acid. Sections were counterstained in Loefflers methylene blue for 1 minute, rinsed in water and dehydrated through alcohols to xylene. Sections were mounted in Entellen.

2.10 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on paraffin wax-embedded sections using the citrate buffer antigen retrieval method modified from the Dako guide to antigen retrieval (24). Sections were dewaxed O/N at 65°C and then rehydrated for 1 x 3 minutes in xylene, 2 x 1 minute in xylene, 2 x 1 minute in 100% ethanol, 2 x 1 minute in 96% ethanol, 2 x 1 minute in 70% ethanol and finally 1 minute in running tap water. The sections were microwaved (Model DM 129, Defy) at medium heat for 2x5 minutes in citrate buffer pH 6, then left to cool in the

buffer at room temperature for 20 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2/methanol for 30 minutes. Sections were then washed thoroughly in 1xPBS before being incubated in 0.1% avidin for 30 minutes, then 0.01 % biotin for 20 minutes to block endogenous biotin (biotin blocking kit, X0590, Dako). Sections were incubated in 1.5% normal goat serum for 30 minutes and left in primary antibody O/N at 4°C. Rabbit anti-mouse iNOS antibody (from Prof. J Pfeilschifter, University of Frankfurt, Germany) was used at 1:2000 and rabbit anti-mouse nitrotyrosine antibody (cat no. 06-284, Upstate Biotechnology) was used at 1:200. After the primary antibody incubation, sections were washed in PBS and the biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector) was added for 30 minutes. After a wash in PBS, sections were incubated in ABC Vector (PK-6100, Vector) for 30 minutes before addition of the liquid DAB substrate chromogen system (R3455, Dako) for 10 minutes. Sections were washed in running tap water before being incubated in 1% $CuSO_4$ for 2 minutes, running tap water for 2 minutes, haematoxylin for 4 minutes and a final wash in running tap water for 5 minutes. Sections were dehydrated and mounted in Entellen. All incubations were done at room temperature in a humidifying chamber, unless otherwise stated.

2.11 BRONCHOALVEOLAR LAVAGE

After 3 and 6 months post-infection, bronchoalveolar lavage (BAL) was done to assess cytokine production and cellular activation state in the lungs. Mice were given a lethal injection of Xylazine/Ketamine. The trachea was exposed and a 20-gauge cannula (Introcan, Braun) was carefully inserted. Lungs were lavaged twice, using 300µl 1xPBS, and each time the lungs were aspirated 5 times. The BAL fluid was used for cytokine ELISAs. All lavage procedures were carried out in a biohazard level 3 facility and all samples were kept on ice.

The BAL supernatant was centrifuged (5415D, Eppindorf) at 2000rpm for 5 minutes, aliquoted and stored at -70°C.

2.12 LUNG TISSUE HOMOGENISATION

The left lobe of all mice were weighed and homogenised in 0.5ml sterile 1xPBS pH 7.4. The homogenised samples were centrifuged at 10000 rpm for 10 seconds. Supernatants were aliquoted and frozen at –70°C until cytokine analysis using ELISA.

2.13 CYTOKINE ELISA

All ELISA procedures involving H37Rv was performed in a laminar flow hood in a biohazard level 3 isolation unit. Before reading of the ELISA, 50µl/well absolute ethanol was added and the plates were incubated for 30 minutes at room temperature to kill the mycobacteria. Cytokines detected were IL-12, IFNγ, TNF and TGFβ. Antibodies used were obtained from Pharmingen and R&D Systems (Table 6).

ANTIBODY	CAPTURE Purified rat α-mouse	RECOMBINANT MOUSE	DETECTING biotinylated
IL-4	18031D	550067	18042D
IL-5	18051D	19241V	18062D
IL-12	18491 (p40) 20011D (p35/p70) 20171D (p70)	19361V	18482D (p40/p70)
IFNγ clone AN18	18181D	554587	18112D
TNF	18131A	19321T	clone MP6-XT3.11
TGFβ	23201D	555104	23212D

Table 6. Antibodies used for ELISA. All antibodies are from Pharmingen except TGFβ, which is from R&D Systems.

For IL-12, IFNγ and IL-4, 96-well microtiter plates (Maxisorb, Nunc) were coated with 100µl/well rat anti-mouse capture antibody diluted to 2µg/ml in coating buffer. After 2 hour incubation at 37°C or O/N incubation at 4°C, the plates were washed 4 times with 200µl/well washing buffer. Plates were then blocked with 200µl blocking buffer for 1 hour at 37°C or O/N at 4°C. Three-fold dilutions of recombinant mouse standards were made in dilution buffer starting at 100ng/ml. Samples (BAL fluid supernatant, lung homogenates) were also diluted in dilution buffer and 50µl/well were added to the plates. Plates were incubated for 3 hours at 37°C or O/N at 4°C. Plates were then washed 4 times with 200µl/well washing buffer. Biotin-labeled rabbit anti-mouse secondary antibody was diluted to 0.5µg/ml in dilution buffer, 50µl/well were added and the plate was incubated for at least 3 hours at 37°C or O/N at 4°C. Streptavidin alkaline phosphatase (cat no. 13043E, Pharmingen) was diluted 1:1000 in dilution buffer and 50µl/well were added. Plates were incubated for 1 hour at 37°C, and then washed 4 times with 200µl/well washing buffer. The substrate p-nitrophenylphosphate (PNPP) (cat no. 738 352, Boehringer Mannheim) was diluted to 1mg/ml in substrate buffer. After addition of 50µl/well, the reaction was read on an ELISA reader (Versamax, Molecular Devices) at 405nm, with a reference wavelength of 492nm. The reaction was stopped by the addition of 50µl of 1M NaOH.

For TNF, 96-well microtiter plates (Maxisorb, Nunc) were coated with 50µl/well rat anti-mouse TNF capture antibody diluted to 10µg/ml in coating buffer. After O/N incubation at 4°C, the plates were washed 4 times with 200µl/well washing buffer. Plates were then blocked with 200µl blocking buffer for 1 hour at 37°C or O/N at 4°C. Three-fold dilutions of recombinant mouse TNF standard were made in dilution buffer starting at 100ng/ml. Samples were also diluted in dilution buffer and 50µl/well were added to the plates. Plates were

incubated for 3 hours at 37°C or O/N at 4°C. Plates were then washed 4 times with 200µl/well washing buffer. Biotin-labeled rabbit anti-mouse TNF secondary antibody was diluted to 2µg/ml in dilution buffer, 50µl/well were added and the plate was incubated for at least 3 hours at 37°C or O/N at 4°C. Before the addition of 50µl/well goat anti-rabbit IgG-AP (cat no. 12153E, Pharmingen) (diluted 1:10000 in dilution buffer) and 1 hour incubation at 37°C, the plate was first washed 4 times with 200µl/well washing buffer. After the addition of goat anti-rabbit IgG-AP, the plate was washed 4 times with 200µl/well washing buffer. The substrate PNPP was diluted in substrate buffer to a concentration of 1mg/ml and 50µl was added per well. 50µl of 1M NaOH was added to each well to stop the reaction, and the microtiter plate was read on an ELISA reader at 405nm.

3 RESULTS

3.1 RESULTS OF IL-4/IL-13 STUDY

3.1.1 GENOTYPING OF MICE

All mice were genotyped to confirm the genetic complement of the mice. PCR analysis of IL-$4^{-/-}$ mice indicated that these mice only contain the 1208bp band (Figure 10A, lanes 1-6), whereas WT mice only contain the 180bp band (lane 8). The heterozygous control (lane 7) did not work but should contain both the 1208bp and 180bp band. The negative control was negative (lane 9).

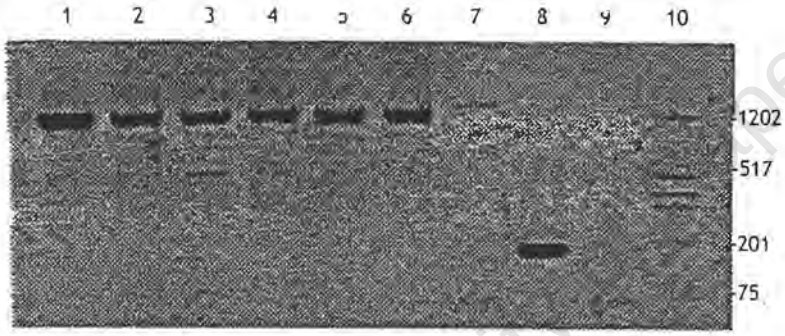


Figure 10A. Genotyping of IL-$4^{-/-}$ mice using IL-4 primers. Lanes 1-6 are IL-$4^{-/-}$ digests. Lane 7 is the IL-$4^{-/-}$ positive control. Lane 8 is the WT positive control and lane 9 is the negative control. Lane 10 is the bacterial DNA marker.

IL-4R$\alpha^{-/-}$ mice were genotyped with both the WT alleles and the deleted alleles (Table 2). IL-4R$\alpha^{-/-}$ mice should be negative for the WT allele of 800bp and should contain the deleted alleles of 1300bp (Figures 10B and 10C).

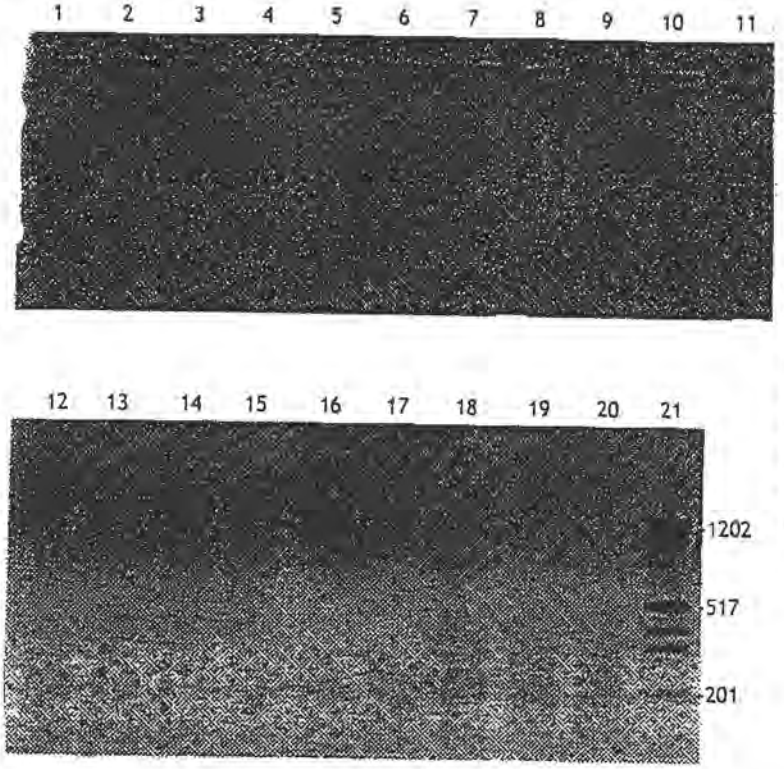


Figure 10B. Genotyping of IL-4Rα$^{-/-}$ mice using deleted allele primers. Lanes 1-16 are IL-4Rα$^{-/-}$ mice digests. Lane 17 is the IL-4Rα$^{-/-}$ positive control. Lane 18 is the WT control. Lane 19 is the heterozygous control and Lane 20 is the negative control. Lanes 11 and 21 are the bacterial DNA markers.

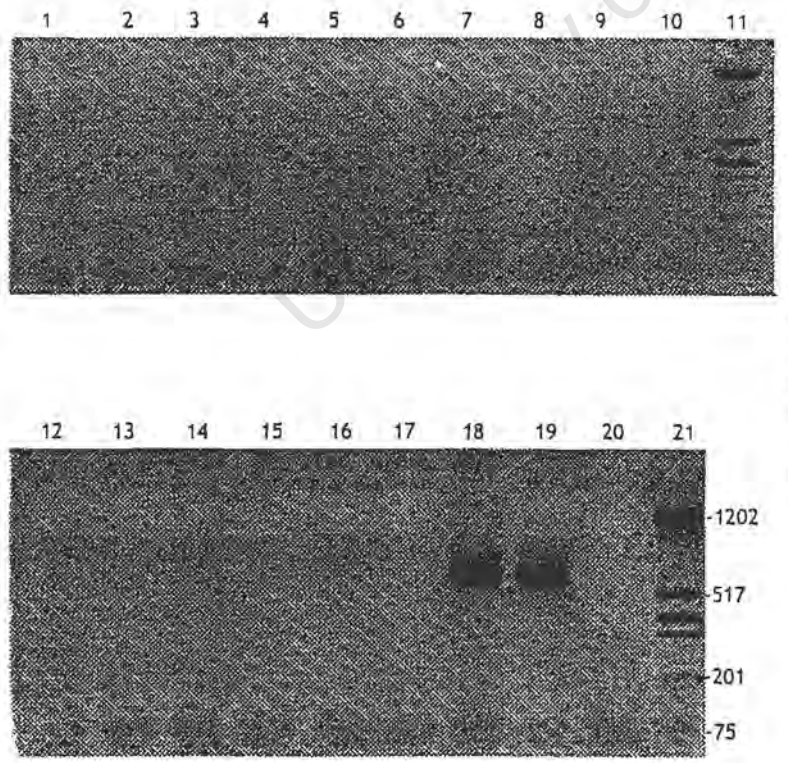


Figure 10C. Genotyping of IL-4Rα$^{-/-}$ mice using WT primers. Lanes 1-16 are IL-4Rα$^{-/-}$ mice digests. Lane 17 is the IL-4Rα$^{-/-}$ positive control. Lane 18 is the WT control. Lane 19 is the heterozygous control and Lane 20 is the negative control. Lanes 11 and 21 are the bacterial DNA markers.

3.1.2 CLINICAL SIGNS

To determine the susceptibility of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice to an aerosol infection, ten WT mice between the ages of 8-12 weeks were infected with either 10, 100 or 350 CFU/lung of H37Rv, monitored regularly and weighed weekly. The same experiment was repeated using IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice. Mice infected with 10 CFU displayed no clinical or pathological signs of infection after week 2 therefore this part of the experiment was terminated (data not shown). No changes in weights were observed between the three strains after a normal dose of infection (100 CFU/lung) (data not shown). Weights remained stable for more than six months and no deaths were observed. After the high dose infection (350 CFU/lung), all the WT mice had succumbed to infection by week 20 (Figure 11). Only one IL-4$^{-/-}$ mouse survived up to week 21. In contrast, 30% of IL-4Rα$^{-/-}$ mice survived up to week 24, but the study was terminated shortly afterwards due to the morbidity of the remaining mice. The difference in the mortality of the three strains after a high dose of H37Rv indicates that there may be slight differences in their immune response. Elimination of both IL-4 and IL-13 could have contributed to the slightly longer survival time of the IL-4Rα$^{-/-}$ mice.

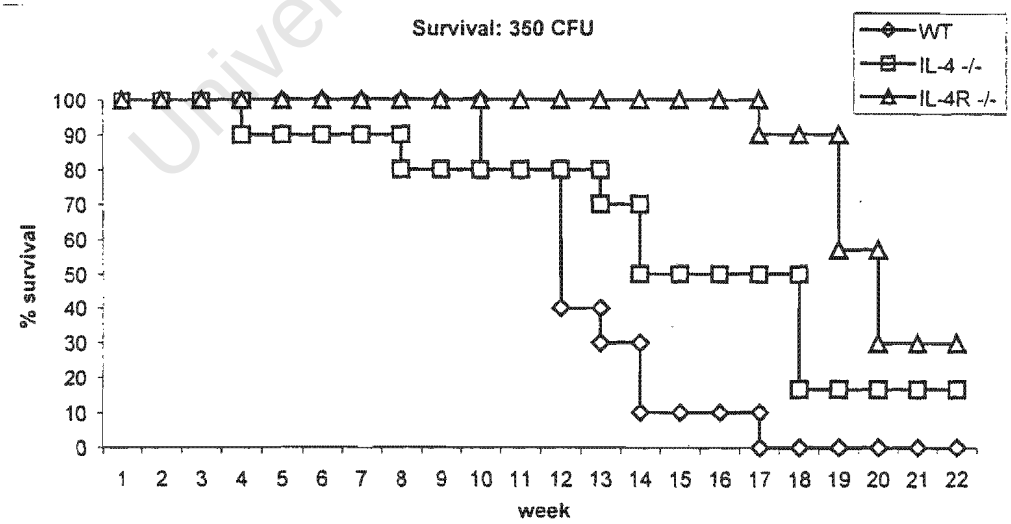
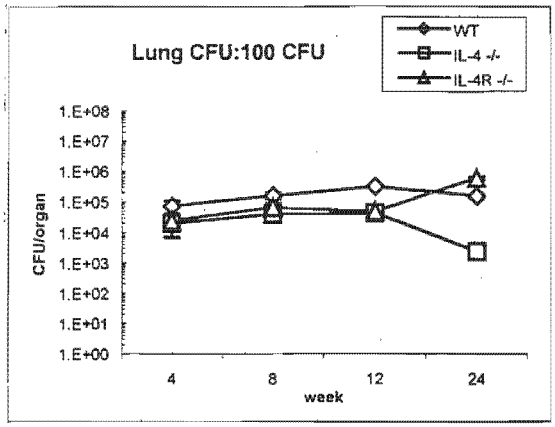
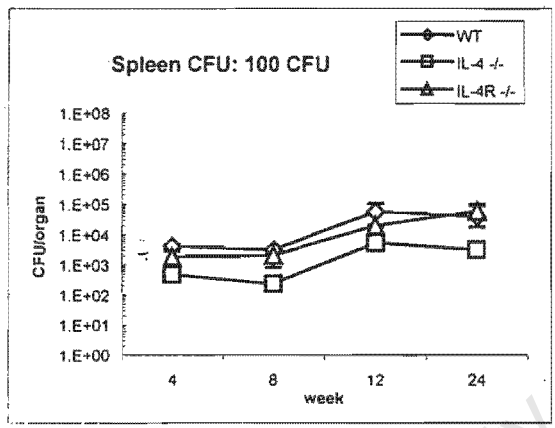


Figure 11. Mortality study of WT, IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice infected with 350 CFU *Mycobacterium tuberculosis* H37Rv, via the aerosol route. Each group contained 4 mice and the experiment was repeated twice.

