In Situ Expression of Cytokines and Cellular Phenotypes in the Lungs of Mice with Slowly Progressive Primary Tuberculosis

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The cellular phenotypes and the expression of cytokines were studied in the lungs of mice, using immunohistochemistry, during different phases of slowly progressive primary murine tuberculosis infection. During the first phase the small focal lesions in healthy mice contained predominantly interleukin-2 (IL-2)-expressing cells. A small number of tumour necrosis factor-α (TNF-α)-, monocyte chemoattractant protein-1 (MCP-1)- and IL-10-expressing cells were also present. IL-4-expressing cells were not detected. During the second phase the mice became unwell, but the bacterial counts and the size of focal lesions stabilized. IL-4-expressing cells appeared. The IL-10-, TNF-α- and MCP-1-expressing cells increased in number. On progression to phase three, the mice became seriously unwell and died rapidly. The inflammation spread to ≈ 80% of the lung parenchyma. There was a marked increase in the number of IL-10-expressing cells. Expression of other cytokines was similar to that observed in the second phase. In the lesions, 3–6% of the macrophages (Mφ) containing mycobacterial antigens expressed high levels of IL-10 and TNF-α. The absolute numbers of CD3-, CD4- and CD11b-expressing cells in the lesions increased with the progression of infection. The numbers of CD8$^+$ cells were reduced in the last phase of infection. The kinetics of T-lymphocyte subsets and the pattern of cytokine expression changed with the type and degree of tissue injury. The small number of Mφ with a heavy load of mycobacterial antigens may be the cause of this disturbance in cytokine balance, thus leading to progression of inflammation.

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INTRODUCTION

Acquired resistance to *Mycobacterium tuberculosis* infection is believed to depend upon cell-mediated immunity, with major effectors being mononuclear phagocytes and T lymphocytes [1, 2]. The immune response, however, contributes both to control of infection and tissue damage. In murine tuberculosis (TB), both CD4$^+$ [3, 4] and CD8$^+$ [3–6] T cells play a major role in protective immunity. The T helper 1 (Th1) cytokines − interleukin (IL)-2 and interferon-γ (IFN-γ) − promote cellular immunity by activating macrophages (Mφ), while T helper 2 (Th2) cytokines (IL-4, −5, −6 and −10) induce B-cell differentiation and promote humoral immunity [7]. Th2 cytokines are known to down-regulate the Th1 response to a number of microbial pathogens, consequently preventing infection from being resolved [8–10]. During the first 3–4 weeks of *M. tuberculosis*

infection, it has been shown that mice predominantly generate a Th1 response, whereas in the following weeks they also generate a Th2 response. This is interpreted as evidence that the Th1 cells are protective, whereas the Th2 cells are associated with the chronic and progressive disease [11, 12]. In human TB the presence of an increased level of Th2 cytokines in blood is shown to be associated with severe disease [13, 14]. A Th2-dominated immune response has been found in tuberculin-negative patients as compared with tuberculin-positive patients or healthy donors [15]. In leprosy, the Th1 cytokines are correlated with the tuberculoid form of disease while Th2 cytokines dominate in the lepromatous form [16].

We have previously established a mouse model of slowly progressive primary tuberculosis [17, 18]. Mice infected intraperitoneally (i.p.) with a moderate dose of *M. tuberculosis* strain H37Rv developed slowly progressive disease, which passed

through three distinctive phases [17]. The first phase was characterized by lack of clinical signs of disease, small granulomas in the lungs and progressive increase in bacterial counts. During the second phase, mice developed signs of disease. The bacterial counts and granuloma size, however, remained stable. The third phase was characterized by severe disease, high mortality and a sudden loss of the focal nature of the lesions and spread of inflammatory infiltrates to about 80% of lung parenchyma. This dramatic deterioration took place without significant change in the bacterial counts. Thus, the increase in morbidity and mortality appears to occur as a result of sudden changes in the host immune response.

The aim of this study was to analyse the composition of inflammatory cells and the cytokine profile *in situ* in the lungs throughout the course of infection, with particular emphasis on the immunological changes coinciding with a shift to different phases of infection.

MATERIALS AND METHODS

Mouse model. The mouse model for slowly progressive primary tuberculosis has been described previously [17]. Briefly, B6D2F1Bom hybrid female mice of approximately 12 weeks of age were used. Each mouse was inoculated i.p. with 200 µl of phosphate-buffered saline (PBS) containing $\approx 1.5 \times 10^6$ colony-forming units (CFU) of *M. tuberculosis* strain H37Rv. Mice were killed at four-weekly intervals up to week 41 and later at 52, 57 and 70 weeks after infection. Time-points selected for this study were weeks 8 and 12 from phase 1, weeks 24 and 29 from phase 2, and weeks 41 and 70 from phase 3.

