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In Situ Expression of CD40, CD40L (CD154), IL-12, TNF-α, IFN-γ and TGF-β1 in Murine Lungs during Slowly Progressive Primary Tuberculosis

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Abstract

The distribution and expression of CD40, its ligand CD40L (154) and related cytokines interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and transforming growth factor-β1 (TGF-β1) were studied in the lungs of B6D2F1 hybrid mice during slowly progressive primary tuberculosis (TB) by immunohistochemistry. CD40 and CD40L are implicated in cell-mediated immunity (CMI) causing activation or apoptosis of infected cells. The phenomenon of apoptosis is associated with *Mycobacterium tuberculosis* survival. In this study, using frozen lung sections ($n = 33$), our results showed increased CD40, IL-12 and TGF-β1 expression in macrophages with progression of disease. High percentages of mycobacterial antigens (M.Ags), CD40L and IFN-γ expression were maintained throughout infection, and TNF-α-expressing cells were decreased. In lymphocytes, the percentage of IFN-γ-positive cells was increased, but CD40L and IL-12 were maintained with the progression of disease. M.Ags, CD40 and CD40L were expressed in the same areas of the lesions. We conclude that changes in the expression of CD40–CD40L and cytokines associated with *M. tuberculosis* infection favour the hypothesis that *M. tuberculosis* causes resistance of host cells to apoptosis causing perpetuation of infection.

Introduction

A mouse model of slowly progressive *Mycobacterium tuberculosis* was developed in our laboratory [1]. In the model, different phases of infection were described on the basis of bacillary numbers, clinical signs and extent of inflammation in the lungs. During early phase 1 (weeks 8–12), bacillary numbers increased and the mice started to show signs of illness. Later, in phase 2 (weeks 16–37), the mice were moderately sick but mortality was low and the granulomas occupied one-third of the lung parenchyma. During phase 3 (weeks 40–70), the mice became sick and mortality was high despite no increase in bacillary numbers. The inflammatory cells infiltrated two-thirds of the lung parenchyma, and the bronchi became dilated with loss of epithelial cells and architectural pattern compared to phase 1. With progression of time, the distribution of inflammatory cells became more defined in the lesions. Cells staining with macrophage (Mϕ) marker CD11 increased in size and attained a vacuolated appearance and formed separate aggregates referred to as Mϕ-predominant aggregates (MPDAs). Small cells with scanty cytoplasm formed separate aggregates referred to as lymphocyte-predominant aggregates (LPDAs). During initial infection at week 8, cell aggregates were mixed and became more organized into separate MPDA and LPDA from week 16. In the LPDAs, only about 20–35% of the lymphocytes expressed CD3. Some of the lymphocytes did not express CD3, CD4 and CD8 [2]. These might be B lymphocytes or natural killer cells. The MPDAs were formed around the LPDAs. About 5–7% of the vacuolated Mϕs containing large amounts of mycobacterial antigens (M.Ags) also expressed FasL, while the expression of Fas was weak on these cells [3]. We also showed that the M.Ags containing Mϕs had increased the expression of Bcl-2, while Bax was reduced in the same cells [4]. These findings supported a hypothesis that *M. tuberculosis* can modulate the regulators of apoptosis in order to survive in Mϕs causing perpetuation of infection.

Following *M. tuberculosis* infection, Mϕs, lymphocytes and other antigen-presenting cells (APCs) are activated resulting in the release of cytokines enhancing inflammatory cell activation and granuloma formation [5–8].

Tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) increase microbicidal efficiency of Mϕs with synergistic effects in Mϕs activation during *M. tuberculosis* infection [7]. TNF-α has a crucial role in granuloma formation during the acute phase of *M. tuberculosis* infection [9]. However, transforming growth factor-β1 (TGF-β1) released later has anti-inflammatory functions [10, 11]. TGF-β1 has an important role in the alteration of production of TNF-α, decreasing the expression of interleukin-12 (IL-12) receptor, IFN-γ-induced class II expression and Mϕs activation resulting in diminished oxidative responses [11–13]. IL-12 produced is associated with augmenting CD40L (CD154) expression on T cells [14].

CD40L has paracrine activation of the CD40 signalling along with IL-12 inducing the expression of CD40 and costimulatory signals [14–16]. CD40 is a receptor belonging to the TNF family [17, 18]. It is constitutively expressed on Mϕs, B cells and other APCs, while its ligand, CD40L, is expressed on lymphocytes and other APCs [19]. Ligation of CD40 was shown to provide costimulatory signals for the development of T-cell-dependent immunity, regulating both humoral and cell-mediated immune (CMI) response [20]. The role of CD40 and CD40L signalling is controversial in apoptosis. Some data suggest that CD40 signalling induces FasL expression, nuclear factor-kappa B and AP-1 signalling, resulting in susceptibility to Fas-dependent apoptosis in human primary cultures of intrahepatic biliary epithelial cells, whereas in cancer cell lines CD40 ligation has been associated with resistance to apoptosis [21, 22]. Similarly, during infection with *M. tuberculosis*, the roles of CD40 and CD40L in mediating immune responses are unclear [23, 24].