3.1.3 BACTERIAL BURDEN

To investigate progression of disease with respect to the mycobacterial load, lungs, spleen and liver were removed and bacterial burden was determined by the count of bacterial colonies (CFU per organ). For all experiments, the day 1 CFU determination confirmed that there was an initial dose of 10 CFU, 100 CFU and 350 CFU, respectively. After an initial dose of 100 CFU, WT lung CFU remained slightly higher than the knockout strains (Figure 12A). Lung CFU from the knockout strains remained similar until week 24. At this point, IL-$4^{-/-}$ mice appeared to control the infection better than IL-4R$\alpha^{-/-}$ mice. Spleen CFU of all the strains increased between weeks 8-12. WT liver CFU was undetectable between weeks 6 and 8 and no bacteria could be detected in the livers of IL-$4^{-/-}$ mice at week 8. Mycobacteria could be detected for both strains at week 12. IL-4R$\alpha^{-/-}$ liver CFU was detected at week 24 after having no bacteria between weeks 8 and 12. Despite the slight changes in bacterial burden between the strains, this had no impact on mortality. Twenty-four weeks after a high dose infection, IL-4R$\alpha^{-/-}$ mice had decreased CFU in the lung and at extrapulmonary sites such as liver and spleen (Figure 12B). Since IL-4R$\alpha^{-/-}$ mice but not IL-$4^{-/-}$ mice showed increased elimination of mycobacteria at week 24, the data suggests that endogenous IL-13 in chronic infection may reduce cell-mediated immunity.





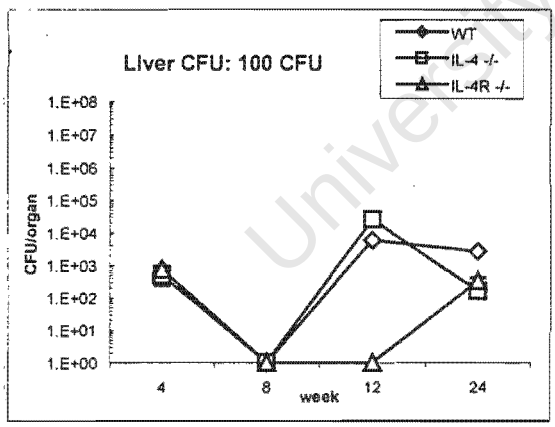
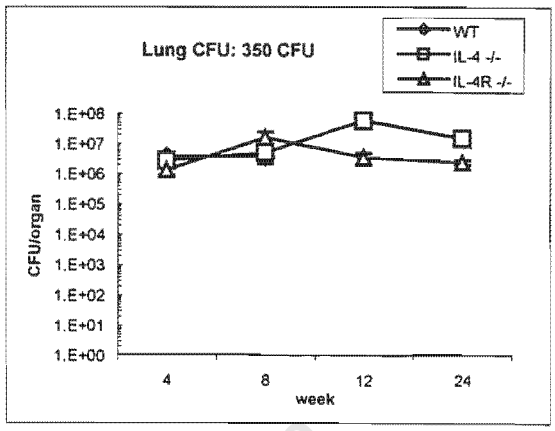
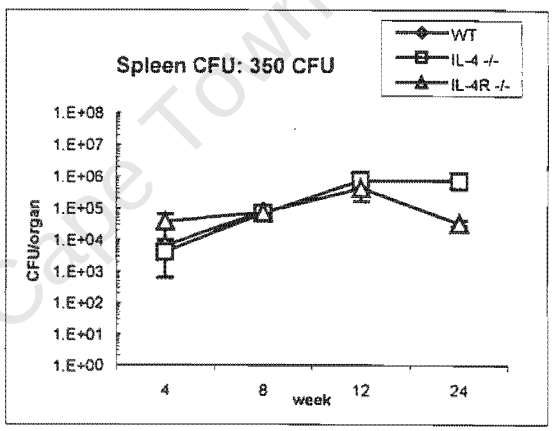


Figure 12A. Mycobacterial burden in organs of mice infected with 100 CFU *Mycobacterium tuberculosis* H37Rv. The bacterial burden in lung, spleen and liver (CFU) was determined 4, 8, 12 and 24 weeks after aerosol infection. The results are expressed as the mean +/- SD from 4 mice and the data are representative of 2 independent experiments.





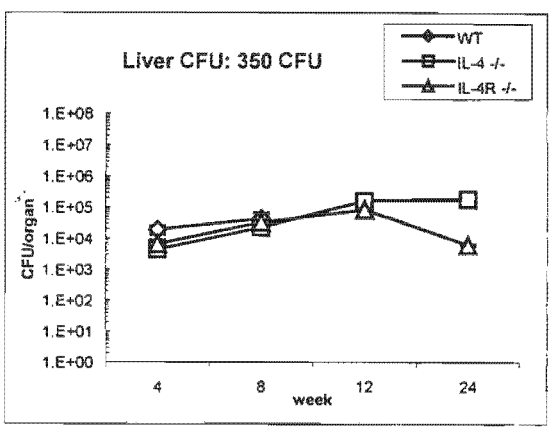


Figure 12B. Mycobacterial burden in organs of mice infected with 350 CFU *Mycobacterium tuberculosis* H37Rv. The bacterial burden in lung, spleen and liver (CFU) was determined 4, 8, 12 and 24 weeks after aerosol infection. The results are expressed as the mean +/- SD from 4 mice and the data are representative of 2 independent experiments.

3.1.4 HISTOLOGY

3.1.4a HAEMATOXYLIN AND EOSIN STAIN

Histology was performed on 2 non-serial sections per mouse, 4 mice per group. The haematoxylin and eosin stain shows morphological alterations that may have occurred in the organs due to infection. All three strains exhibited extensive granulomatous structures regardless of the infective dose. With a dose of 100 CFU, there were little differences in the morphology at week 4 and week 8 although IL-4Rα$^{-/-}$ mice had slightly more cellular infiltration than the other strains (data not shown). At week 12 post-infection, the histological differences between the three strains were still minimal and at week 24 post-infection, IL-4Rα$^{-/-}$ lungs had slightly larger granulomas than the other strains (Figure 13A). At week 24, the histology for the high dose showed that the lungs from IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice had a granular appearance, increased weight and reduced alveolar air space. (Figure 13B). IL-4Rα$^{-/-}$ mice had slightly larger lung granulomas than IL-4$^{-/-}$ mice. We further quantified the granuloma response in lungs and livers and demonstrated that IL-4Rα$^{-/-}$ mice had the most prominent cellular response in the lungs whereas IL-4$^{-/-}$ mice had more liver granulomas (Figure 14). These results indicate that IL-4Rα$^{-/-}$ mice had a slightly stronger granuloma response in the lung, which resulted in less spread of bacilli to extrapulmonary sites such as the liver. The final outcome to the increased resistance is reduced mortality.

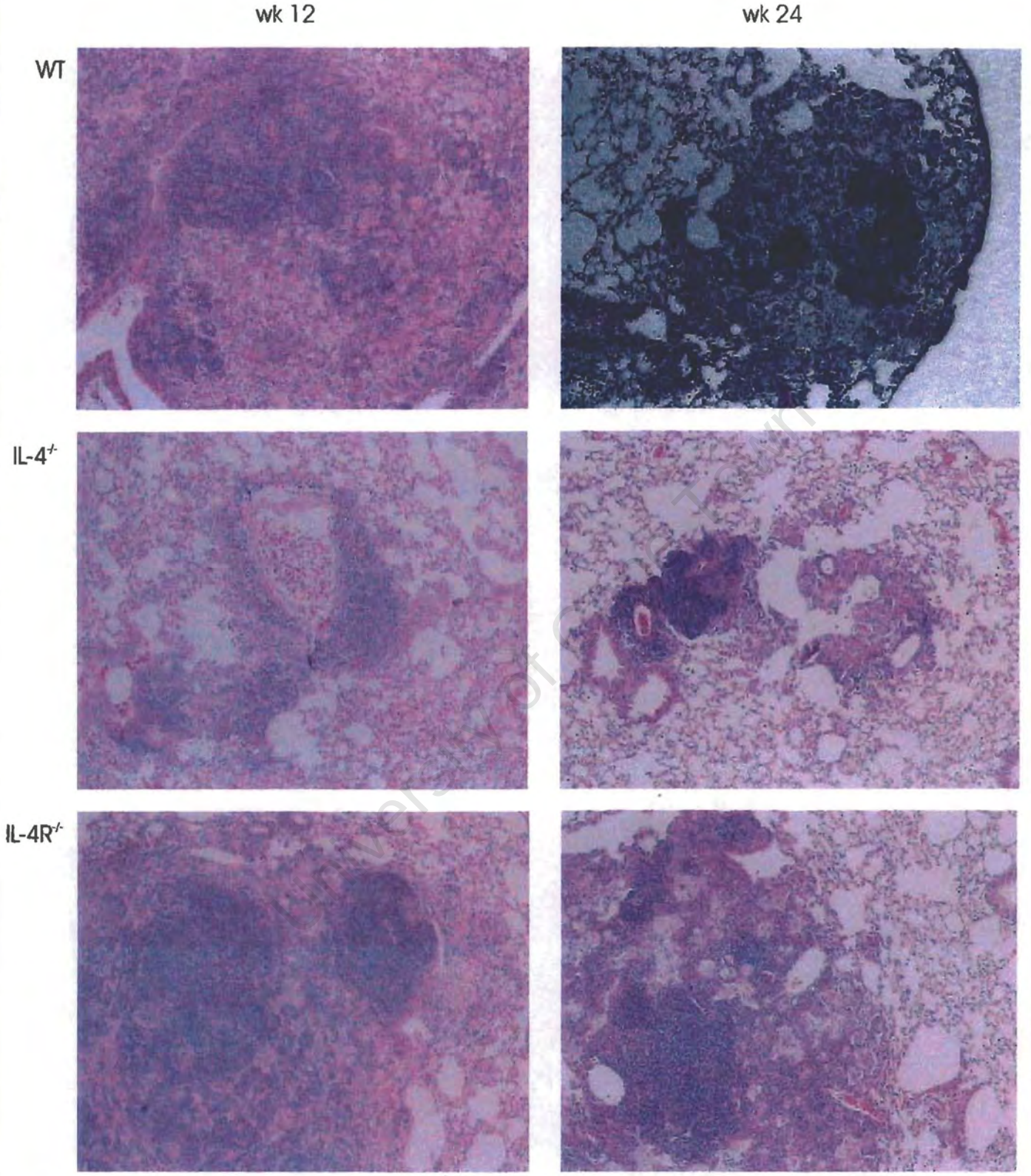


Figure 13A. Haematoxylin and eosin representations of lung sections from *Mycobacterium tuberculosis* H37Rv-infected mice sacrificed 12 and 24 weeks after infection with 100 CFU. Magnification 40x.

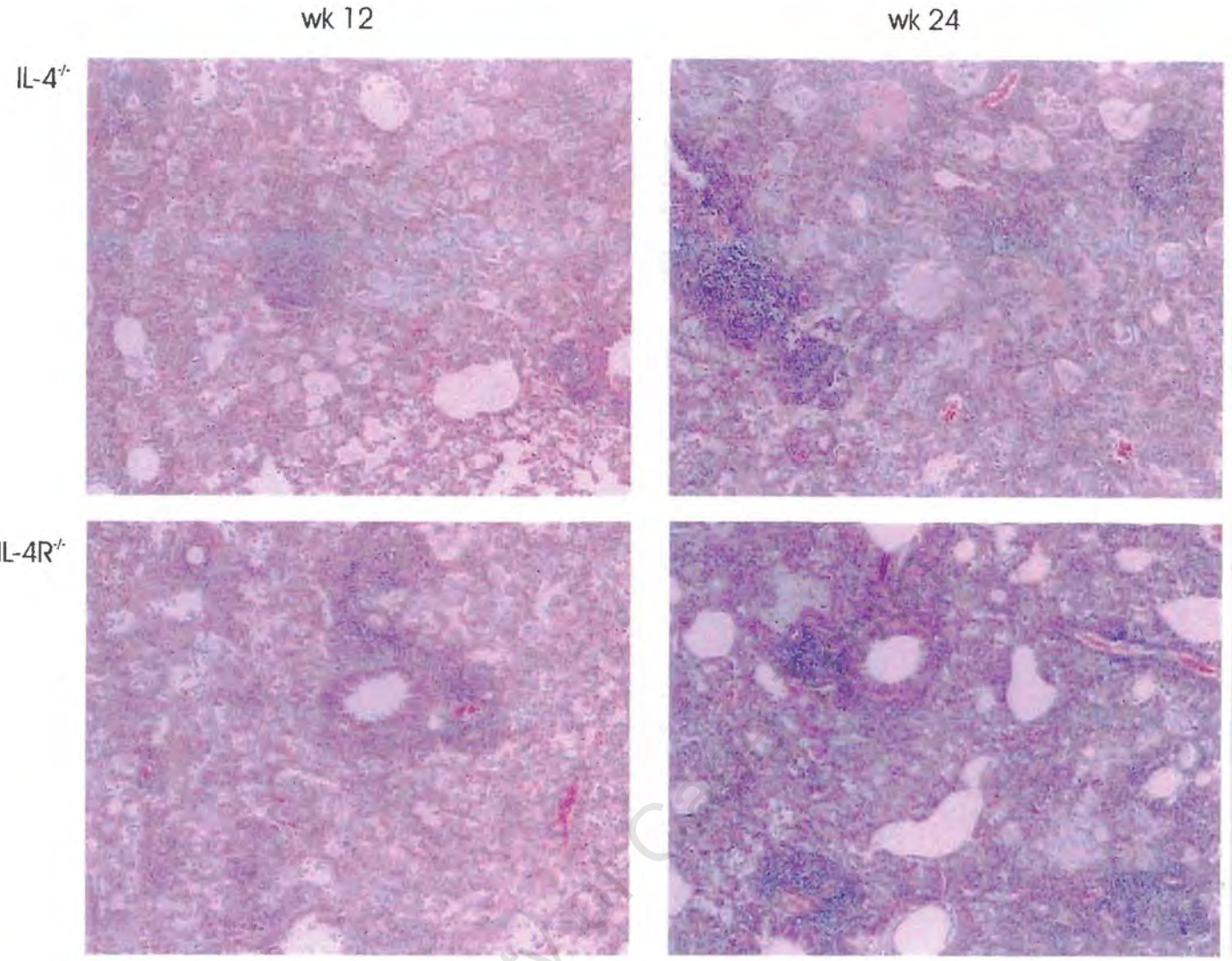


Figure 13B. Haematoxylin and eosin representations of lung sections from *Mycobacterium tuberculosis* H37Rv-infected IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice sacrificed 12 and 24 weeks after infection with 350 CFU. Magnificaton 40x.

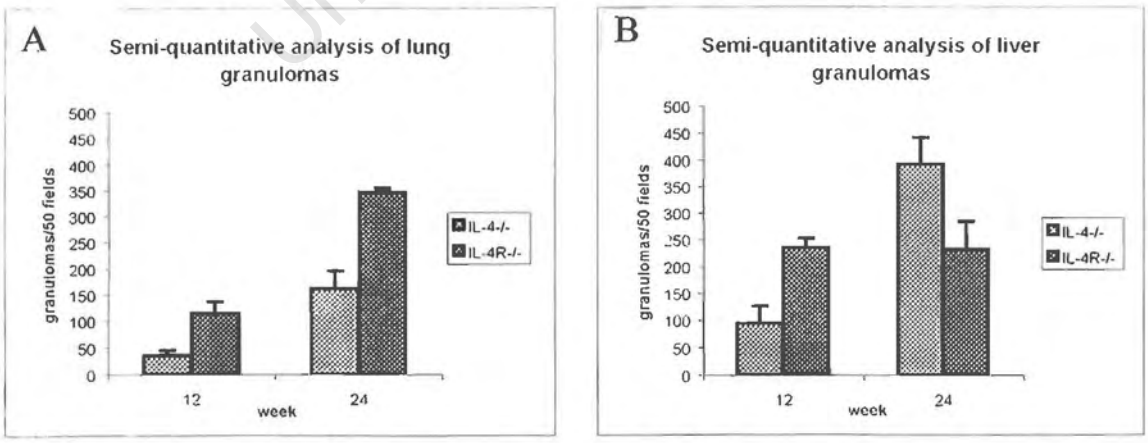


Figure 14. Semi-quantitative analysis of granulomas 12 and 24 weeks after a 350 CFU infection. Mononuclear cell clusters of more than 15 cells were counted as granulomas. The results are expressed as the mean +/- SD from 4 mice. A lung granulomas. B liver granulomas.

3.1.4b ZIEHL-NEELSON STAIN

To determine the number of acid-fast bacilli (AFB) present in the organs, tissue sections were stained with Ziehl-Neelson. Twelve and 24 weeks after a normal dose infection, organs of WT, IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ organs showed similar low levels of AFB (Figure 15A). After a high dose infection, there were more bacilli at week 12 and week 24 in the lungs of IL-$4^{-/-}$ than IL-$4R\alpha^{-/-}$ lungs (Figure 15B). These results correlate with the HE stain, showing that an increased granuloma response contributed to a reduction in AFB.

3.1.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on paraffin sections for the presence of iNOS and nitrotyrosine. iNOS detects inducible nitric oxide synthase activity and the nitrotyrosine stain detects the iNOS reaction end-product. After a normal dose, strong lung iNOS activity was detected in all strains of mice at week 12 and week 24 (Figure 16A). After a high dose infection, strong iNOS activity was detected in all lungs at both week 12 and week 24 but was most prominent in IL-$4R\alpha^{-/-}$ lungs (Figure 16B). iNOS was also detected in spleen and liver. Nitrotyrosine was highest in IL-$4R\alpha^{-/-}$ lungs at week 24 after a high dose (Figure 16C). These data indicate that during high dose infections, endogenous IL-13 inhibits macrophage activation in chronic infection.