Immunohistochemistry. One-half of a lung from each mouse was frozen in isopentane prechilled with liquid nitrogen. From week 29 onwards, perfusion of the lungs was performed, followed by fixation. The right lungs were perfused with a mixture (1 : 1, vol/vol) of distilled water and Tissue-Tek® (OCT compound, Leica Mikroscopi AS, Oslo, Norway) before freezing. The frozen specimens were embedded in OCT compound and stored at $-70\,°C$ until sectioning. Sections of approximately 5 µm were prepared, in a cryostat, from frozen tissues. All the antibodies used are described in Table 1. The sections were stained by using the avidin–biotin–peroxidase complex (ABC; Dako A/S, Glostrup, Denmark), as described previously [17]. Briefly, the sections were fixed in cold acetone for 10 min. Endogenous peroxidase activity was blocked with H_2O_2. Sections were also pretreated with avidin and biotin blocking solution in order to reduce endogenous biotin staining (Vector Laboratories, Burlingame, CA, USA). To block nonspecific binding mainly by Fc receptors, incubation was carried out with normal serum of the animal species in which the secondary antibody was made. Sections were then incubated overnight with primary antibody. Incubation with biotinylated secondary antibody was followed by incubation with ABC (Dako A/S). Location of antigen was visualized by incubating sections with H_2O_2 and 3-amino-9-ethylcarbazol-containing buffer. The sections were washed in Tris-PBS (TBS) between incubations. The slides were slightly counter-stained with Mayer's haematoxylin. As a negative control, 1% BSA/TBS instead of primary antibody, and an irrelevant antibody from the same species with the same immunoglobulin subtype as the primary antibody, were used.

Evaluation of immunostaining. For the evaluation of immunostaining, three to four animals were analysed at each time-point. The positively stained cells were enumerated in a light microscope as:

(1) Percentage of stained cells as evaluated by using a $\times 40$ ocular fitted with a 0.25×0.25 mm graticule. The positively stained cells and the total number of nucleated cells were counted for each field. For each specimen, three to five fields were counted separately from the inflammatory area and morphologically normal parenchyma.

Table 1. Antibodies used in the immunohistochemical staining

Antibodies	Specificity	Source
Rat IgG$_{2a}$, KT-3	CD3 (T cells)	AMS biotechnology, Frankfurt, Germany
Rat IgG$_{2a}$, κ, H129.19	CD4 (T cells)	PharMingen, CA, USA
Rat IgG$_{2a}$, κ, 53-6.7	CD8 α-chain (T cells)	PharMingen, CA, USA
Rat IgG$_{2b}$, M1/70.15	Macrophage CD11b	Seralab, Leicestershire, England
Rat IgG$_{2a}$, S4B6	IL-2	PharMingen, CA, USA
Rat IgG$_{2b}$, BVD4−1D11	IL-4	PharMingen, CA, USA
Rat IgG$_{2b}$, JES5−16E3	IL-10	PharMingen, CA, USA
Hamster IgG, 5H2	MCP-1	PharMingen, CA, USA
Rabbit polyclonal	TNF-α	Genzyme, Mexico
Rabbit polyclonal	Mycobacterial antigens (BCG antigens)	Dako A/S, Denmark
Rabbit anti-rat	Secondary antibody	Vector Laboratories, Burlinghame, CA
Swine anti-rabbit	Secondary antibody	Dako A/S, Denmark
Mouse anti-hamster	Secondary antibody	PharMingen, CA, USA

BCG, *M. bovis* bacille Calmette–Guérin; IL, interleukin; MCP-1, monocyte chemo-attractant protein-1; TNF-α, tumour necrosis factor-α.
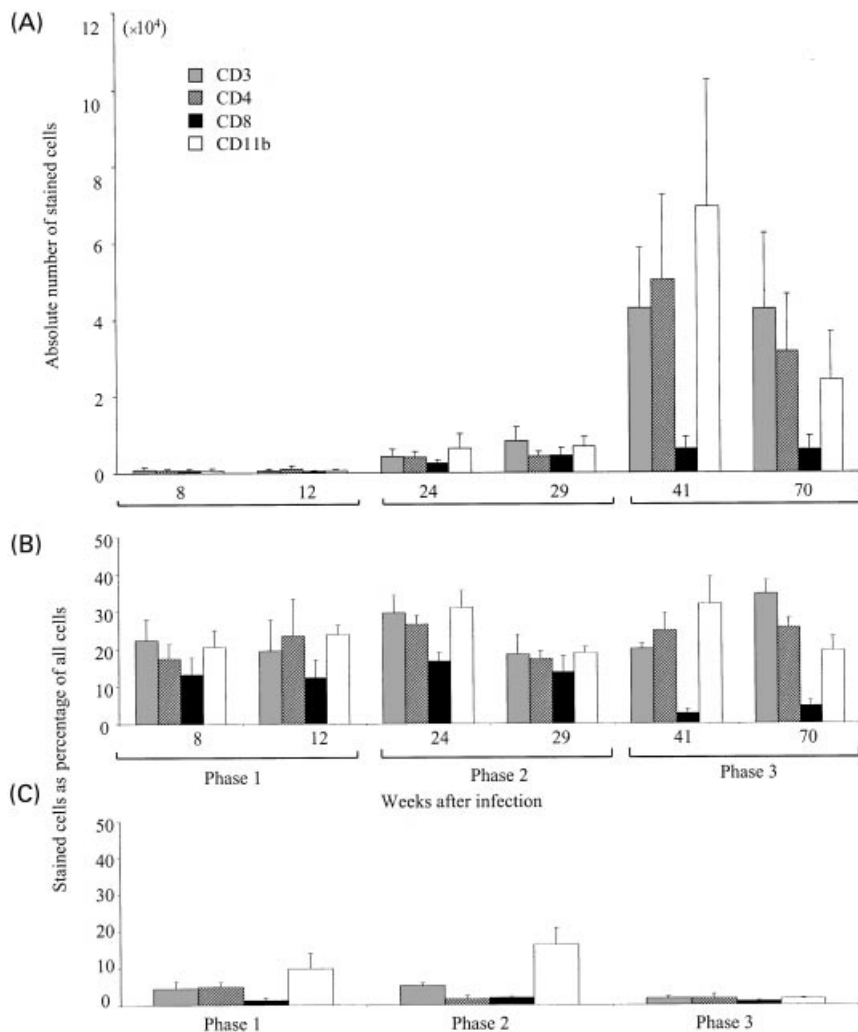
Fig. 1. *In situ* distribution of CD3 (▨), CD4 (▨), CD8 (■), and CD11b (□) positive cells in lung sections of mice with slowly progressive primary tuberculosis analysed by using immunohistochemical staining. (A) Absolute number of stained cells in the lesions. The number of all the inflammatory cells increased as the size of the focal lesions increased except for CD8$^+$ cells, which did not increase with shift to phase 3.

(B) Stained cells as a percentage of all cells in the lesions. The proportion of CD8$^+$ cells was significantly lower in phase 3 as compared to phase 2 ($P = 0.003$) and phase 1 ($P = 0.03$). The mice at week 41 were moribund (dying mice). At week 70 they constituted a selected population of late survivors owing to natural loss of all the moribund mice. The percentage of CD3$^+$ cells in dying mice at week 41 was low as compared to the late survivors at week 70 ($P = 0.03$).

(C) Stained cells as a percentage of all cells in the morphologically normal lung parenchyma. The proportion of CD11b$^+$ cells was significantly lower in phase 3 as compared to phase 2 ($P = 0.003$).

In each group, $n = 3$ or $n = 4$ mice except for normal parenchyma (C) where the number of mice in each group varied from two to seven. Bars represent standard error of the mean (SEM).

(2) Absolute number of stained cells in the inflammatory lesions in the lung section from each mouse, which was evaluated as: (average number of stained cells in three to five fields/area of each field) × area of the focal lesions in the section.

The area of the focal lesions was measured using a Leitz Dialux 22 EB microscope with ×1 and ×10 objective lens connected to a Sony CCD video camera and using the image processing system Videoplan (Kontron Bild-analyse, Eching, Germany), as described previously [17].

Statistical analysis. The Kruskal–Wallis test for three-group comparisons and the Mann–Whitney *U*-test for two-group comparisons were used.

RESULTS

The phenotype of mononuclear cells in the lungs during the course of infection

Figure 1(A) shows the absolute number of CD3$^+$, CD4$^+$, CD8$^+$ and CD11b$^+$ cells in the focal lesions during the course of infection. The low level of inflammatory cells corresponded to the small focal lesions observed during phase 1. During phase 2,

Table 2. Ratios of the percentage of CD11b$^+$ cells to CD3$^+$ cells and CD4$^+$ to CD8$^+$ cells in the lungs of mice with slowly progressive primary tuberculosis

	Weeks after infection	CD11b/CD3	CD4/CD8
Inflammatory lesions			
Phase 1	8	0.9	1.9
	12	1.7	2.1
Phase 2	24	1.3	1.6
	29	1.2	1.5
Phase 3	41	1.6	6.8*
	70	0.6	6.8*
Normal areas†			
Phase 1		2.8	3.9
Phase 2		1.9	0.8
Phase 3		1.3	2.9