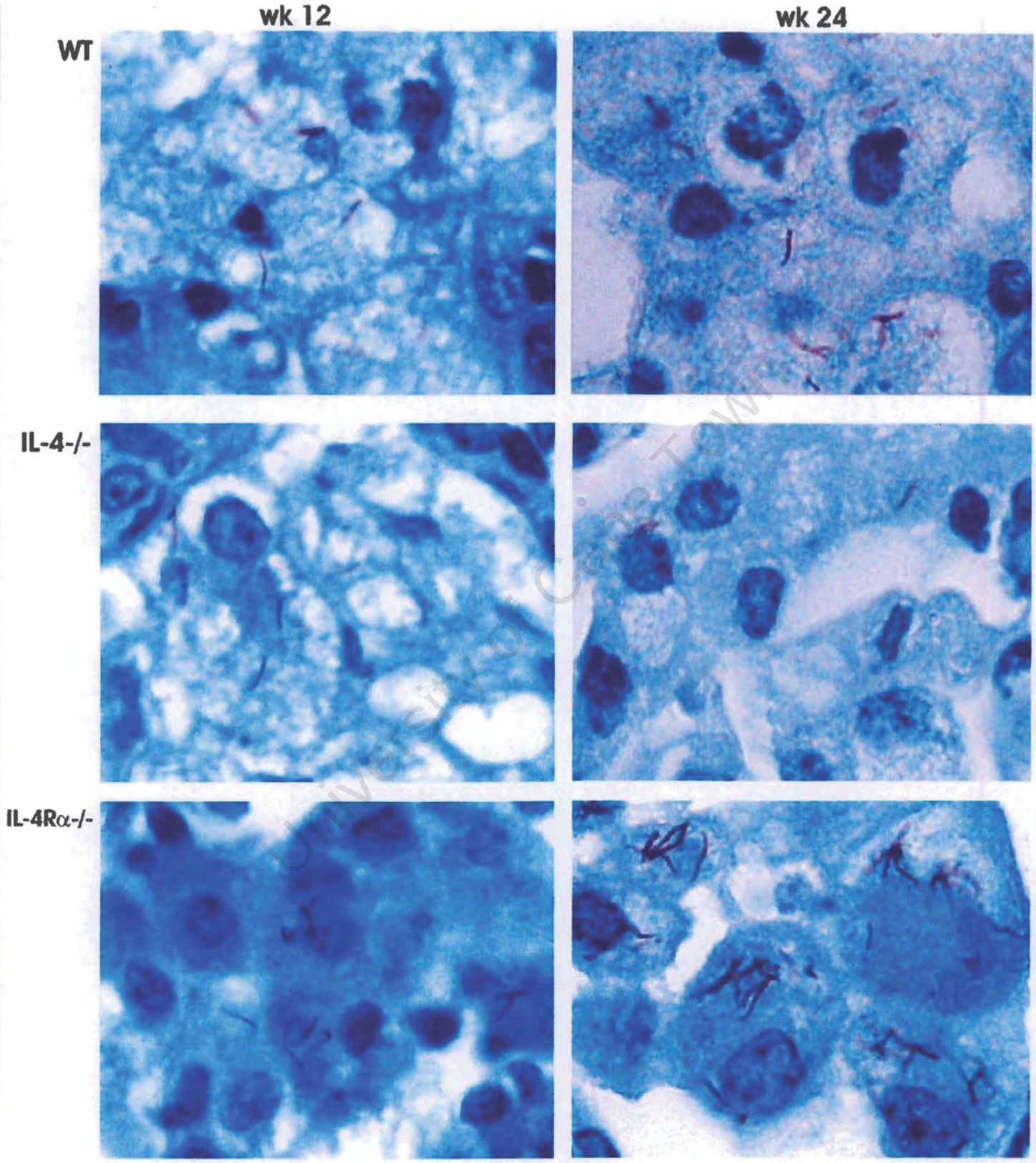


Figure 15A. Lung Ziehl-Neelson staining of sections from mice 12 and 24 weeks after *Mycobacterium tuberculosis* H37Rv infection with 100 CFU. Magnification 1000x.

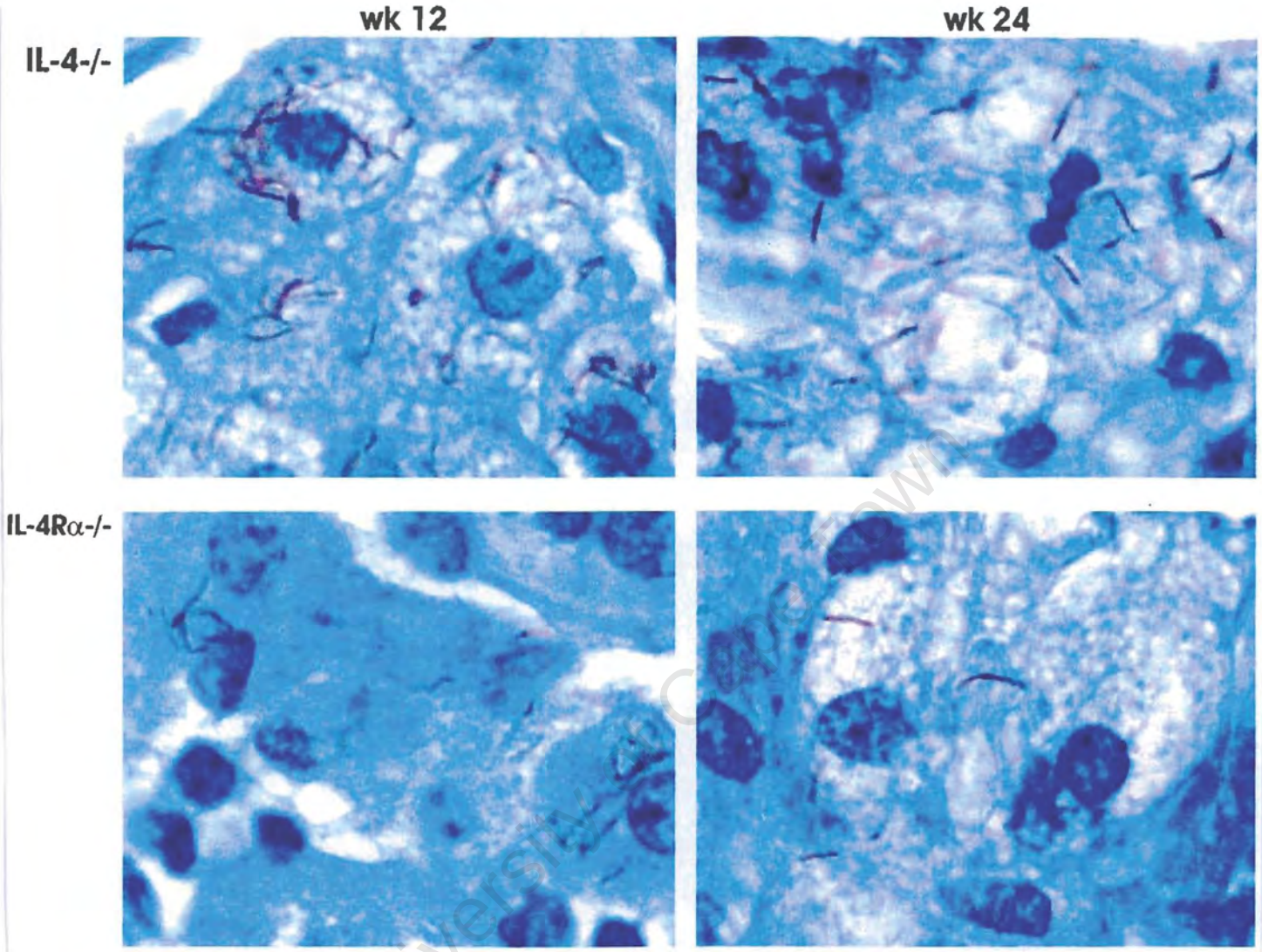


Figure 15B. Lung Ziehl-Neelson staining of sections from *Mycobacterium tuberculosis* H37Rv-infected IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice sacrificed 12 and 24 weeks after infection with 350 CFU. Magnification 1000x.

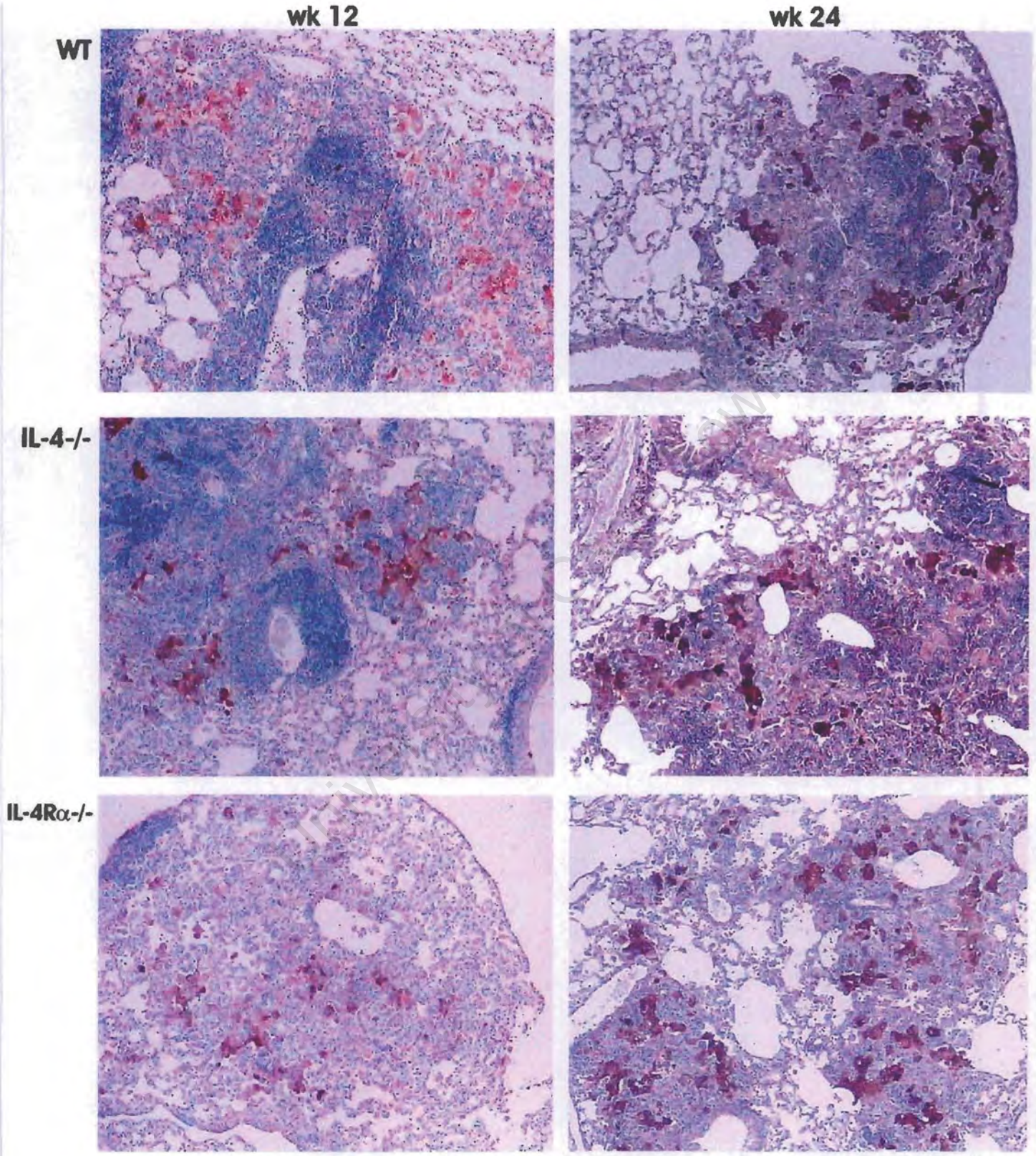


Figure 16A iNOS staining of lung sections from *Mycobacterium tuberculosis* H37Rv-infected mice sacrificed 12 and 24 weeks after infection with 100 CFU. Magnification 40x.

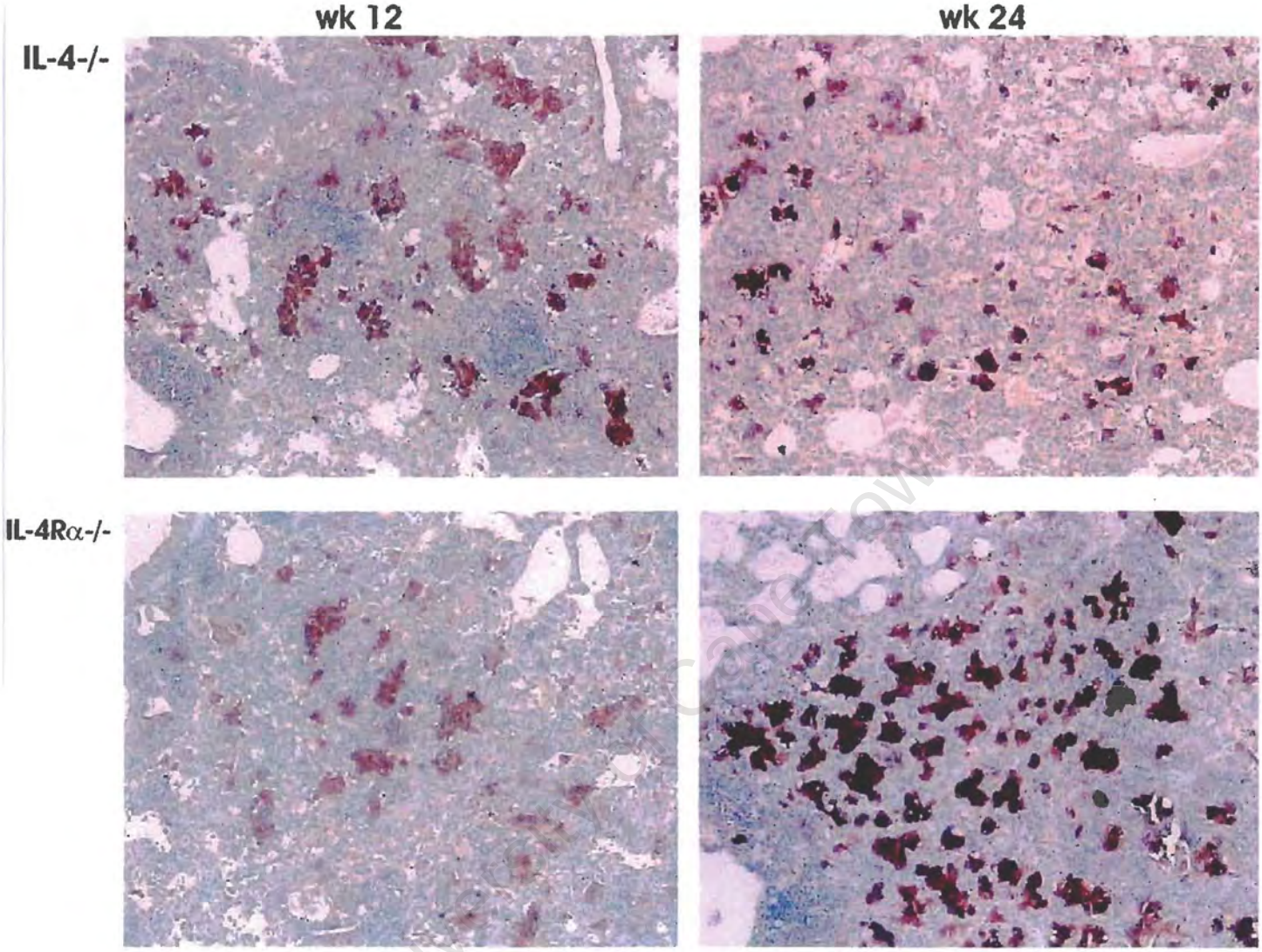


Figure 16B. iNOS staining of lung sections from *Mycobacterium tuberculosis* H37Rv-infected IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice sacrificed 12 and 24 weeks after infection with 350 CFU. Magnification 40x.

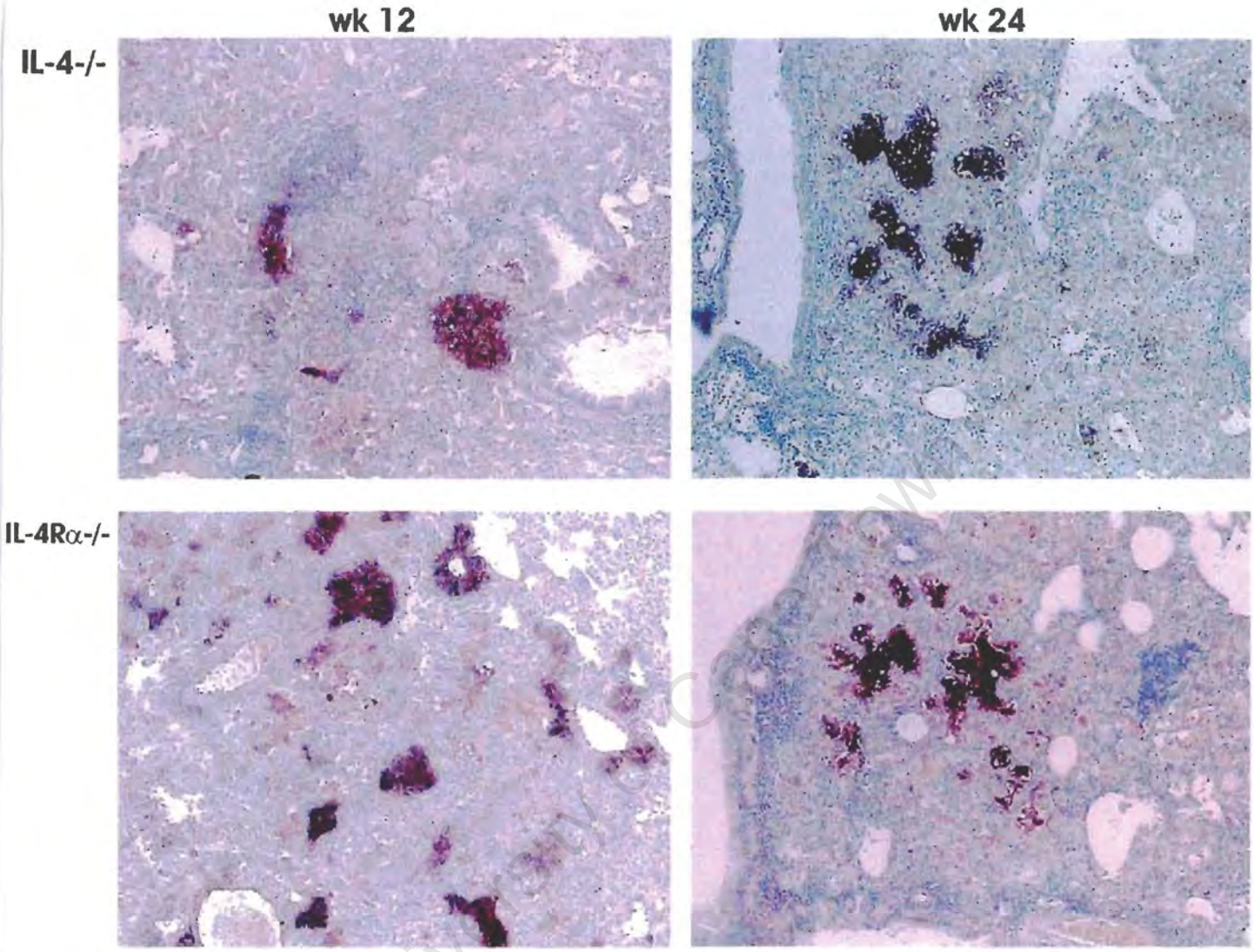


Figure 16C. Nitrotyrosine staining of lung sections from *Mycobacterium tuberculosis* H37Rv-infected IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice sacrificed 12 and 24 weeks after infection with 350 CFU. Magnification 40x.