* CD4/CD8 in phase 3 versus phase 1 and phase 2, *P*-value = 0.003.
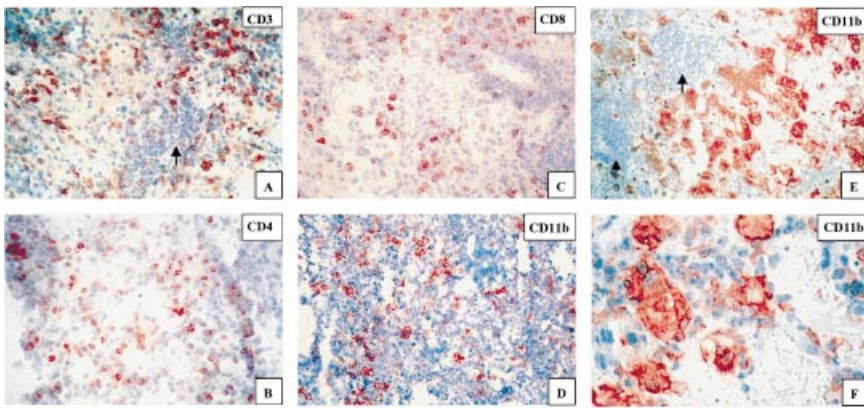† Morphologically normal parenchyma in infected lungs.

as the lesions became larger, the total number of all cell types increased. With shift to phase 3, when the inflammatory infiltrates spread to about 80% of the lung parenchyma, the total number of CD3$^+$, CD4$^+$ and CD11b$^+$ cells increased but the number of CD8$^+$ cells was largely unchanged.

Figure 1(B) shows the percentage of stained cells in the focal lesions during the course of infection. There was no significant change in the percentage of cells in the first and second phases of infection. With the shift to phase 3, the percentage of CD8$^+$ cells was lower than in phase 2 ($P = 0.003$) and phase 1 ($P = 0.03$). The percentage of CD4$^+$ cells was at some time-points higher or equal to the percentage of CD3$^+$ cells. This may be because CD4 molecules are expressed on some monocytes and Mϕ in addition to T cells [19]. The ratio of the percentage of CD11b$^+$ to CD3$^+$ cells was similar in all the phases. The ratio of CD4$^+$ to CD8$^+$ cells was similar in phases 1 and 2, while in phase 3 was significantly higher ($P = 0.003$) (Table 2). The main change was the reduced number of CD8$^+$ cells in the lesions in phase 3. In phase 3 at week 41, the mice were moribund, whereas mice killed at week 70 constituted a selected population of late survivors as all the moribund mice were lost by this time, as described in our previous study [17]. The ratio of CD11b$^+$ to CD3$^+$ cells in the dying mice was 1.6, but in the late survivors this ratio was reduced to 0.6 (Table 2). This difference was mainly the result of a lower percentage of CD3$^+$ cells in the dying mice as compared to the late survivors ($P = 0.03$).

Figure 1(C) shows the percentage of stained cells in the morphologically normal parenchyma of infected lungs. There was no difference in the number of stained cells between different phases of infection except for CD11b$^+$ cells which were significantly lower in number in phase 3 as compared to phase 2 ($P = 0.003$). The ratios of CD11b$^+$ to CD3$^+$ cells and CD4$^+$ to CD8$^+$ cells were not significantly different in different phases of infection (Table 2).

Figure 2 shows the pattern of staining of CD3-, CD4-, CD8- and CD11b-expressing cells. The staining was restricted to membranes in the case of CD3$^+$, CD4$^+$ and CD8$^+$ cells (Fig. 2A, 2B,

2C). In the lesions, only ≈ 20–35% of the lymphocytes expressed CD3 molecules. Some of the lymphocyte aggregates did not express CD3, CD4 or CD8 (Fig. 2A, 2B, 2C). These might thus be B lymphocytes or natural killer (NK) cells. With CD11b antiserum, both monocyte-like cells (Fig. 2D) and large vacuolated Mϕ (Fig. 2E, 2F) were stained. On the monocyte-like cells the stain was restricted to the membranes, whereas on large vacuolated Mϕ the stain was also found intracellularly. The large vacuolated Mϕ expressed CD11b strongly compared with the monocyte-like cells.

Kinetics of cells expressing IL-2, IL-4, IL-10, tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) in the lesions

Figure 3 shows the percentage of cells expressing IL-2, IL-4, IL-10, TNF-α and MCP-1 in the lungs during the course of infection. IL-2 was expressed throughout the course of infection. In phase 1, 30–35% of the cells expressed IL-2. In phase 2 a lower percentage of cells ($\approx 0.06\%$ at week 24 and $\approx 16\%$ at week 29) expressed IL-2 as compared to phase 1 ($P = 0.002$). In phase 3, the percentage of IL-2-expressing cells was higher (≈ 21–25%) compared with phase 2 ($P = 0.004$), but not significantly different from phase 1. IL-2 was expressed on cells both in Mϕ and lymphocyte aggregates, while some lymphocyte aggregates did not express IL-2 (Fig. 4A).