3.1.6 CYTOKINE ELISA

Cytokine and chemokine levels in lung homogenates and BAL fluid were assessed to determine whether the increased response to H37Rv infection in IL-$4R\alpha^{-/-}$ mice could be due to better or increased recruitment or activation of cells. Cytokine/chemokine levels in BAL fluid were minimal, regardless of the infective dose. Levels of cytokines/chemokines in lung homogenates after a normal dose was significantly lower than that obtained after a high dose

infection. Comparing IL-12 levels 24 weeks after a high dose infection revealed that IL-4Rα$^{-/-}$ mice had more IL-12 than IL-4$^{-/-}$ mice (Figure 17A). TNF levels in IL-4Rα$^{-/-}$ mice were markedly reduced ($p < 0.001$) compared to IL-4$^{-/-}$ mice (Figure 17B). IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice had similar high levels of IFNγ and TGFβ (Figure 17C and 17D respectively). Chronic TB infection is associated with increased TH2 cytokines when compared to individuals who control the disease (64). It has been speculated that this failure is due to the suppression of the protective TH1 response by TH2 cytokines. During the absence of IL-13, the IL-4Rα$^{-/-}$ mice have increased IL-12 levels and this could be due to the absence of suppression by IL-13. The increased IL-12 levels also could account for the increased survival of the IL-4Rα$^{-/-}$ mice.

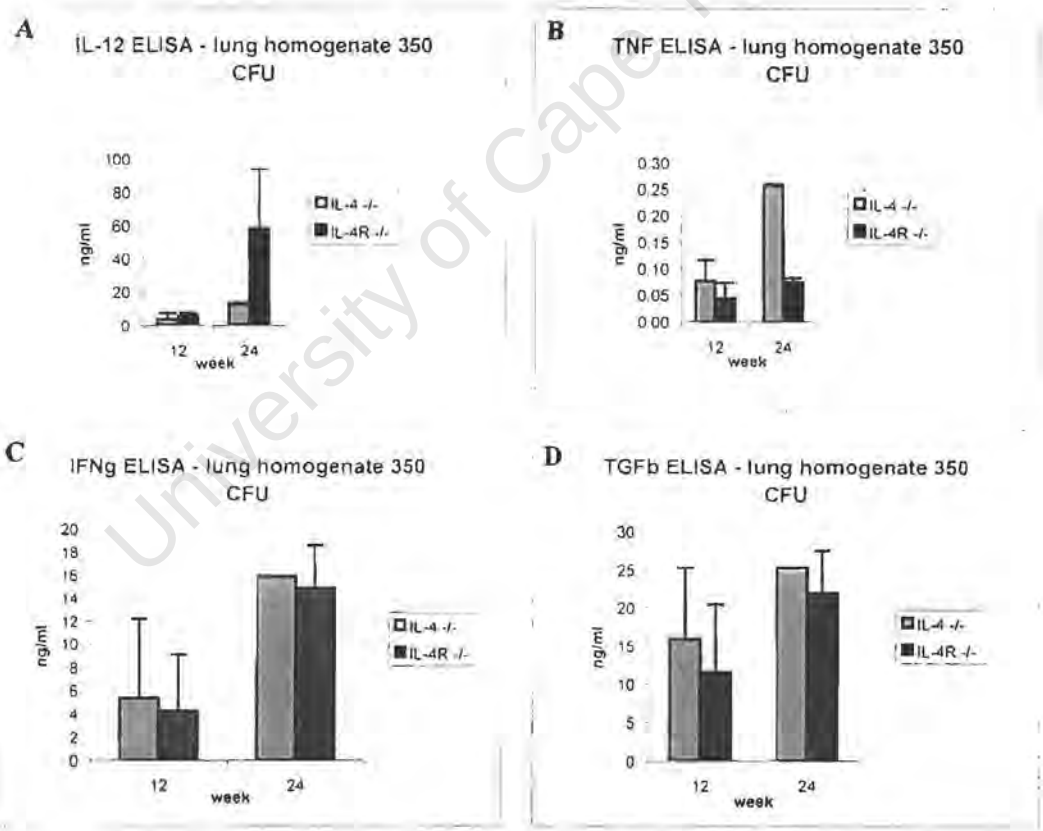


Figure 17. Cytokine and chemokine secretion in the supernatant from lung homogenates of IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice at weeks 12 and 24 after a high dose infection. The results are expressed as the mean +/- SD from 4 mice.

3.2 RESULTS OF THE PROTECTIVE EFFICACY STUDY

3.2.1 MORTALITY

Since purC mutant mycobacteria reportedly are attenuated, we asked whether this mutant has reduced virulence in an immune-suppressed host. Therefore, IFNγR$^{-/-}$ mice were infected with WT and purC$^{-/-}$ H37Rv respectively, by aerosol (100 CFU per lung). The immune-deficient IFNγR$^{-/-}$ mice infected with WT H37Rv started to lose body weight within 2 weeks and succumbed to infection between week 4 and 6, whereas 80% the WT mice survived (Figure 18). IFNγR$^{-/-}$ mice infected with the attenuated purC strain of H37Rv rapidly lost weight within 5 weeks, and all mice succumbed by week 7. WT mice survived after infection with purC$^{-/-}$ H37Rv.

In the vaccination study, there were no deaths and no reduction in body weight of any of the mice, regardless of the vaccination given (data not shown).

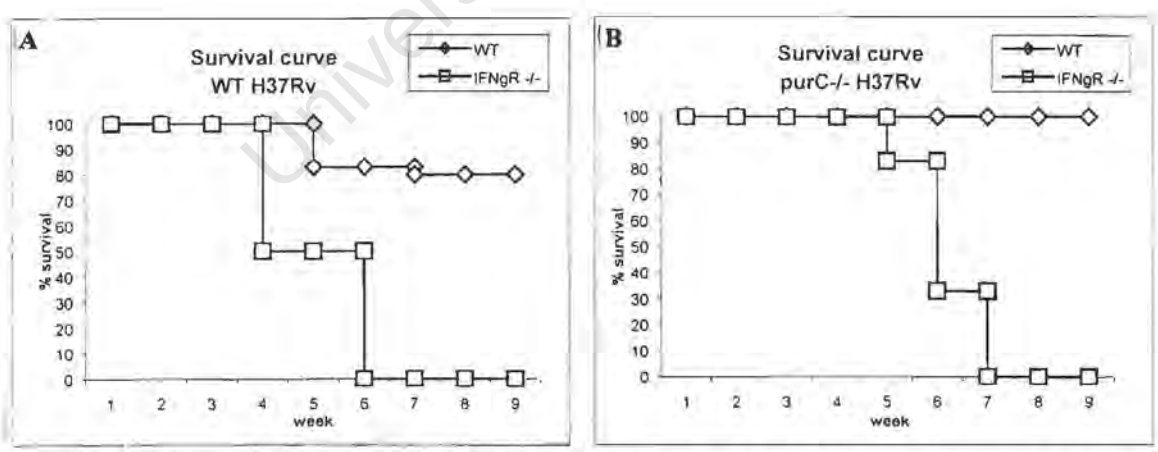


Figure 18. Mortality study of WT and IFNγR$^{-/-}$ mice infected with 100 CFU *Mycobacterium tuberculosis* H37Rv, via the aerosol route. Each group contained 4 mice. A Infection with WT H37Rv. B Infection with purC$^{-/-}$ H37Rv.

3.2.2 CFU DETERMINATION

Organs of infected mice were assessed to determine the mycobacterial growth. As expected, the WT H37Rv grew more prolifically in all the organs than the purC$^{-/-}$ H37Rv (Figure 19A). This gives an indication that there is a reduction in virulence after deletion of the purine gene. In WT mice, WT H37Rv CFU had been reduced at week 8. The purC$^{-/-}$ H37Rv CFU had an apparent increase at week 8 that could be due to the delayed growth kinetics of this strain. At week 4, more WT H37Rv and purC$^{-/-}$ H37Rv were recovered from the organs of IFNγR$^{-/-}$ mice compared to WT mice. The IFNγR$^{-/-}$ mice died shortly before week 8, which is consistent with the higher bacterial load. Therefore, even though the purC$^{-/-}$ H37Rv had initial reduced growth, this did not affect its ability to cause morbidity and eventually, mortality in IFNγR$^{-/-}$ mice.

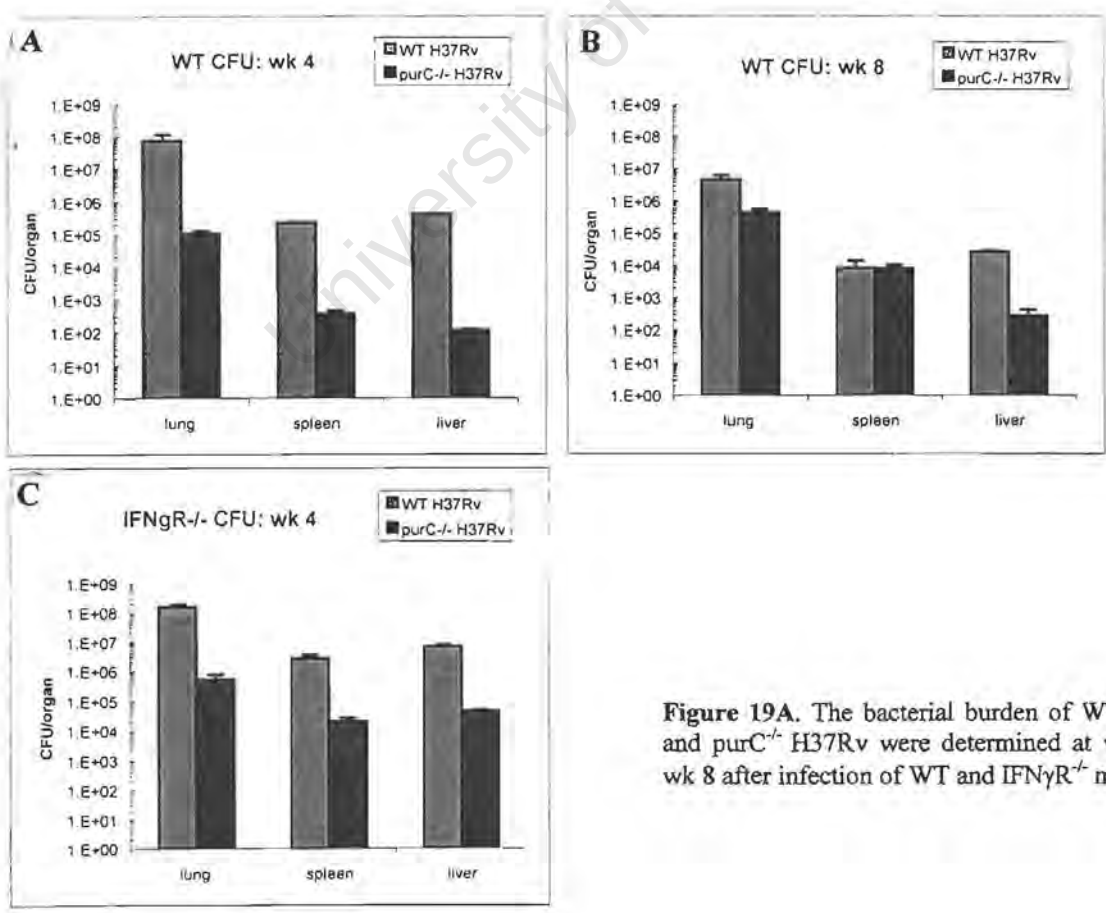


Figure 19A. The bacterial burden of WT H37Rv and purC$^{-/-}$ H37Rv were determined at wk 4 and wk 8 after infection of WT and IFNγR$^{-/-}$ mice.

Four weeks after vaccination, bacteria could be recovered from all the organs of mice vaccinated with WT BCG. Mice vaccinated with purC$^{-/-}$ BCG also had some bacteria in the organs but this was significantly lower than the WT BCG detected (Figure 19B). Four weeks after vaccination, the mice were exposed to WT H37Rv. This infection was continued for a further four weeks (week 8 after initial infection), after which the numbers of WT H37Rv were assessed (Figure 19C). Mice vaccinated with purC$^{-/-}$ BCG and saline had similar numbers of H37Rv in the lungs. This was slightly higher than that obtained for mice vaccinated with WT BCG. The spleen and liver CFU of mice infected with purC$^{-/-}$ BCG was similar but slightly higher than that obtained from mice vaccinated with WT BCG. The results obtained with purC$^{-/-}$ BCG vaccination are similar to that obtained with unvaccinated controls and indicates that WT BCG vaccination provides some protection from subsequent H37Rv infection.

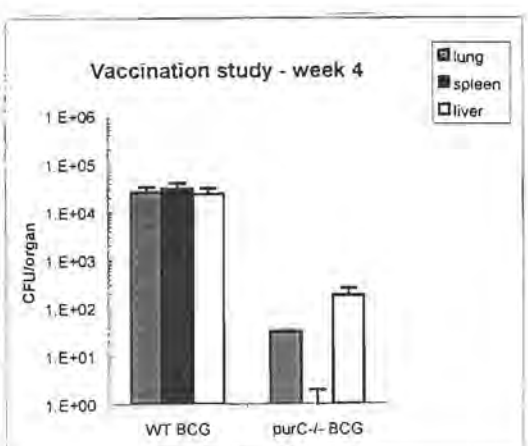


Figure 19B. WT or purC$^{-/-}$ BCG recovered at week 4 from the organs of WT mice vaccinated with either strain.

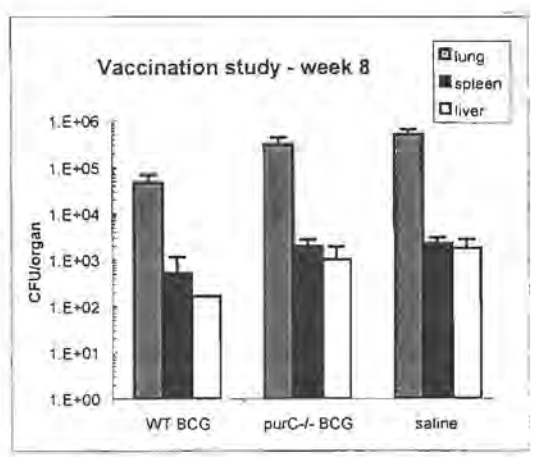


Figure 19C. WT mice were vaccinated with either WT BCG or purC$^{-/-}$ BCG. Four weeks after vaccination, the mice were challenged via aerosol.

3.2.3 HISTOLOGY

3.2.3a Haematoxylin and eosin stain

The inflammatory granuloma response was investigated by light microscopy (Figure 20A). WT H37Rv caused typical lung granulomas consisting of activated macrophages and lymphocytes in WT mice. The lungs of IFNγR$^{-/-}$ mice infected with WT H37Rv exhibited extensive necrosis, with extracellular growth of the mycobacteria. In addition, there were many small granulomas in the liver. WT mice, after purC$^{-/-}$ H37Rv infection, had less pathology than after WT H37Rv infection and displayed an increase in peribronchial cellular infiltration after 8 weeks. PurC$^{-/-}$ H37Rv caused less severe lung lesions in IFNγR$^{-/-}$ mice at week 4, although much of the alveolar space was occupied by mononuclear cellular infiltrates. All mice died shortly after week 4. Thus these observations taken together with the CFU data suggest that purC$^{-/-}$ H37Rv exhibits a reduced virulence but that it is not attenuated enough to be used in immunocompromised mice.

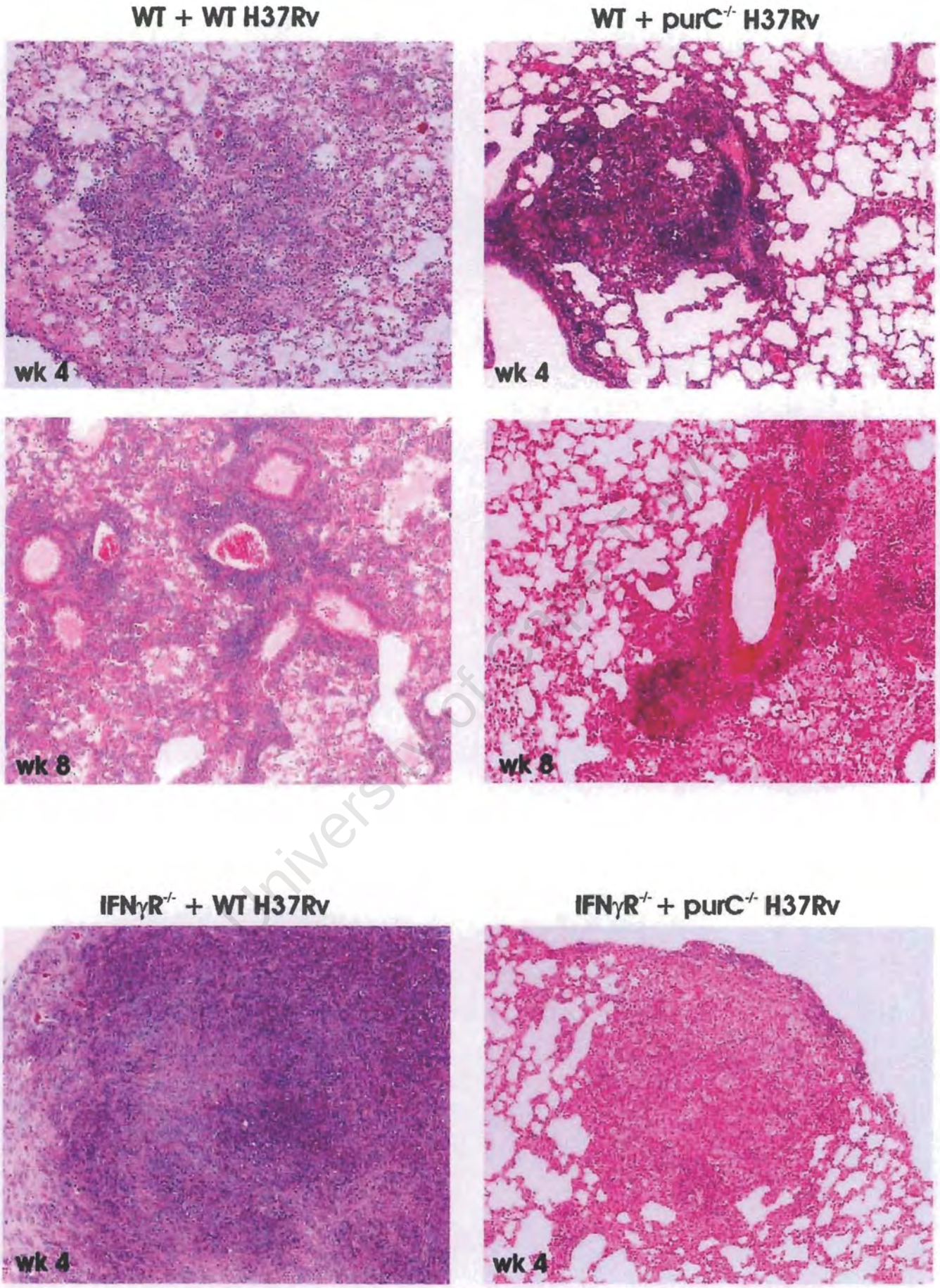


Figure 20A. Representations of lung sections from WT and IFNγR$^{-/-}$ mice 4 and 8 weeks after infection. Sections were stained with haematoxylin and eosin. Magnification 40x.

Fours weeks after vaccination, mice vaccinated with WT BCG had well-developed granulomas in all organs while those vaccinated with purC$^{-/-}$ BCG had much less infiltration and only very small granulomas (Figure 20B). Eight weeks after the WT BCG vaccination (four weeks after challenge with WT H37Rv) the granulomas were smaller than at week 4. Control mice or mice vaccinated with purC$^{-/-}$ BCG had much larger granulomas in all organs (Figure 20C). These results indicate that purC$^{-/-}$ BCG does not provide as good protection against H37Rv challenge as WT BCG does.

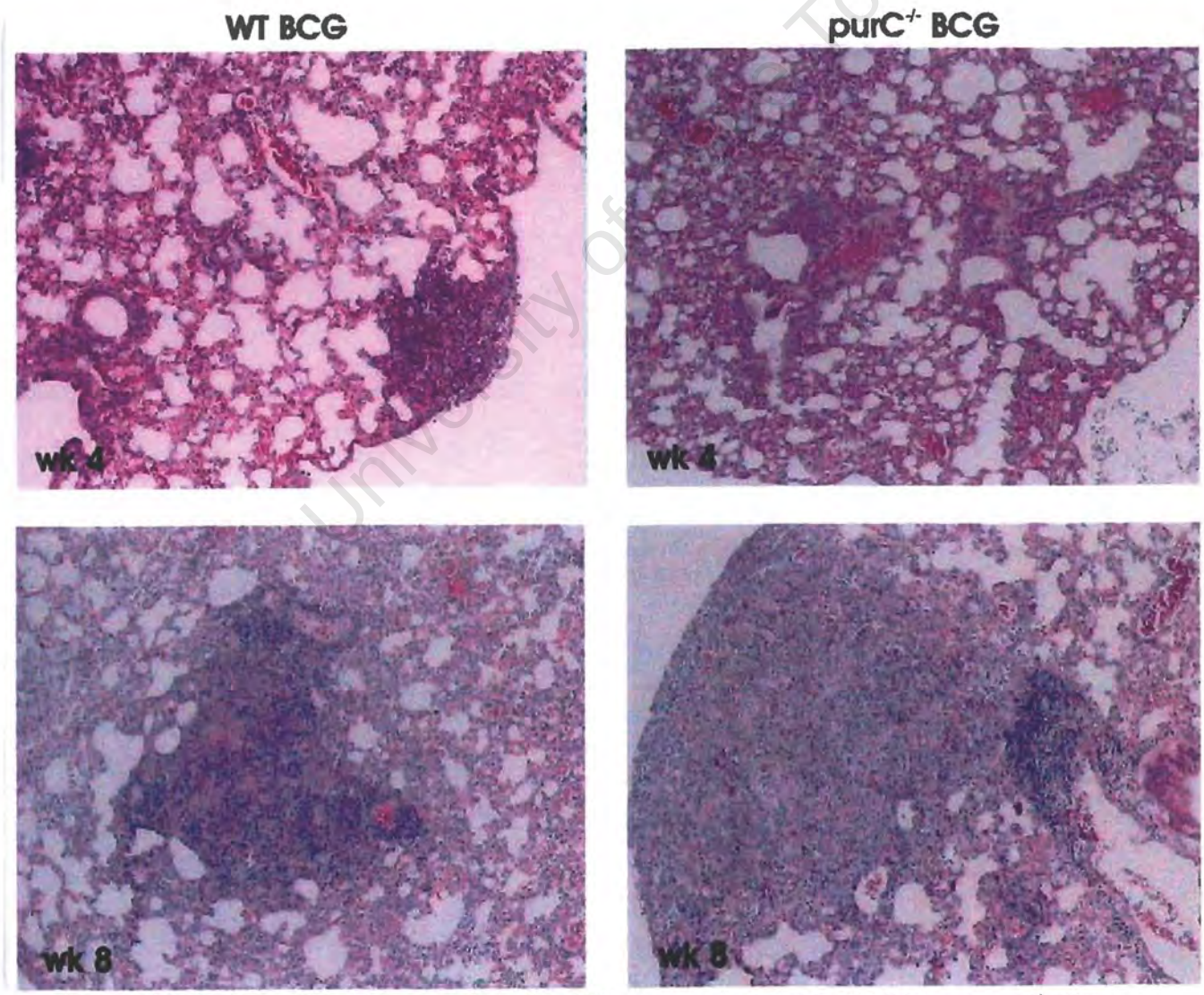


Figure 20B. Representations of lung sections from WT mice vaccinated with WT BCG and purC$^{-/-}$ BCG at 4 and 8 weeks. Sections were stained with haematoxylin and eosin. Magnification 40x.

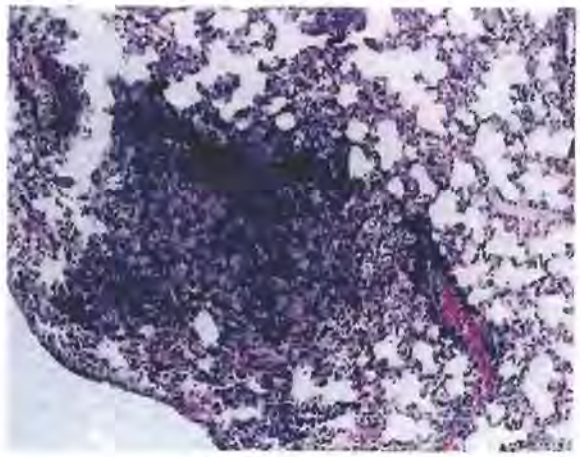


Figure 20C. HE staining of lung sections from saline-vaccinated controls. 4 weeks after WT H37Rv challenge. Magnification 40x.

3.2.3b Ziehl-Neelson stain

The uninhibited growth of mycobacteria was further visualised by the Ziehl-Neelson stain. Growth of mycobacteria in WT lungs decreased between weeks 4 and 8 after a WT H37Rv infection (Figure 21A). Spleen and liver had minimal acid-fast bacilli. Four weeks after infection with WT H37Rv, IFNγR$^{-/-}$ mice had a dramatic increase in acid-fast bacilli in the lungs, with some bacteria having spread to the liver and spleen. In contrast, fewer bacteria were found in lung sections from WT mice infected with purC$^{-/-}$ H37Rv at weeks 4 and 8 when compared to an infection with WT H37Rv. At week 4, IFNγR$^{-/-}$ mice infected with purC$^{-/-}$ H37Rv had more acid-fast bacilli in the lungs compared to WT mice infected with purC$^{-/-}$ H37Rv but less than IFNγR$^{-/-}$ mice infected with WT H37Rv. Therefore the growth of purC$^{-/-}$ H37Rv was reduced in both WT and IFNγR$^{-/-}$ mice.

Fewer acid-fast bacilli were observed in the organs of purC$^{-/-}$ BCG-vaccinated animals than in WT BCG-vaccinated mice (Figure 21B). After being challenged with WT H37Rv, mice vaccinated with purC$^{-/-}$ BCG or saline had more bacilli in their organs than mice vaccinated with WT BCG (Figure 21B and 21C). This indicated that the protective efficacy of purC$^{-/-}$ BCG was not as good as that of WT BCG.

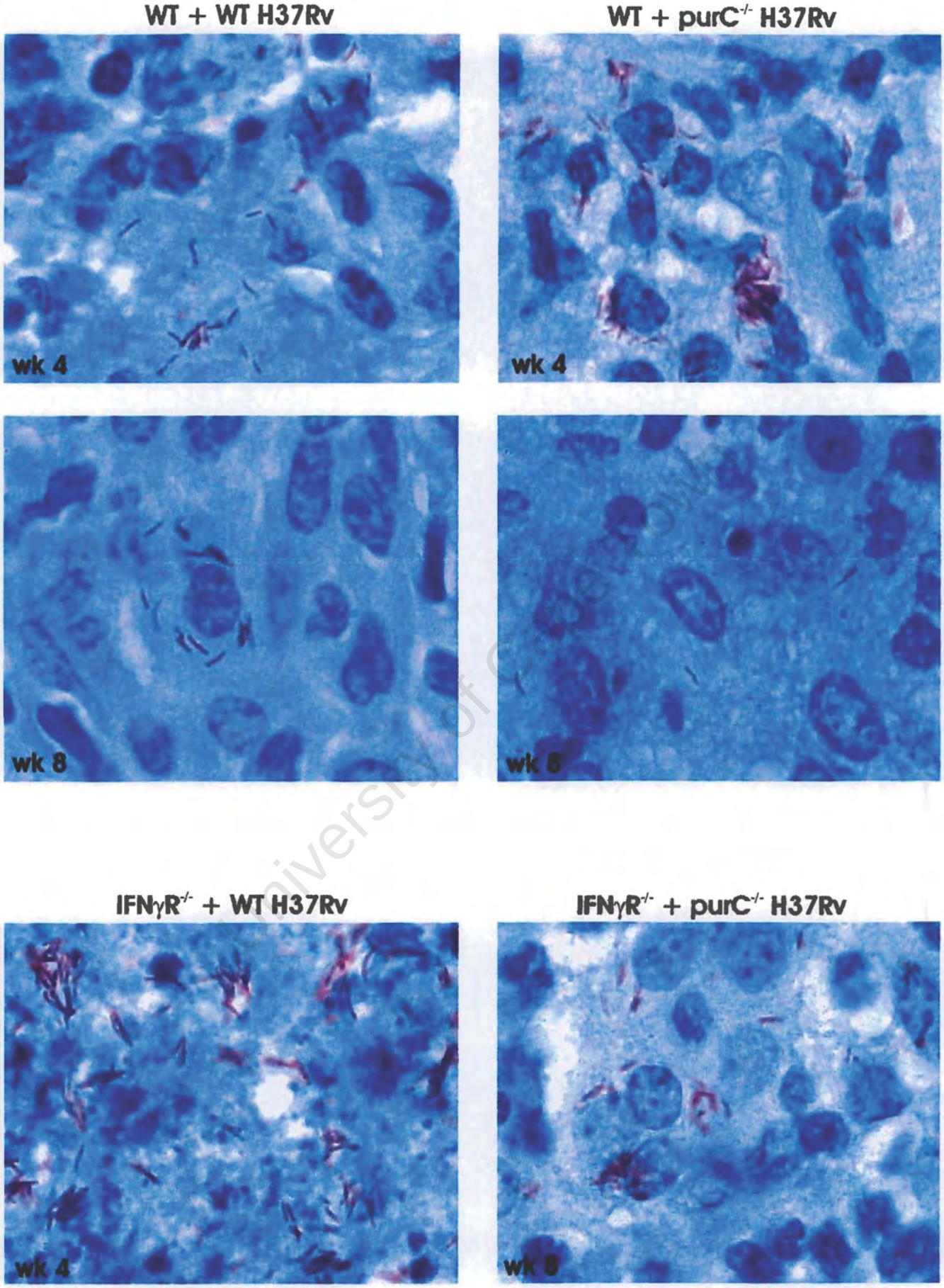


Figure 21A. Lung Ziehl-Neelson staining of sections from WT and IFNγR$^{-/-}$ mice 4 and 8 weeks after infection. Magnification 1000x.

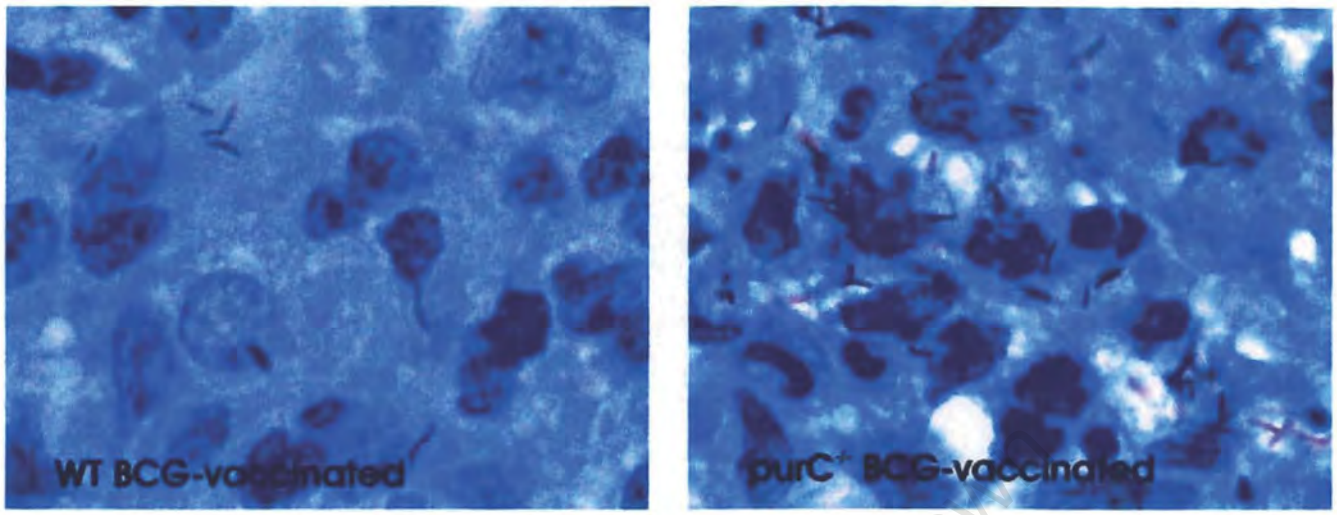


Figure 21B Lung ZN staining of WT mice vaccinated with WT and purC$^{-/-}$ BCG 4 weeks after aerosol challenge of WT H37Rv. Magnification 1000x.

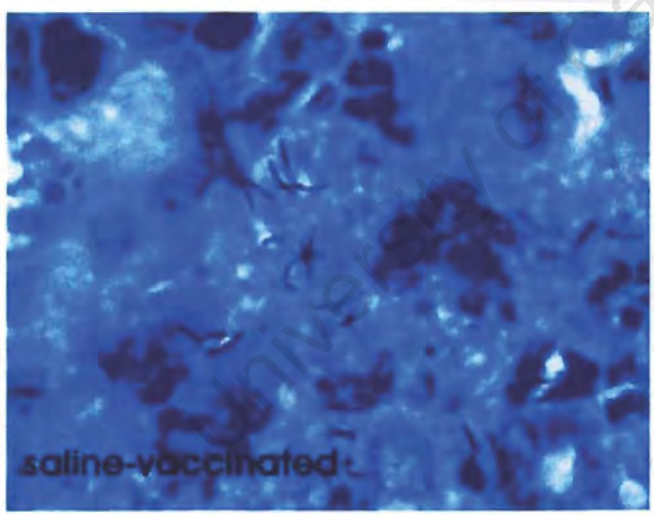


Figure 21C. Lung ZN sections of saline-vaccinated controls 4 weeks after an aerosol challenge with WT H37Rv. Magnification 1000x.

3.2.4 IMMUNOHISTOCHEMISTRY

Activated macrophages can be detected in organs by staining for their inducible nitric oxide synthase (iNOS) immunoreactivity. At week 4, and to a lesser extent at week 8, WT mice infected with either WT H37Rv or the purC mutant displayed strong iNOS immunoreactivity in the lungs, as well as in liver and spleen (Figure 22A). In contrast, no iNOS staining was found in IFNγR$^{-/-}$ mice infected with either WT or purC$^{-/-}$ H37Rv. Nitrotyrosine, which is the end product of the iNOS reaction, was detected in lungs of WT mice and was very similar to iNOS staining of these tissues (Figure 22B). As expected, IFNγR$^{-/-}$ mice stained negative for nitrotyrosine since there was no iNOS reactivity. Therefore, purC$^{-/-}$ H37Rv has a reduced capacity to induce iNOS expression, and in the absence of IFNγR signalling, no iNOS expression was observed, suggesting absence of proper macrophage activation, hence poor granuloma activation.

iNOS staining of unvaccinated and purC$^{-/-}$ BCG-vaccinated mice was more prominent than that observed for WT BCG-vaccinated mice (Figure 22C). This correlates with the HE and ZN data which indicate that these mice have a more extreme infection than WT BCG-vaccinated mice. The increased iNOS expression would be a result of increased macrophage activation to cope with the increased AFB. With WT BCG-vaccinated mice, the mycobacteria have been controlled and therefore less activation of the macrophages occurs at this time point.