IL-4 was found on a very small number of cells (Fig. 4B). During phase 1, IL-4-expressing cells were not seen (Fig. 3). During phase 2, 0.1–0.2% of the cells expressed IL-4 ($P = 0.01$). With a shift to phase 3 there were no changes in the number of IL-4-expressing cells as compared to phase 2, and were higher compared with phase 1 ($P = 0.002$).

IL-10 was expressed by 0.15–0.3% of cells during phase 1 (Fig. 3). In phase 2, a higher number of cells expressed IL-10 (0.6–1.2%) as compared to phase 1 ($P = 0.016$). With the shift to phase 3 there was a marked increase in IL-10-expressing cells as compared to phase 2 ($P = 0.008$) and phase 1
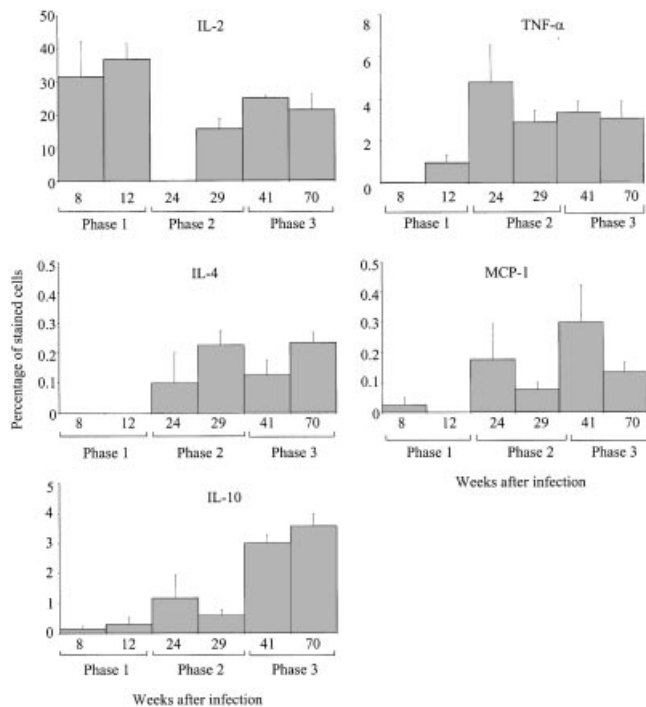
Fig. 3. *In situ* distribution of cells expressing interleukin (IL)-2, IL-4, IL-10, tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) in the lesions in lung sections of mice with slowly progressive primary tuberculosis analysed by immunohistochemistry. In each group, $n = 3$ or $n = 4$ mice. Bars indicate the SEM. Note the different scale on the *y*-axis for different cytokines. In phase 2 of infection as compared to phase 1, there were lower numbers of IL-2-expressing cells ($P = 0.002$) and a higher number of IL-4- ($P = 0.01$), IL-10- ($P = 0.016$), TNF-α- ($P = 0.001$) and MCP-1- ($P = 0.03$) expressing cells. In phase 3 as compared to phase 2 there was a significant increase in numbers of IL-10- ($P = 0.008$) and IL-2- ($P = 0.004$) expressing cells. Other cytokines were not significantly different from phase 2, and were significantly higher as compared to phase 1 (IL-4, $P = 0.002$; IL-10, $P = 0.001$; TNF-α, $P = 0.002$; MCP-1, $P = 0.005$) except for IL-2 which was at the same level as in phase 1.

($P = 0.001$). IL-10 was expressed by cells both in lymphocyte aggregates and by a small number of the vacuolated Mϕ in Mϕ aggregates (Fig. 4D, 4E). The bronchiolar epithelial cells and the bronchus-associated lymphoid tissue (BALT) cells also expressed IL-10.

TNF-α was expressed by 0.02–1% of the cells during phase 1 (Fig. 3). In phase 2 a higher number of TNF-α-positive cells (3–5%) were found compared with phase 1 ($P = 0.001$). In phase 3, the number of cells expressing TNF-α was not different compared with phase 2, and higher compared with phase 1 ($P = 0.002$). TNF-α was expressed exclusively by cells in the Mϕ aggregates (Fig. 4G, 4H). The lymphocyte aggregates did not contain TNF-α-expressing cells. TNF-α and IL-10 seemed to be expressed by the same cells in the Mϕ aggregates as the staining co-localized in the neighbouring sections (Fig. 4F, 4I).