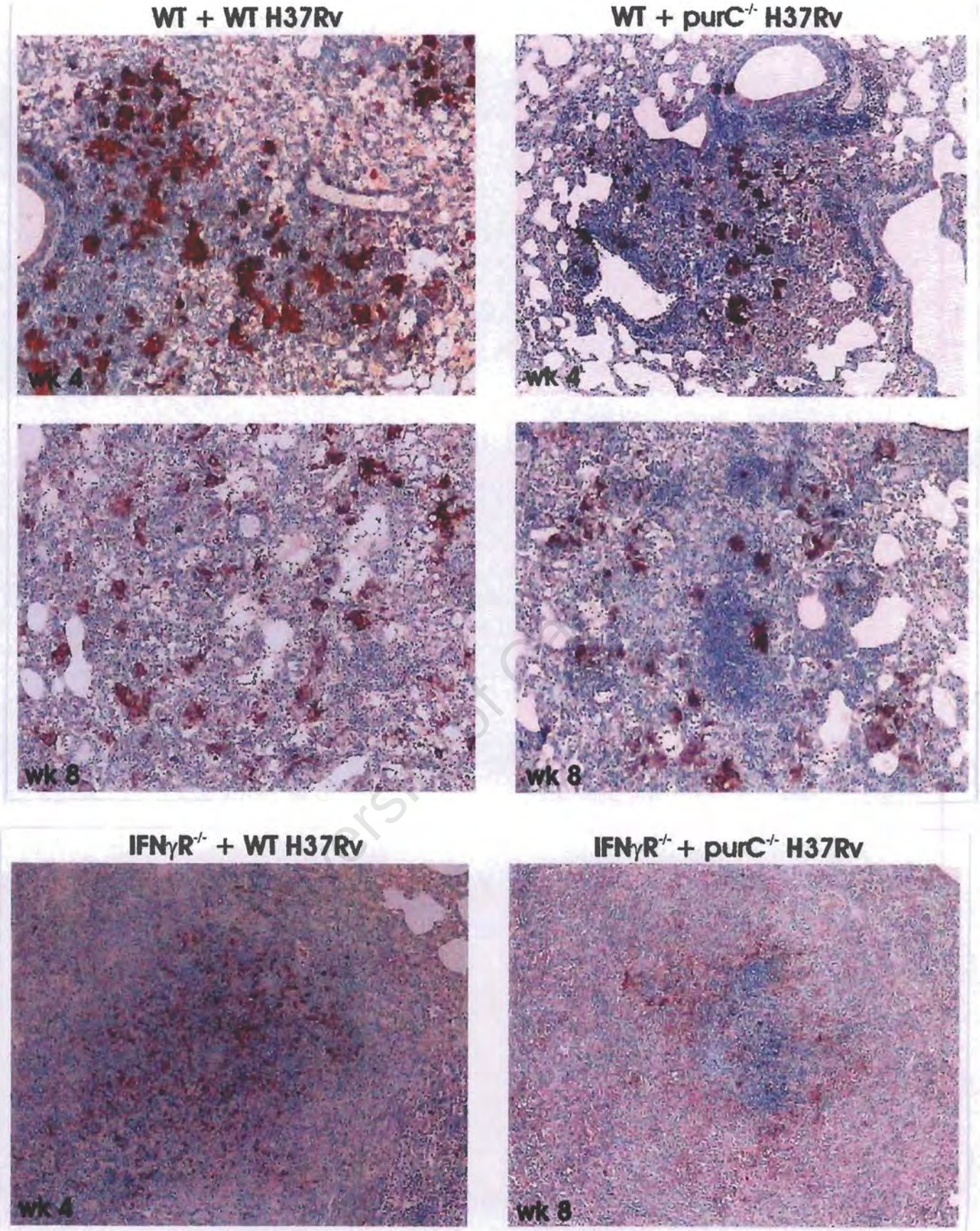


Figure 22A. iNOS staining of lung sections from WT and IFNγR$^{-/-}$ mice 4 and 8 weeks after infection. Magnification 40x.

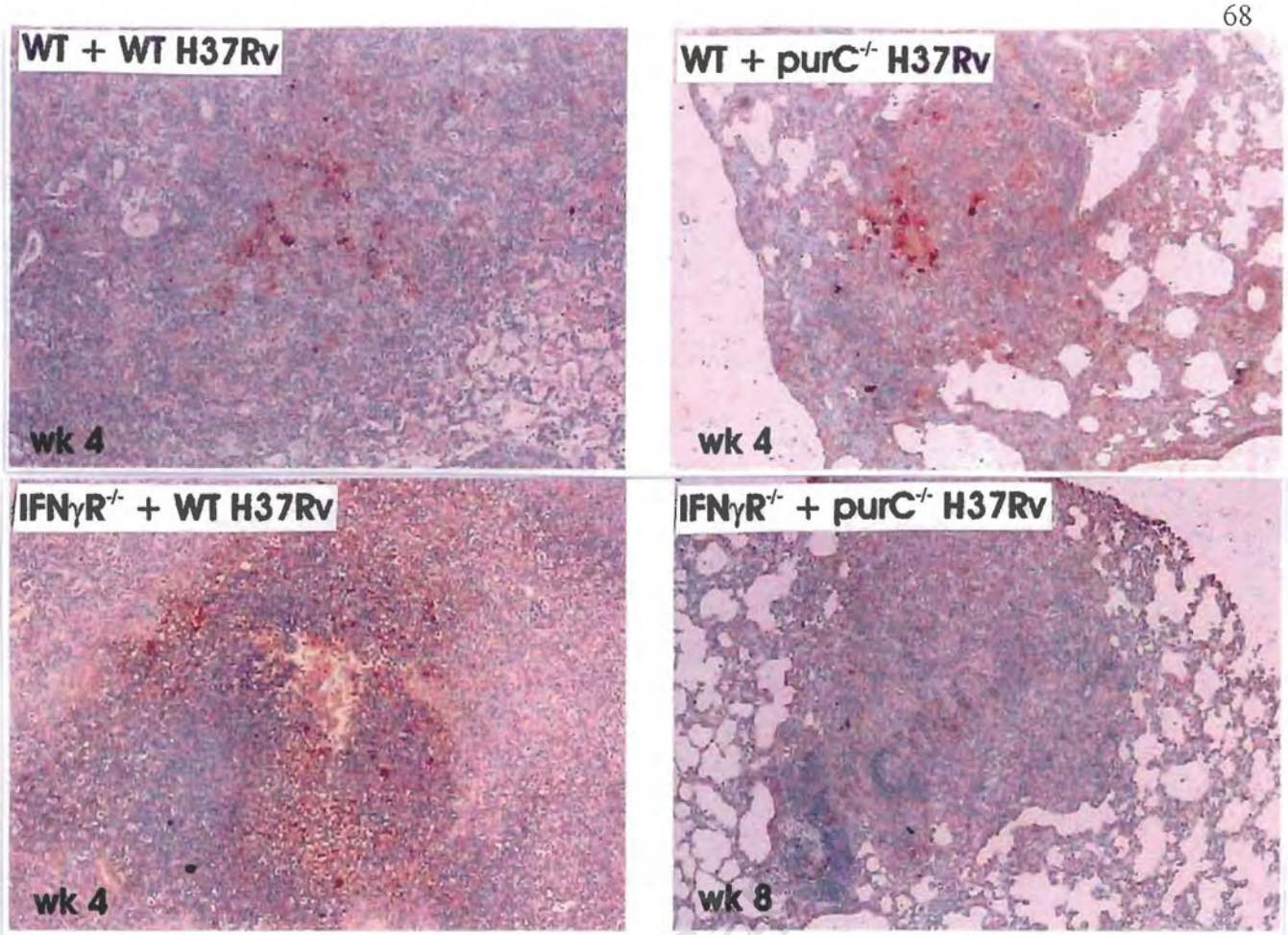


Figure 22B. Nitrotyrosine staining of lung sections from WT and IFNγR$^{-/-}$ mice 4 and 8 weeks after infection. Magnification 40x.

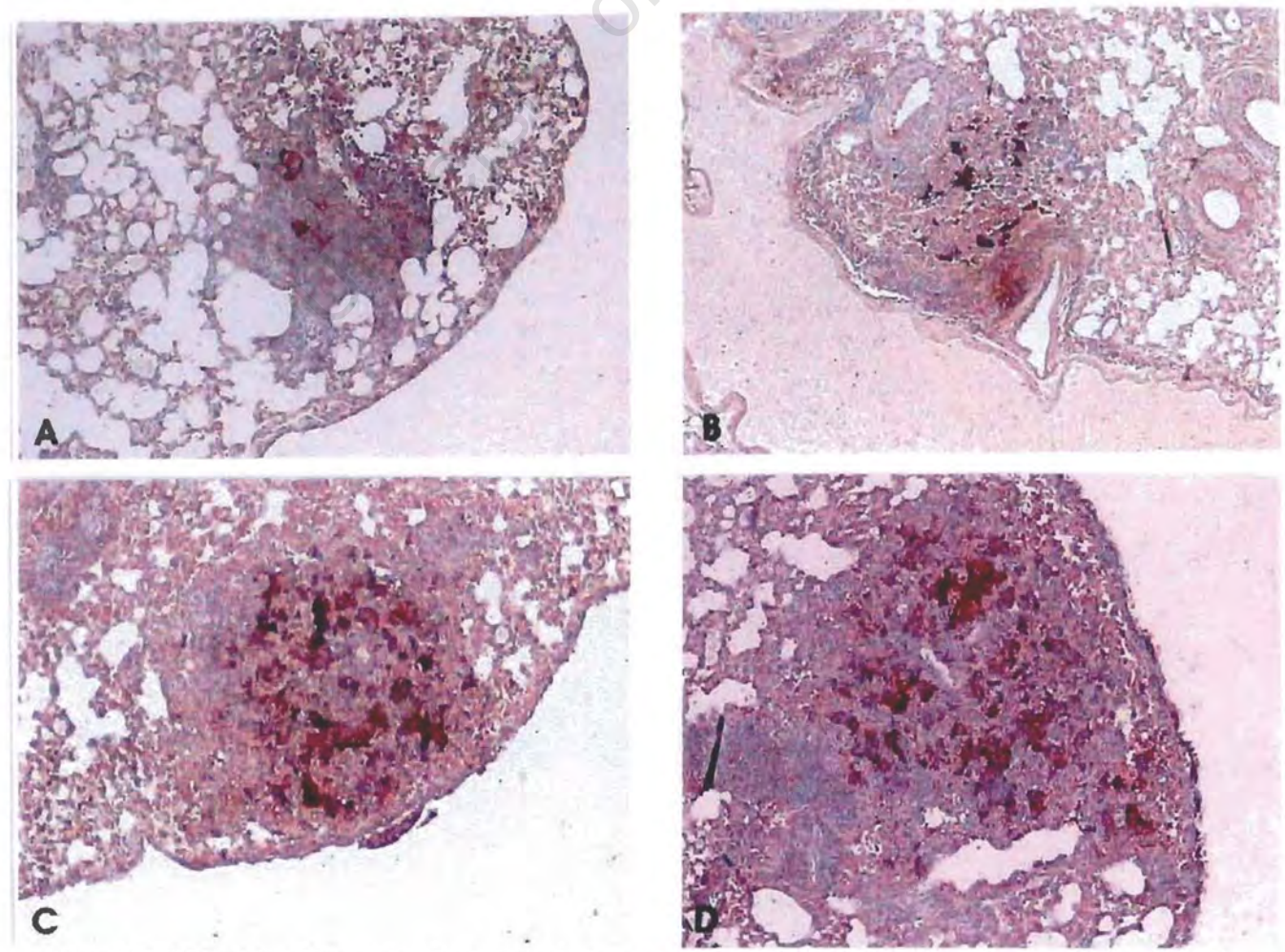


Figure 22C. iNOS staining of lung sections from vaccinated mice at 4 and 8 weeks. **A**. WT BCG-vaccinated mice at week 4. **B**. WT BCG-vaccinated mice at week 8. **C**. purC$^{-/-}$ BCG-vaccinated mice at week 8. **D**. Saline-vaccinated control mice at week 8. Magnification 40x.

4 DISCUSSION

4.1 IL-4/IL-13 STUDY

It is well known that TH1 cytokines are critical for protection against TB infection, whileTH2 cytokines play only minor roles (6, 19, 20, 25, 28, 34, 35, 42, 80). Here, for the first time, it is shown that there is an increased protective cell-mediated response in the absence of IL-13.

IL-4 and IL-13 share a receptor component and therefore it was thought there would be some redundancy concerning the functions of these two cytokines (10, 82). Due to the common receptor component, IL-4 and IL-13 share some properties but they also assume distinct functions. Both IL-4 and IL-13 have a role in TH2 differentiation (69, 70). It is believed that IL-13 exerts its effects on T cells indirectly since T cells do not express IL-13 receptor components on their surface although IL-13 mRNA have been detected in these cells (122).

Work on the role of IL-4 and IL-13 in infectious diseases has revealed distinct functions for these two cytokines. During *L major* infection, resistant mice develop a dominant TH1 response with high IFNγ and low IL-4 (74). In acute leishmaniasis, IL-$4^{-/-}$, IL-$13^{-/-}$ and IL-$4R\alpha^{-/-}$ were resistant to *L major* and could control infection (65, 79). IL-4/IL-13 double knockouts were most resistant (65). Although IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice had similar numbers of parasites during the late chronic phase of infection, IL-$4R\alpha^{-/-}$ could not control dissemination of the disease indicating that signals mediated through the IL-4Rα, but which exclude IL-4, are essential for maintaining immune control in chronic infections (75). Therefore IL-13 is needed to control dissemination of the parasite.

IL-13 plays a critical role in resistance to various nematode infections. IL-13$^{-/-}$ mice cannot expel *Trichus muris* despite a delayed TH2 response and comparable IL-4 production to WT mice (3). IL-4$^{-/-}$ mice are also susceptible to *T. muris* due to the inadequate production of TH2 cells and therefore their ability to produce TH2 cytokines including IL-13. In *N brasiliansis* infections, IL-4$^{-/-}$ mice had higher egg counts than WT mice but could expel the worms (5). IL-4Rα$^{-/-}$ had high egg counts and high worm counts. This indicates that IL-4 is sufficient but not necessary for protection against *N brasiliensis* and that IL-13 and IL-4Rα are needed for expulsion of *N brasiliensis*.

IL-13 is essential for pulmonary granuloma formation in *S mansoni* infections. WT mice given a soluble IL-13Rα2 conjugated to an antibody (sIL-13Rα2-Fc) had a reduction in granuloma volume which was similar to that observed in IL-4$^{-/-}$ mice (16). IL-4$^{-/-}$ mice given sIL-13Rα2-Fc had an almost complete abrogation of granuloma formation which indicates that IL-13 has a role in egg-induced granuloma formation. IL-4Rα$^{-/-}$ mice infected with *S mansoni* had a similar complete impairment of granulomatous inflammation and fibrosis showing that IL-4 was not essential for hepatic fibrosis in pathogenesis (51). IL-13 was implicated in hepatic fibrosis (30). These results indicate that there are distinct functions for IL-4 and IL-13.

There are only a few investigations on the role of the TH2 cytokines IL-4, IL-13 and IL-10 with respect to resistance in TB infection. Some studies demonstrated that the TH2 cytokines like IL-4, IL-5 and IL-10 have a minimal impact in TB protection (28, 80). Using a low dose infection (100 CFU), this study confirms the above data since very little difference between WT, IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice were found at this infective dose. However, a recent study suggested that IL-4 might be required for the defence against mycobacterial infection (116).

It has previously been shown that the TH1 response to mycobacteria is enhanced in the absence of IL-10 (50). The present study determined whether this enhancement would apply when IL-4 and IL-13 are absent. Differences in the host resistance between IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice were found with the higher dose of infection (350 CFU) and the data suggest that endogenous IL-13 may inhibit the protective immune response to TB. Firstly, in the absence of IL-4 and IL-13, the survival was increased as found in IL-$4R\alpha^{-/-}$ mice. The increased survival is likely due to a reduced bacterial load in all organs of IL-$4R\alpha^{-/-}$ mice. Enhanced bacterial clearance in IL-$4R\alpha^{-/-}$ mice was associated with stronger granuloma response, and increased macrophage activation (increased iNOS activity). The increased macrophage activation in IL-$4R\alpha^{-/-}$ mice could be due to the higher levels of IL-12 in these mice. IL-4 and IL-13 are known to inhibit IL-12 production so in the absence of both IL-4 and IL-13 we expect more IL-12 production. However, TNF and IFNγ production was reduced or similar in IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice. The data suggest that endogenous IL-13 may have a greater suppressive effect on IL-12 production than IL-4.

During early TB infection, TH1 cytokines are critical to contain infection, while IL-4 and IL-13 may have minor roles. However, the TH2 cytokines may be important during the chronic stage of infection, when bacterial numbers increase and disease persists. During *L major* infection, the effect of the absence of IL-13 is only observed in the chronic stage of infection when IL-13 appears to be needed to control dissemination of the disease (75). Similar to *L major* infection, the effect of IL-13 in this study of the TB model was observed in the chronic stage of infection. The absence of IL-13 at this stage and the resultant improved immune response suggests that the effect of IL-13 on the TH1 immune response is important in the containment of MTB infection. This data therefore support the concept that the inadequacy of

TH1-mediated anti-tuberculosis immunity may be due to the down-regulation by TH2 cytokines.

In conclusion, the data indicate that increased survival and reduced bacterial load is due to a stronger cell-mediated immune response resulting in a better confinement of the mycobacteria into well-formed granulomas and more efficient killing in IL-4R$\alpha^{-/-}$ mice as compared to IL-4 and WT mice.