During the course of slowly progressive TB, mycobacterial antigens were detected in only 3–6% of the Mϕ in the lesions, as has been described previously [17]. Neighbouring sections stained for TNF-α, mycobacterial antigens and IL-10 were analysed during phase 3. Cells with high levels of mycobacterial antigens in the Mϕ aggregates seemed to express high levels of IL-10 and TNF-α (Fig. 5).

The chemokine MCP-1 was expressed during all phases of the infection, but the number of positive cells was, in general, low, resembling that of IL-4-positive cells (Fig. 4C). In phase 2, the number of cells expressing MCP-1 (0.08–0.2%) increased as compared to phase 1 ($P = 0.03$) (Fig. 3). During phase 3, the number of MCP-1-expressing cells did not change significantly as compared to phase 2, and were higher as compared to phase 1 ($P = 0.005$).

DISCUSSION

The immunological profile was different in the different phases of infection in mice with slowly progressive primary TB. In the first phase, cells expressing Th1 cytokines dominated small focal lesions. A high number of cells expressed IL-2, but no IL-4-expressing cells and only a small number of IL-10-, TNF-α- and MCP-1-expressing cells were seen. During phase 2 there was a Th0 pattern of cytokines in the lesions. Cells expressing IL-4 appeared. The number of IL-10-expressing cells increased slightly. The number of cells expressing TNF-α and MCP-1 also increased. There was a reduction in the number of IL-2-expressing cells. With a shift to phase 3 there was a marked increase in the number of IL-10-expressing cells. There was still a predominance of IL-4, TNF-α and MCP-1, as in phase 2, and of IL-2, as in phase 1. There was a relative reduction in the number of CD8+ cells.

During phase 1 the mice were healthy. There was a progressive increase in bacterial numbers despite the predominance of Th1 cytokines. The inflammatory lesions were, however, small. TNF-α contributes to protection against *M. tuberculosis* infection in mice [20–23], in particular with Th1 cytokines [24]. MCP-1 is also shown to contribute less to the inflammation in the granulomas with a Th1 pattern of cytokines [25]. In phase 1, IL-2, TNF-α and MCP-1 therefore probably contribute to the granuloma formation and attempt to contain the infection, but are not able to control the bacillary multiplication.