4.2 PROTECTIVE EFFICACY STUDY

Previous studies have shown that auxotrophic mycobacteria grow much slower than their WT counterparts (45, 49, 111). This study confirms a previous observation that the purC$^{-/-}$ H37Rv mutant is attenuated with respect to growth in WT mice (49). Studies using the intravenous route of infection have found that immunocompromised mice could control an infection by certain auxotrophic H37Rv (45, 111). Proline and tryptophan H37Rv mutants could protect against subsequent iv H37Rv infection (111). H37Rv purine-deficient BCG showed positive results in guinea pigs when tested for delayed-type hypersensitivity but could not prevent the dissemination of H37Rv (49). Methionine-deficient BCG protected guinea pigs against aerosol challenge with virulent H37Rv (11). Leucine-deficient mycobacteria, on the other hand, could not protect against aerosol H37Rv challenge although it protected against BCG aerosol challenge and both BCG and H37Rv intravenous challenge (11, 45). A more recent study showed that a pantothenate auxotroph of H37Rv protected mice against an aerosol H37Rv challenge (103).

In the present study, using aerosol infection, IFNγR$^{-/-}$ mice were unable to control purC$^{-/-}$ H37Rv infection and subsequently died. IFNγR$^{-/-}$ signalling is important in the early recruitment and activation of cells involved in granuloma formation and containment of

infection (19, 25, 31, 32, 101). Despite the fact that purC$^{-/-}$ H37Rv had delayed growth kinetics compared to WT H37Rv, it still required the same events that WT H37Rv require for containment. IFNγR$^{-/-}$ mice lack these early events and subsequently cannot control purC$^{-/-}$ H37Rv infection. Although purC$^{-/-}$ H37Rv protected guinea pigs against aerosol H37Rv challenge (49), it may not have been sufficiently attenuated to protect an immune-deficient host, as was the case in this study. Furthermore, some studies show that mutant mycobacteria could not protect against an aerosol challenge (11, 45). Since purC$^{-/-}$ H37Rv could protect guinea pigs, it could be a combination of the use of an immune-deficient host together with the aerosol challenge that may explain the death of the IFNγR$^{-/-}$ mice.

The rationale for using an attenuated MTB strain rather than BCG is the fact that MTB expresses a substantial number of additional genes which may be critical for acquired immunity. It has been suggested that BCG vaccines conferred more protection against *M bovis* challenge than against MTB challenge (11). BCG is derived from *M bovis* so protective antigens in BCG is similar to *M bovis* and could explain the greater protection against *M bovis* compared to MTB.

It has been postulated that different antigens may be the target for preventing pulmonary disease as opposed to haematogenous disease (11). It has also been suggested that the ability of some auxotrophs to protect against haematogenous but not pulmonary TB infection could be due to the auxotroph not being sufficiently metabolically active to synthesise antigens needed for protection against MTB in the lung (11, 45). This may be the reason why numerous studies report protection against haematogenous but not aerosol infection. It does not, however, apply in this study since high numbers of the mutant were recovered from

organ homogenates and the purC$^{-/-}$ H37Rv was sufficiently virulent to cause death in IFNγR $^{-/-}$ mice.

BCG auxotrophs as vaccine candidates against TB have yielded promising results and it is hoped that similar safe MTB vaccines would be produced. Thus far, a number of MTB auxotrophs have protected mice and guinea pigs from haematogenous spread of MTB although pulmonary spread of TB has yielded varying results (11, 45, 49, 111). The difficulty in producing live vaccines is to reach a balance between attenuation and immunogenicity (114). Results from other studies indicate that the inefficient expression of immunity in the lungs are likely to prove an obstacle to successful vaccination against TB in resistance and susceptible mouse strains but more so in the latter strains (72). From this study it can be concluded that although the purine-deficient mutant of H37Rv had delayed growth kinetics, it was only partially attenuated and therefore lethal in an immune-deficient host. It is therefore not an ideal candidate for vaccine development.

5 REFERENCES

1. Andersson A, Grunewald SM, Duschl A, Fischer A and DiSanto JP. Mouse macrophage development in the absence of the common gamma chain: defining the receptor complexes for IL-4 and IL-13 signalling. Eur. J. Immunol. (1997) 27:1762-1768
2. Bancroft AJ, Artis D, Donaldson DD, Sypek JP and Grencis RK. Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependant. Eur. J. Immunol. (2000) 30:2083-2091
3. Bancroft AJ, McKenzie AN and Grencis RK. A critical role for IL-13 in resistance to intestinal nematode infection. J. Immunol. (1998) 160:3453-3461
4. Bange FC, Brown AM and Jacobs WR Jr. Leucine auxotrophy restricts growth of *Mycobacterium bovis* BCG in macrophages. Infect. Immun. 1996 64(5): 1794-1799
5. Barner M, Mohrs M, Brombacher F and Kopf M. Differences between IL-4Rα-deficient and IL-4-deficient mice reveal a role for IL-13 in the regulation of TH2 responses. Current Biol. (1998) 8:669-672
6. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD and Britton WJ. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. J. Immunol. (1999) 162:3504-3511
7. Bloom BR. *Tuberculosis, pathogenesis, protection and control*, ed. BR Bloom, pps 1-43. 1994. ASM Press, New York

8. Bogdan C, Thuring H, Dlaska M, Rollinghoff M and Weiss G. Mechanism of suppression of macrophage nitric oxide release by IL-13. J. Immunol. (1997) 159:4506-4513

9. Brandt L, Feino Cuhna J, Weinreich Olsen A, Chilima B, Hirsch P, Appelberg R and Andersen P. Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect. Immun. (2002) 70:672-678

10. Brombacher F. The role of interleukin 13 in infectious diseases and allergy. Bioassays (2000) 22:646-656

11. Chambers MA, Williams A, Gavier-Widen D, Whelan A, Hall G, Marsh PD, Bloom BR, Jacobs WR and Hewinson RG. Identification of a *Mycobacterium bovis* BCG auxotrophic mutant that protects guinea pigs against *Mycobacterium bovis* and haematogenous spread of *Mycobacterium tuberculosis* without sensitisation to tuberculin. Infect. Immun. (2000) 68: 7094-7099

12. Chan J and Kaufmann SHE. Immune mechanisms of protection. In *Tuberculosis: pathogenesis, protection and control*, ed. BR Bloom, pps389-415. 1994. ASM Press, New York

13. Chan J, Xing Y, Magliozzo RS and Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated macrophages. J. Exp. Med. (1992) 175:1111-1122

14. Chatterjee D, Roberts AD, Lowell K, Brennan PJ and Orme IM. Structural basis of capacity of lipoarabinomannan to induce secretion of tumour necrosis factor. Infect. Immun. (1992) 60:1249-1253

15. Chensue SW, Ruth JH, Warmington K, Lincoln P and Kunkel SL. *In vivo* regulation of macrophage IL-12 production during type 1 and type 2 cytokine-mediated granuloma formation. J. Immunol. (1995) 155:3546-3551

16. Chiaramonte MG, Schopf LR, Neben TY, Cheever AW, Donaldson DD and Wynn TA. IL-13 is a key regulatory cytokine for TH2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. J. Immunol. (1999) 162:920-930

17. Chilton PM and Fernandez-Botran R. Production of soluble IL-4 receptors by murine spleen cells is regulated by T cell activation and IL-4. J. Immunol. (1993) 151:5907-5917

18. Collins FM. Pathogenesis of *M tuberculosis* in experimental animals. In: *Tuberculosis*, eds WN Rom and S Garay, pps 259-268. 1996. Little, Brown and Company

19. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG and Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. (1993) 178:2243-2247

20. Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM and Orme IM. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology (1995) 84:423-432

21. Crawford RM, van der Verg L, Yuan L, Hadfield TL, Warren RL, Drazek ES, Houng HSH, Hammack C, Sasala K, Polsinelli T, Thompson J and Hoover DL. Deletion of *purE* attenuates *Brucella melitensis* infection in mice. Infect. Immun. (1996) 64: 2188-2192

22. Culling CFA (a). In: *Handbook of histopathological and histochemical techniques*, 3rd edition, pg 214. 1974. Butterworths, London

23. Culling CFA (b). In: *Handbook of histopathological and histochemical techniques*, 3rd edition, pg 396. 1974. Butterworths, London

24. Dako guide to demasking of antigens on formalin-fixed, paraffin-embedded tissue, 2nd edition, pgs 3-5. 1997

25. Dalton D, Pitts-Meek S, Keshav S, Figaro IS, Bradley A and Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon γ genes. Science (1993) 259:1793-1742

26. Doffinger R, Dupuis S, Picard C, Fieschi C, Feinberg J, Barcenas-Morales G and Casanova JL. Inherited disorders of IL-12 and IFN gamma-mediated immunity: a molecular genetics update. Mol Immunol. (2002) 38:903-909

27. Durum SK and Muegge K. Cytokine knockouts. 1998. Humana Press, New Jersey

28. Erb KJ, Kirman J, Delahunt B, Chen W and Le Gros G. IL-4, IL-5 and IL-10 are not required for the control of *M. bovis*-BCG infection in mice. Immunol. Cell. Biol. (1998) 76:41-46

29. Eugster HP, Muller M, Karrer U, Car BD, Schnyder B, Eng VM, Woerly G, Le Hir M, Di Padova F, Agnes M, Zinkernagel R, Bluethmann H and Ryffel B. Multiple immune abnormalities in tumour necrosis factor and lymphotoxin-alpha double-deficient mice. Int. Immunol. (1996) 8:23-36

30. Fallon PG, Richardson EJ, McKenzie GJ and McKenzie AN. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J. Immunol. (2000) 164:2585-2591

31. Flesch IEA and Kaufmann SHE. Mycobacterial growth inhibition by interferon-γ-activated bone-marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J. Immunol. (1987) 138:4408-4413

32. Flesch IEA and Kaufmann SHE. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumour necrosis factor. Infect. Immun. (1990) 58:2675-2677

33. Flynn JL and Chan J. Immunology of tuberculosis. Ann. Rev. Immunol. (2001) 19:93-129

34. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA and Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. (1993) 178:2249-2254

35. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak K and Bloom BR. Tumour necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity (1995) 2:561-572

36. Flynn JL, Goldstein MM, Triebold KJ, Koller B and Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA. (1992) 89:12013-12017

37. Gauchat JF, Schlagenhauf E, Feng N-P, Moser R, Yamage M, Jeanin P, Alouani S, Elson G, Notarangelo LD, Wells T, Eugster H-P and Bonnefoy J-Y. A novel 4kb interleukin-13 receptor α mRNA is expressed in human B, T and endothelial cells encoding an alternate type-II interleukin-4/interleukin-13 receptor. Eur. J. Immunol (1997) 27:971-978

38. Gazzinelli RT, Oswald IP, James SL and Sher A. IL-10 inhibits parasite killings and nitrogen oxide production by IFN-gamma-activated macrophages. J. Immunol. (1992) 148:1792-1796

39. Global alliance for TB drug development, www.tballiance.org

40. Graber P, Gretener D, Herren S, Aubry JP, Elson G, Poudrier J, Lecounet-Henchas S, Alouani S, Losberger C, Bonnefoy J-Y, Kosco-Vilbois MH and Gauchat JF. The distribution of IL-13 receptor alpha1 expression on B cells, T cells and monocytes and its regulation by IL-13 and IL-4. Eur. J. Immunol. (1998) 28:4286-4298

41. Guleria I, Teitelbaum R, McAdam RA, Kaplan G, Jacobs WR Jr and Bloom BR. Auxotrophic vaccines for tuberculosis. Nat Med. (1996) 2:334-337

42. Hernandez-Pando R, Orozcoe H, Sampieri A, Pavon L, Velasquillo C, Larriva-Sahd J, Alcocer JM and Madrid MV. Correlation between the kinetics of TH1, TH2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology (1996) 89:26-33

43. Hilton D, Zhang JG, Metcalf, D, Alexander W, Nicola N and Wilson T. Cloning and characterisation of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc. Acad. Natl. Sci. USA (1996) 93:497-501

44. Holscher C, Atkinson RA, Arendse B, Brown N, Myburgh E, Alber G and Brombacher F. A protective and agonistic function of homodimeric interleukin-12p40 in mycobacterial infection. J. Immunol. (2001) 167:6957-6966

45. Hondalus MK, Bardarov S, Russell R, Chan J, Jacobs WR Jr and Bloom BR. Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. Infect. Immun. (2000) 68: 2888-2898

46. Huebner RE. Bacillus Calmette and Guerin (BCG) vaccine. In: *Tuberculosis*, eds WN Rom and S Garay, pps 892-905. 1996. Little, Brown and Company

47. Huygen K, Content J, Denis O, Montgomery DL, Yawmen AM, Deck RR, DeWitt CM, Orme IM, Baldwin S, D'Souza C, Drowatt A, Lozes E, Vandenbussche P, Van

Vooren JP, Liu MA and Ulmer JB. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nature Med. (1996) 2:893-898

48. Jackson M, Berthet FX, Otal I, Rauzier J, Martin C, Gicquel B and Guilhot C. The *Mycobacterium tuberculosis* purine biosynthetic pathway: Isolation and characterisation of the purC and purL genes. Microbiology (1996) 142: 2439-2447

49. Jackson M, Phalen SW, Lagranderie M, Ensergueix D, Chavarot P, Marchal G, McMurray DN, Gicquel B and Guilhot C. Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. Infect. Immun. (1999) 67: 2867-2873

50. Jacobs M, Brown N, Allie N, Gulert R and Ryffel B. Increased resistance to mycobacterial infection in the absence of interleukin-10. Immunology (2000) 100:494-501

51. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Ward JM, Cheever AW, Paul WE and Sher A. Schistosome-infected IL-4 receptor knockout mice, in contrast to IL-4 knockout mice, fail to develop granulomatous pathology while maintaining the same lymphokine expression profiles. J. Immunol. (1999) 163:337-342

52. Kamijo R, Le J, Shapiro D, Havell EA, Huang S, Aguet M, Bosland M and Vilcek J. Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide J. Exp. Med. (1993) 178: 1435-144

53. Kamijo R, Shapiro D, Le J, Huang S, Aguet M and Vilcek J. Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon-gamma receptor. Proc. Natl. Acad. Sci. USA (1993) 90: 6626-6630

54. Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K and Sugawara I. Role of tumour necrosis factor-α in mycobacterium-induced granuloma formation in tumour necrosis factor-α-deficient mice. Lab. Invest. (1999) 79:379-386

55. Kindler V, Sappino AP, Grau GE, Piquet P-F and Vassalli P. The inducing role of tumour necrosis factor in the development of bactericidal granulomas during BCG infection. Cell (1989) 56:731-740

56. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B and Trichieri G. Identification and purification of natural killer cell-stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. J. Exp. Med. (1989) 170:827-845

57. Kopf M, LeGros G, Coyle AJ, Kosco-Vilbois M and Brombacher F. Immune responses in IL-4, IL-5 and IL-6-deficient mice. Immunol. Rev. (1995) 148:45-69

58. Kubo M, Yamashita M, Abe R, Tada T, Okumura K, Ransom RT and Nakayama T. CD28 costimulation accelerates IL-4 receptor sensitivity and IL-4 mediated TH2 differentiation. J. Immunol. (1999) 163: 2432-2442

59. Kumararatne DS, Pithie AS, Drysdale P, Gaston JS, Kiessling R, Iles PB, Ellis CJ, Innes J and Wise R. Specific lysis of mycobacterial antigen-bearing macrophages by Class II MHC-restricted polyclonal T cell lines in healthy donors or patients with tuberculosis. Clin. Exp. Immunol. (1990) 80:314-323

60. Ladel CH, Flesch IE, Arnoldi J and Kaufmann SH. Studies with MHC-deficient knockout mice reveal impact of both MHC I and MHC II-dependant T cell response on *Listeria monocytogenes*. J. Immunol. (1994) 153:3116-3122

61. MacMicking J, Xie QW and Nathan C. Nitric oxide and macrophage function. Ann. Rev. Immunol. (1997) 15:323-350

62. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK and Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA (1997) 94:5243-5248

63. Malakoff D. The rise of the mouse, biomedicine's model mammal. Science (2000) 288:248-253

64. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, Barry CE, Freedman VH and Kaplan G. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce TH1 type immunity and is associated with induction of IFN-α/β. Proc. Natl. Acad. Sci. USA (2001) 98:5752-5757

65. Matthews DJ, Emson CL, McKenzie GJ, Jolin HE, Blackwell JM and McKenzie AN. IL-13 is a susceptibility factor for *Leishmania major* infection. J. Immunol. (2000) 164:1458-1462

66. Matthews DJ, Hibbert LM, Friedrich K, Minty A and Callard RT. X-SCID B cell responses to interleukin 4 and interleukin 13 are mediated by a receptor complex that induces the interleukin 4 receptor α chain (p140) but not the γc chain. Eur. J. Immunol. (1997) 27:116-121

67. McDonough KA, Kress Y and Bloom BR. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect. Immun. (1993) 61:2763-2773

68. McKenzie AN, Culpepper JA, de Waal Malefyt R, Briere T, Punnonen J, Aversa G, Sato A, Drury W, Cocks BG and Menon S. Interleukin 13, a T cell-derived cytokine that regulates human monocyte and B cell function. Proc. Natl. Acad. Sci. USA (1993) 90:3735-3739

69. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fellon P, Zurawski G, Murray R, Grencis R and McKenzie AN. Impaired development of TH2 cells in IL-13-deficient mice. Immunity (1998) 9:423-432

70. McKenzie GJ, Fallon PG, Emson CL, Grencis RK and McKenzie AN. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type-2-mediated responses. J. Exp. Med. (1999) 189:1656-1572

71. McNeil MR, Besra GS and Brennan PJ. Chemistry of the mycobacterial cell wall. In: *Tuberculosis*, eds WN Rom and S Garay, pps 171-185. 1996. Little, Brown and Company

72. Medina E and North RJ. Genetically susceptible mice remain proportionally more susceptible to tuberculosis after vaccination. Immunology (1999) 96:16-21

73. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B, Minty C, Casellas P, Loison G, Lupker J, Shire D, Terrara P and Caput D. Interleukin 13 is a new human lymphokine regulating inflammatory and immune responses. Nature (1993) 362:248-250

74. Mohrs M and Brombacher F. Interleukin-4 receptor alpha-deficient BALB/c mice show an unimpaired T helper 2 polarisation in response to *Leishmania major* infection. Infect. Immun. (2000) 68:1773-1780

75. Mohrs M, Ledermann B, Köhler G, Dorfmüller A, Gessner A and Brombacher F. Differences between IL-4 and IL-4 receptor α-deficient mice in chronic leishmaniasis reveals a protective role for IL-13 receptor signalling. J. Immunol. (1999) 162:7302-7308

76. Murata T, Noguchi PD and Puri RK. IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signalling. J. Immunol. (1996) 156:2972-2978

77. Murata T, Taguchi J and Puri RK. Interleukin-13 receptor α', but not α: a functional component of interleukin-4 receptors. Blood (1998) 91:3884-3891

78. Nakamura T, Kamagawa K, Bottomly K and Flavell RA. Polarisation of IL-4- and IFNγ-producing $CD4^+$ T cells following activation of naïve $CD4^+$ T cells. J. Immunol. (1997) 158:1085-1094

79. Noben-Trauth N, Shultz LD, Brombacher F, Urban Jr JF, Gu H and Paul WE. An interleukin 4 (IL-4) independent pathway for $CD4^+$ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. Proc. Natl. Acad. Sci. USA (1997) 94:10838-10843

80. North RJ. Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis*. Clin. Exp. Immunol. (1998) 113:55-58

81. Novick R. Mycobacteria: growth, metabolism and molecular biology. In: *Tuberculosis*, eds WN Rom and S Garay, pps 187-198. 1996. Little, Brown and Company

82. Obiri NI, Leland P, Murata T, Debinski W and Puri RK. The IL-13 receptor structure differs in various cell types and may share more than one component with IL-4R. J. Immunol. (1997) 158:756-764

83. Oettinger T and Andersen AB. Cloning and B cell-epitope mapping of MPT64 from *Mycobacterium tuberculosis*. Infect. Immun. (1994) 62:2058-2064

84. Orme IM. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. J. Immunol. (1987) 138:293-298

85. Orme IM, Andersen P and WH Boom. T cell response to *Mycobacterium tuberculosis*. J. Infect. Dis. (1993) 167:1481-1497

86. Orme IM and Cooper AM. Cytokine/chemokine cascades in immunity to tuberculosis. Immunol. Today (1999) 20:307-312

87. Orme IM and McMurray DN. The immune response to tuberculosis in animal models. In: *Tuberculosis*, eds WN Rom and S Garay, pps 269-280. 1996. Little, Brown and Company

88. Orme IM, Miller ES, Roberts AD, Furney SK, Griffin JP, Dobos KM, Chi D, Rivoire R and Brennan PJ. T lymphocytes mediating protection and cellular cytolysis during the course of *Mycobacterium tuberculosis* infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. J. Immunol. (1992) 148:189-196

89. Orme IM, Roberts AD, Griffin JP and Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. (1993) 151:318-325

90. Parranchi P, de Carli M, Manetti R, Simonelli C, Sampognaro S, Piccirini MP, Macchia D, Maggi E, Del Preta G and Romagnia IS. IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by TH1 or TH2 human T cell clones. J. Immunol. (1992) 149:2977-2983

91. Paul WE. Interleukin 4: a prototypic immunoregulatory lymphokine. Blood (1991) 77:1859-1870

92. Pelicic V, Jackson M, Reyrat J-M, Jacobs WR Jr, Gicquel B and Guilhot C. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA. (1997) 94: 10955-10960

93. Poudrier J, Graber P, Herren S, Gretener D, Elson G, Berney C, Gauchat J-F and Kosco-Vilbois MH. A soluble form of the IL-13 receptor α1 promotes IgG2a and IgG2b production by murine germinal centre B cells. J. Immunol. (1999) 163:1153-1161

94. Power CA, Wei G and Bretscher PA. Mycobacterial dose defines the TH1/TH2 nature of the immune response independently of whether immunisation is administered by the intravenous, subcutaneous, or intradermal route. Infect. Immun. (1998) 66:5743-5750

95. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, De Waal Malefyt R and De Vries JE. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc. Natl. Acad. Sci. USA (1993) 90:3730-3734

96. Rakasz E, Blum AM, Metwali A, Elliot DE, Li J, Ballas ZK, Qadir K, Lynch R and Weinstock JV. Localisation and regulation of IFNγ production within the granulomas of murine schistosomiasis in IL-4 deficient and control mice. J. Immunol. (1998) 160:4994-4999

97. R&D Systems catalogue. TH1/TH2: outcome of adaptive immunity. 2000

98. Roach DR, Bean AGD, Demangel C, France MP, Briscoe H and Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation and clearance of mycobacterial infections. J. Immunol. (2002) 168:4620-4627

99. Robinson DS, Hamid G, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR and Kay AB. Predominant TH2-like bronchoalveolar lavage T lymphocyte population in atopic asthma. N. Engl. J. Med. (1992) 326:298-304

100. Rogers PR and Croft M. CD28, Ox40, LFA-1 and CD4 modulation of TH1/TH2 differentiation is directly dependent on the dose of antigen. J. Immunol. (2000) 164:2955-2963

101. Rook GA, Steele J, Ainsworth M and Champion BR. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparisons of the effects of

recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology (1986) 59:333-338

102. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri R, Paul WE and Leonard WJ. Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science (1993) 262-1880-1883

103. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM, Morris SL and Jacobs WR Jr. A pantothenate auxotroph *of Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. Nat. Med. (2002) 8:1171-1174

104. Scanga CA, Mohan VP, Tanaka K, Alland D, Flynn JL and Chan J. The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of *Mycobacterium tuberculosis* in mice. Infect. Immun. (2001) 69:7711-7717

105. Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J and Flynn JL. Depletion of $CD4^+$ T cells causes reactivation of murine persistent tuberculosis despite continued expression of IFNγ and NOS2. J. Exp. Med. (2000) 192:347-358

106. Schaible UE, Collins HL and Kaufmann SH. Confrontation between intracellular bacteria and the immune system. Adv. Immunol. (1991) 71:267-377

107. Seder RA and Paul WE. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. Annu. Rev. Imm. (1994) 12:635-673

108. Serbina NV and Flynn JL. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. Infect. Immun. (1999) 67:3980-3988

109. Shalekoff S, Pendle S, Johnson D, Martin DJ and Tiemessen CT. Distribution of the human immunodeficiency virus coreceptors CXCR4 and CCR5 on leukocytes of persons with human immunodeficiency virus type 1 infection and pulmonary tuberculosis: implications for pathogenesis. J. Clin. Immunol. (2001) 21:390-401

110. Simmons CP, Hodgson AL and Strugnell RA. Attenuation and vaccine potential of *aroQ* mutants of *Corynebacterium pseudotuberculosis*. Infect. Immun. (1997) 65: 3048-3056

111. Smith DA, Parish T, Stoker NG and Bancroft GJ. Characterisation of auxotrophic mutants of *Mycobacterium tuberculosis* and their potential as vaccine candidates. Infect. Immun. (2001) 69: 1142-1150

112. Sonnenberg MG and Belisle JT. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing and electrospray mass spectrometry. Infect. Immun. (1997) 65:4515-4524

113. Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, Van Kaer L and Bloom BR. Relative contributions of distinct MHC class I-dependant cell populations in protection to tuberculosis in mice. Proc. Natl. Acad. Sci. USA (2000) 97:4204-4208

114. Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH and Hatfull GF. New use of BCG for recombinant vaccines. Nature (1991) 351:456-460

115. Stover CK, Hanson MS and Lagermann S. Recombinant BCG vaccines and the development of mycobacterial biology. In: *Tuberculosis*, eds WN Rom and S Garay, pgs 911-925. 1996. Little, Brown and Company

116. Sugawara I., Yamada H., Mizuno S. and Iwakura Y. IL-4 is required for defence against mycobacterial infection. Microbiol. Immunol. (2000) 44: 971-979

117. Surcell HM, Troye-Blomberg M, Paulie S, Andersson G, Moreno C, Pasvol G and Ivanyi J. TH1/TH2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology (1994) 81:171-176

118. Tighe H, Corr M, Roman M and Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. Immunol. Today (1998) 19:89-97

119. Urban Jr JF, Noben-Trauth N, Donaldson DD, Madden KR, Morris SL, Collins M and Finkelman FD. IL-13, IL-4Rα and STAT6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. Immunity (1998) 8:255-264

120. WHO Report 2002. Global TB/HIV Working Group, 2nd meeting, Durban, South Africa, June 2002, www.who.int

121. Yeager Jr H, Azumi N and Underhill CB. Fibrosis: The formation of the granuloma matrix. In: *Tuberculosis*, eds WN Rom and S Garay, pps 363-370. 1996. Little, Brown and Company

122. Zhang JG, Hilton DJ, Willson TA, McFarlane C, Roberts BA, Simpson RJ, Alexander WS, Metcalf D and Nicola NA. Identification, purification and characterisation of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned IL-13 receptor and IL-4 receptor-alpha chain. J. Biol. Chem. (1997) 272:9474-9480

123. Zurawski G and De Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes, but not on T cells. Immunol. Today (1994) 15:19-26

124. Zurawski G, Hoshino T, Winkler-Pickett RT, Mason AT, Ortaldo JR and Young HA. IL-13 production by NK cells: IL-13-producing NK and T cells are present *in vivo* in the absence of IFNγ. J. Immunol. (1999) 162:51-59

6. ACKNOWLEDGEMENTS

The following individuals had a big role in this dissertation.

Profs. Ryffel and Brombacher	who provided the incentive for the study and who provided support and input through the project
Prof Gicquel	for providing us with the purine-deficient mycobacteria
UCT Animal Unit Staff	who weathered many questions and complaints, and still provided an excellent service
Dhuraiyah	for sorting out our endless administrative queries
Muazzam	who was my mentor when I started in the lab and who taught me most of the techniques which were so valuable for this project
Wendy, Lizette, Marilyn and Zoë	for their expertise with the histology
Reagon and Michelle	who did a great job with the genotyping of the mice
Nasiema	for much-needed assistance with the ELISA work
Aldina	who worked on the purine-deficient BCG project from which the purine-deficient MTB work followed
Barbara	for not giving me too much work while I was completing my thesis
Support staff	for provision of numerous luxuries like clean glassware, custom-made storage bags, that we often took for granted
And most importantly, Joseph	who gave of his time, and provided constructive input throughout the drafting of this dissertation.

To everyone who had any part in this dissertation – MANY MANY THANKS

APPENDIX A

LIST OF FIGURES AND TABLES

page no.

TABLES

APPENDIX B

ABBREVIATIONS

AFB	acid-fact bacillus
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
BMDM	bone marrow-derived macrophages
CFU	colony forming unit
DNA	deoxyribonucleic acid
DOTS	directly observed therapy system
ELISA	enzyme-linked immunosorbant assay
γc	common gamma chain
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
H37Rv	*Mycobacterium tuberculosis* H37Rv
HE	haematoxylin and eosin stain
HIV	human immunodeficiency virus
IFNγ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
IRS	insulin response element
iv	intravenous infection
kDa	kiloDalton
L major	*Leishmania major*

M-CSF	macrophage colony stimulating factor
MDR	multiple drug resistant
MHC	major histocompatibility complex
M bovis	*Mycobacterium bovis* BCG
MTB	*Mycobacterium tuberculosis*
N brasiliansis	*Nippostrongylus brasiliansis*
NK	natural killer
O/N	overnight
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNPP	p-nitrophenyl phosphate
$purC^{-/-}$	purine-deficient mutant
rBCG	recombinant BCG
rRNA	ribosomal ribonucleic acid
SCID	severe combined immune-deficient
STAT	signal transducer and activation of transcription
TB	tuberculosis
TBE	tris borate EDTA
TGFβ	transforming growth factor β
TH1	T helper type 1
TH2	T helper type 2
TNF	tumour necrosis factor
WHO	World Health Organisation
WT	wild type
ZN	Ziehl-Neelson stain

APPENDIX C

PREPARATION OF BACTERIAL DNA MARKER

Preparation of Luria broth and agar

In 1 litre of water, the following is added	Tryptone	10g
	Yeast extract	5g
	NaCl	10g

pH solution to 7, aliquot and autoclave
Before use, add ampicillin to a working concentration of 100µg/ml
For agar, add 15g of agar before autoclaving

Preparation of mini- and maxi-preps of *E. coli* XL Blue containing plasmid PTZ18R

Day 1 *E. coli* is streaked onto a Luria plate containing 100µg/ml ampicillin and incubated O/N at 37°C

Day 2 One of the colonies are picked and transferred into a vial containing 4ml of Luria broth with 4µl ampicillin. This is incubated with shaking at 37°C O/N

Day 3 200ml of Luria broth and 200µl ampicillin are added to 400µl of the mini culture from day 2 and incubated with shaking at 37°C O/N

Day 4 Using the ax500 Nucleobond kit (Wizard Plus maxipreps DNA purification system, cat no. A7270, Promega), plasmid PTZ18R is isolated from the maxiprep according to the manufacturer's recommendations

After the plasmid purification, the plasmid integrity is checked using A260/A280 absorbance readings

Digestion of PTZ18R

Plasmid DNA	33µg
10 x RE buffer	10µl
HinF	100U
Total volume	100µl

The digestion mixture is incubated O/N at 37°C and 5µl of the digest is added to each gel as a PCR DNA marker

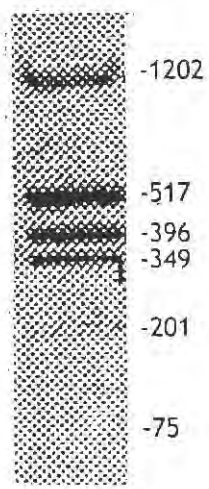


Figure. Size demarcations of bacterial DNA marker, plasmid PTZ18R digested with HinF I.

APPENDIX D

SOLUTIONS/REAGENTS USED

Acid alcohol 1%
1% HCl in 70% ethanol

Carbol fuchsin
Stock – 6% carbol fuchsin in 100% ethanol
Working solution – 10ml stock in 90ml 5% carbolic acid
Filter before use

Carbolic acid (phenol) 5%
5g carbolic acid in 100ml absolute alcohol

Citrate buffer, pH 6
Solution A – 0.01M citric acid
Solution B – 0.1M sodium citrate
Working solution – 9ml solution A, 41ml solution B in 500ml distilled water

Eosin 1%
1% eosin, 1% phloxine, 1 crystal of thymol as preservative

ELISA blocking buffer
2% instant milk powder or 4% BSA, 0.02% NaN_3 in 1xPBS
Solution is stirred until properly dissolved, filtered through Whatmann filter paper no. 1 and stored at 4°C. For BSA, solution was filtered through a 0.45µm filter

ELISA coating buffer
0.02% NaN_3 in 1xPBS
Filter sterilise through a 0.45µm filter, store at 4°C

ELISA dilution buffer
1% BSA (fraction V, #735 086, Boehringer Mannheim), 0.02% NaN_3 in 1xPBS
Filter sterilise through a 0.45µm filter, store at 4°C

ELISA substrate buffer

0.3g NaN_3, 97ml diethanolamine (#03116, Merck, liquify in 37°C water bath), 0.8 $MgCl_2.6H_2O$, 700ml distilled water, pH 9.8 with 10M HCl, then make to 1 litre
Sterile filter through a 0.45µm filter and store at 4°C in the dark

ELISA washing buffer 20x

20g KCl, 20g KH_2PO_4, 144g $Na_2HPO_4.2H_2O$, 800g NaCl, 50ml Tween-20, 100ml 10% NaN_3 were dissolved in 5 litres distilled water
The solution is sterile filtered through a 0.45µm filter and stored at 4°C. The buffer was diluted 1:20 as a working stock

H_2SO_4 25%

25ml H_2SO_4 in distilled water

Immunohistochemistry blocking serum 1.5%

150µl normal serum in 10ml 1xPBS

Immunohistochemistry secondary antibody

150µl normal mouse serum and 50µl biotinylated goat anti-rabbit IgG, mouse adsorbed, in 10 ml 1xPBS

KOH 1%

1g KOH in 100ml water

Lethal anaesthetic

500µl Rompun (20mg xylazine/ml, Bayer), 2ml Brevinase (100mg ketamine/HCl in 10ml, Anaket V), 9ml saline

Loefflers methylene blue

Stock – 0.8% methylene blue in 100% ethanol
Working solution – 30ml stock, 1ml 1% KOH and 99ml distilled water
Filter before use

Mayers haematoxylin

1g Mayers haematoxylin, 50g ammonium alum, 0.2g sodium iodate, 1g citric acid in 1 litre of distilled water

Methylene blue 0.8%

0.8g methylene blue in 100ml water

Middlebrook agar (cat no. 262710, Difco)
19g agar, 5ml glycerol in 900ml distilled water, autoclave at 121°C for 10 minutes, cool to 56°C in a water bath, add 100ml OADC before use
For $purC^{-/-}$ mutants, add 20µg/ml kanamycin (cat no. 1074466, Boehringer Mannheim) and 20µg/ml hypoxanthine (cat no. H-9636, Sigma)

Middlebrook broth (cat no. 0713-01, Difco)
4.7g broth, 2ml glycerol in 900ml distilled water, autoclave at 121°C for 10 minutes, add 100ml OADC before use
For $purC^{-/-}$ mutants, add 20µg/ml kanamycin (cat no. 1074466, Boehringer Mannheim) and 20µg/ml hypoxanthine (cat no. H-9636, Sigma)

Paraformaldehyde 4%
4g NaOH in 100ml water (1M), 40g paraformaldehyde
Add PBS to make up 1 litre, pH to 7.2, aliquot and freeze

PCR loading buffer 6X
0.25% bromophenol blue, 0.25% xylene cyanol, 50% glucose

PCR lysis buffer
50mM Tris-HCl pH 8, 100mM EDTA, 100mM NaCl, 1% SDS, 0.5mg/ml Proteinase K
Add 750µl for each mouse tail.

PCR reaction buffer 10X
Dissolve 100mM Tris pH 9, 500mM KCl and 0.1% gelatin. After autoclaving, add 1% Triton x-100 and 15mM $MgCl_2.6H_2O$

PCR running buffer (10X TBE)
108g Tris, 55g boric acid, 40ml 0.5M EDTA

Phosphate-buffered formalin 4%
40% formaldehyde solution in 1x PBS, pH 7.4

Phosphate buffered saline (PBS) 10x, pH 7.4
80g NaCl, 2.4g KH_2PO_4, 2g KCl, 14.4g $Na_2HPO_4.2H_2O$
Made up to 1 litre with distilled water, filter-sterilised through a 0.45µm filter and stored at room temperature. The buffer was diluted 1:10 as a working stock

Saline
0.9%NaCl in distilled water
The solution is autoclaved before use

Tween/saline
0.9% saline, 0.04% Tween-80 in distilled water
The solution is autoclaved before use