During phase 2 the mice start to show signs of illness [17]. The host immunity controlled bacillary multiplication and pathology. There was, however, a reduction in the number of IL-2-producing cells, as compared to phase 1, and the appearance of IL-4-expressing cells. IL-4 belongs to the Th2 group of cytokines [7] and has been associated with severe or chronic disease [9–16]. IL-4 has, however, also been shown to contribute to the control of bacillary multiplication and inflammatory response [26–30]. It has been shown to act as a Mϕ-activating factor [26, 27], can induce monocyte infiltration *in vivo* [28] and multinucleated giant cell formation *in vitro* [29]. Recombinant IL-4 has been shown to bring about the arrest of *M. tuberculosis* multiplication
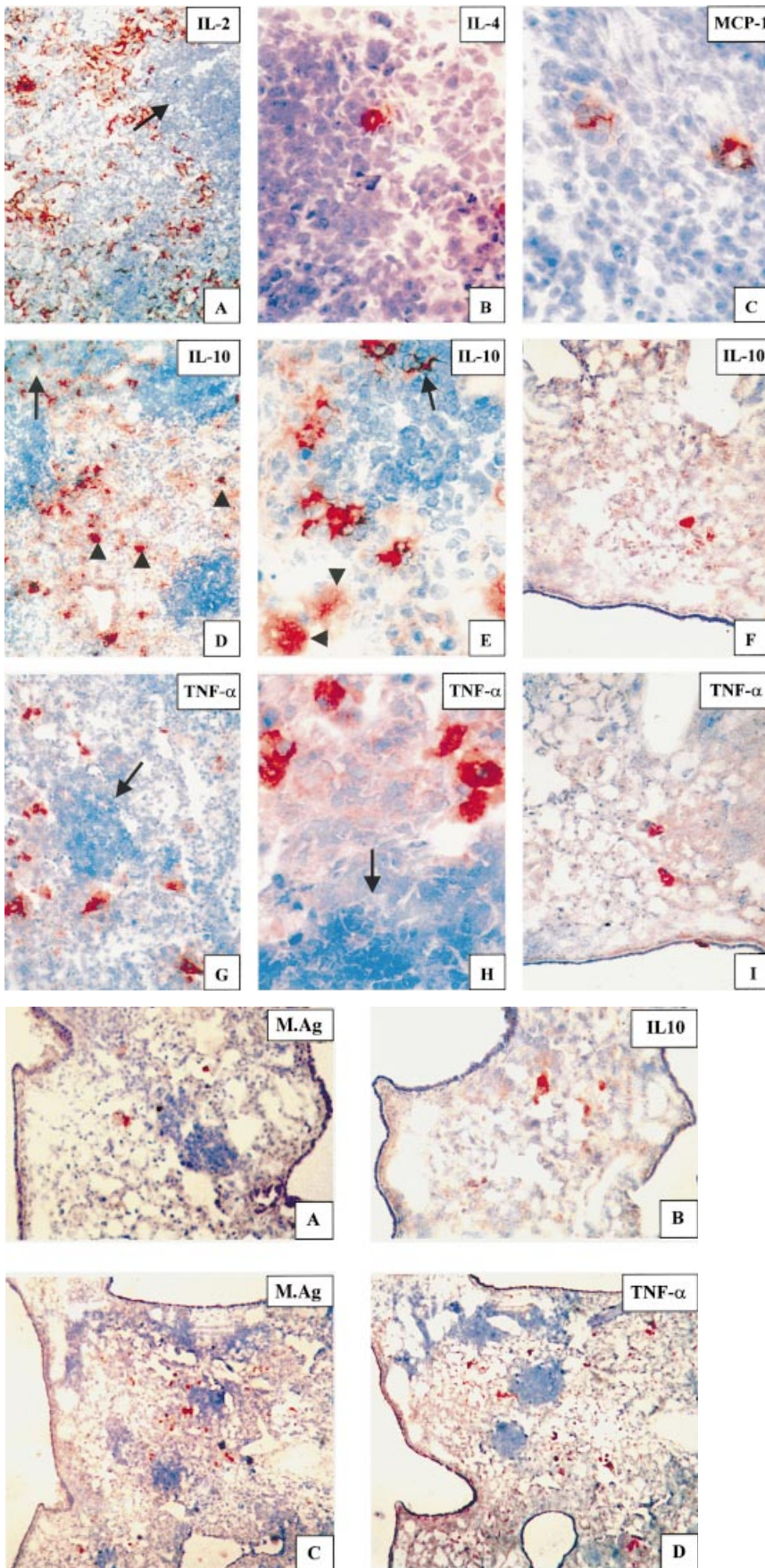
Fig. 4. Lung tissue from mice with slowly progressive primary tuberculosis showing the staining pattern of interleukin (IL)-2-, IL-4-, monocyte chemoattractant protein-1 (MCP-1)-, IL-10- and tumour necrosis factor-α (TNF-α)-positive cells in the focal inflammatory lesions. Frozen sections were stained with immunoperoxidase using the avidin–biotin–peroxidase complex (ABC). (A)(×110) Mainly lymphocytes express IL-2 (week 29 after infection). Note some lymphocyte aggregates without stain for IL-2 (arrow). (B)(×436) IL-4 was detected in a very small number of cells (week 24). (C)(×436) MCP-1 was expressed by a small number of cells, like IL-4 (week 24). (D)(×110) and (E)(×436) IL-10 was expressed by lymphocytes (arrows) and macrophages (arrow heads) (week 24). Note the small number of macrophages with very strong expression of IL-10. (G)(×110) and (H)(×436) TNF-α was expressed by macrophages (week 40). Note the very strongly stained macrophages. Lymphocyte aggregates do not contain TNF-α expressing cells (arrows). (F)(×110) and (I)(×110) TNF-α and IL-10 seemed to be expressed by the same cells as the staining co-localized in neighbouring sections (week 70).

Fig. 5. Lung tissue from mice with slowly progressive primary tuberculosis showing the pattern of co-localization of mycobacterial antigen (M.Ag)-containing cells with interleukin (IL)-10-positive (A)(×108), (B)(×108) and tumour necrosis factor-α (TNF-α)-positive (C)(×60) (D)(×60) cells in the same areas in focal inflammatory lesions (week 70 after infection). Frozen sections were stained with immunoperoxidase using the avidin–biotin–peroxidase complex (ABC).

in murine bone marrow-derived Mϕ [30]. In phase 2 there was a significant increase in the number of TNF-α- and MCP-1-expressing cells. The increase in the number of MCP-1-expressing cells could be promoted by IL-4 [25, 31, 32]. The increase of MCP-1 in afflicted mice is in agreement with the results obtained in human TB [33, 34]. MCP-1 can contribute to control of infection by recruiting monocytes [35] and CD4$^+$ T cells [36] to the lesions. TNF-α stimulates the antimicrobial Mϕ functions [20]. It drives the delayed type hypersensitivity response by inducing a chemokine response, leading to recruitment of Mϕ in the lesions [22, 37, 38]. This restricts the infection and prevents dissemination [38]. The mixture of IL-2, IL-4 and a small amount of IL-10 with TNF-α and MCP-1, as seen in the present study, thus seems to contribute to the control of bacillary multiplication and pathology in phase 2. Increase in the level of proinflammatory cytokines seems to cause the appearance of signs of disease.

When the infection progressed to phase 3 there was an extensive spread of inflammation to about 80% of the lung parenchyma, and a sudden increase in morbidity and mortality was found. Concomitantly there was a marked increase in the number of IL-10-expressing cells. An increased level of IL-10 is found to be associated with advanced human TB [13] and pathogenesis of *M. avium* [39] and *M. bovis* bacille *Calmette–Guérin* (BCG) [40] in mice. But how could the extensive immune pathology in phase 3 be compatible with the suppressive effect of IL-10 on T-cell differentiation, function and proliferation [8, 41–43]? IL-10 has been shown to cause immune stimulation rather than inhibition *in vivo* in IL-10 transgenic diabetic mice [44–46], BCG-infected mice [40] and in IL-10-transfected tumours transplanted in mice [47]. IL-10 has also been shown to have a direct effect on T-cell growth in *in vitro* systems, particularly in concert with other cytokines [48]. The increased level of IL-10 could thus contribute to immune pathology in phase 3 because of its immune stimulatory functions.

There was no significant change in the number of TNF-α-expressing cells in phase 3 as compared to phase 2. The lung cells from these mice produced high levels of IFN-γ [49]. Despite the presence of TNF-α and IFN-γ, which contribute to protection [20–22, 30], there was progression of disease. The effects of these cytokines, to activate Mϕ for killing of intracellular organisms, seem to be overridden by excess IL-10 [23, 40, 50–52]. IL-10 is shown to cause inactivation of TNF-α by inducing the release of soluble TNF-receptor 2 [53]. The sustained production of TNF-α by the infected Mϕ, in the face of reduced antimicrobicidal functions of the Mϕ, can cause continued recruitment of inflammatory cells into the lesions and consolidation of the lung tissue.

Mycobacterial antigens were detected in 3–6% of the Mϕ in the lesions. These antigen-loaded cells expressed high levels of IL-10 and TNF-α. These heavily infected Mϕ also expressed large quantities of Fas ligand [54]. These Mϕ therefore might eliminate Fas-expressing cytotoxic lymphocytes, protecting themselves from being killed and thereby creating an intracellular sanctuary for *M. tuberculosis* [54]. IL-10 is shown to have an antiapoptotic function, causing an increase in the survival of the cell [53, 55, 56]. These infected Mϕ seem to be a continuous

focus for the production of proinflammatory cytokines, leading to an increased accumulation of inflammatory cells. The increase in pathology seems to be the main cause of progression from disease to mortality in the mice.

In spite of the Th0 pattern of cytokines in phases 2 and 3 and the presence of TNF-α, no necrosis was observed, except in some of the dying mice at week 41, as described in our previous morphological description of the model [17]. TNF-α has been proposed as a major mediator of necrosis in *M. tuberculosis*-infected tissue when Th1 cytokines are superimposed by Th2 cytokines [24, 57]. However, in *M. tuberculosis*-infected mice with disruptions in the principal TNF receptor or treatment with anti-TNF-α antibody, tissue necrosis is seen [22]. This shows that mechanisms other than TNF-α contribute to necrosis.

Mycobacteria have evolved mechanisms to evade the phagolysosomal environment and probably escape to the cytoplasm [58]. Cytotoxic CD8$^+$ cells recognize the antigens when presented with major histocompatibility complex (MHC) class I molecules [6]. The perforin-containing cytotoxic granules in CD8$^+$ cells can kill the bacilli directly and are important for the containment of infection during the late stage of murine TB [6]. The shift to phase 3 coincided with a significant decrease in the percentage of CD8$^+$ cells. This could be a result of the change in cytokine pattern on CD8$^+$ cells as shown by some studies. In murine schistosomiasis with a Th0 pattern of cytokines, the generation of CD8$^+$ cells was found to be defective [59]. Mature mouse CD8$^+$ cells were shown to develop into a CD8$^-$ CD4$^-$ population in the presence of IL-4 [60]. In our study the question remains whether the reduction in CD8$^+$ cells is the cause or effect of changes in cytokine patterns.

In conclusion, the kinetics of T-lymphocyte subsets and the pattern of cytokines produced in the lungs changed with the type and degree of tissue injury in mice with slowly progressive primary TB. The mixed Th1/Th2 cytokine pattern was associated with the evolution of disease. A small number of Mϕ with a heavy load of mycobacterial antigens may be the cause of this disturbance in cytokine balance, thus leading to disease and mortality. This model contributes to further understanding of the factors responsible for progression of subclinical infection to disease and to mortality.

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