The main aim of this study was to investigate whether both CD40 and CD40L were expressed in lungs during slowly progressive primary murine tuberculosis and relate this to the immune response to M.Ags and cytokines (IL-12, TNF-α, IFN-γ and TGF-β1) using immunohistochemistry. This might help in the understanding of cellular mechanisms in *M. tuberculosis* infection. Our hypothesis was that *M. tuberculosis* infection modulates CD40–CD40L signalling, in concert with cytokines, in a manner that Mϕs containing bacilli become refractory to activation by altering the expression of these molecules, thereby safeguarding them and the bacilli from CMI killing.

Materials and methods

Mice. B6D2F1 hybrid mice were purchased from Bomholt Gård Breeding and Research Centre Ltd (Bomholtgård, Denmark). They were infected intraperitoneally with 1.5×10^6 CFU of H37Rv *M. tuberculosis* and killed at specific time points, as previously described [1]. Three mice were killed for each time point at weeks 8, 12, 16, 20, 24, 29, 33, 37, 41, 52, 57 and 70. The lungs were fixed with Tissuetek® (OCT Compound, Leica Microscopi AS, Oslo, Norway) and frozen 4 years ago in a deep freezer at −76 °C and used for the current study. Sections were placed onto 0.1% poly L-lysine solution-coated slides (Sigma Diagnostics Inc., St. Louis, MO, USA). The sections were also stored at −76 °C until time of staining. Serial sections were stained with haematoxylin and eosin, anti-bacille Calmette–Guérin (BCG) (Dako A/S, Copenhagen, Denmark), anti-CD40, -CD40L, -IL-12, -TNF-α, -IFN-γ and anti-TGF-β1. Anti-BCG was used for detecting M.Ags. *Mycobacterium bovis* BCG and *M. tuberculosis* are highly similar [25], and antigens detected by anti-BCG were, therefore, referred to as M.Ags in our results.

Procedures for staining. Staining was carried out according to modified standard procedures in use by our laboratory and has been described previously [1]. Briefly, frozen sections were left at room temperature for 30 min and then in 50% acetone for 30 s and absolute acetone for 5 min. Sections were treated with 0.3% H_2O_2 for 20 min to block endogenous peroxidase activity. Nonspecific binding was blocked for 15 min with drops of avidin and biotin solutions (Vector Laboratories Inc., Burlingame, CA, USA), washing in between for 5 min with phosphate-buffered saline (PBS). Further blocking of nonspecific receptors was achieved by either 10% normal swine serum (NSS) in 4% bovine serum albumin (BSA) in Tris-buffered saline (TBS) pH 7.20 for staining with anti-BCG or 10% normal rabbit serum (NRS) in 4% BSA in PBS pH 7.20 for 60 min at room temperature for other antibodies used. Primary antibodies consisted of anti-BCG, -CD40, -CD40L, -IL-12p35a, -TNF-α, -TGF-β1 and anti-IFN-γ at dilutions specified in Table 1, all in 1.5% NSS or NRS/4% BSA in TBS or PBS pH 7.20, respectively. The test and control sections were incubated in a humidified chamber at room temperature for 60 min. Negative controls were test sections treated with PBS instead of primary antibody. After washing as described above, sections were incubated for 30 min with either swine anti-rabbit IgG only for anti-BCG or rabbit anti-goat IgG at dilution of 1 : 100. The slides were washed and incubated with avidin–biotin complex (ABC-Dako A/S, Glostrup, Denmark) for 30 min and washed again. Then, sections were immersed once in 0.1% Triton®X-100 in TBS pH 7.6 solution (Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) and rinsed with PBS. Visualization was achieved with application of distilled 3-amino-9-ethyl carbazol (Vector Laboratories Inc.) prepared just before use. Slides were counter stained with Harris haematoxylin and mounted with cover slides and DPX Vector Immunomount.

Cell counting. The slides were evaluated for immune staining with respective antibodies. Cells were counted using Leitz microscope (Wetzlar, West Germany) at magnification ×40 objective and ocular piece fitted with 10 × 10 mm graticule. Counting was done by random selection of four to five fields with aggregates of moderate

Table 1 Antibodies used in the immunohistochemical staining

	Dilution and specificity	Source
Antibodies		
Rabbit polyclonal IgG	1 : 5000 Mtb antigens (anti-BCG)	Dako A/S, Glostrup, Denmark
Goat polyclonal IgG (T-20), Sc-1731	1 : 50 anti-CD40	Santa-Cruz Biotechnology, Santa Cruz, CA, USA
Goat polyclonal IgG (K-19), Sc-1594	1 : 00 anti-CD40L	Santa-Cruz Biotechnology, Santa Cruz, CA, USA
Goat polyclonal IgG (M-16), Sc-9350	1 : 50 anti-IL-12Ap35	Santa-Cruz Biotechnology, Santa Cruz, CA, USA
Goat polyclonal IgG AF-410NA	1 : 50 anti-TNF-α	R&D Systems, Minneapolis, MN, USA
Goat polyclonal IgG (D-17), Sc-9344	1 : 50 anti-IFN-γ	Santa-Cruz Biotechnology, Santa Cruz, CA, USA
Goat polyclonal IgG Sc-146-G	1 : 50 anti-TGF-β1	Santa-Cruz Biotechnology, Santa Cruz, CA, USA
Secondary antibodies		
Biotinylated rabbit anti-goat IgG (H+L) BA-5000	1 : 200	Vector Laboratories, Burlingame, CA, USA

IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-12, interleukin-12; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α.

or strong staining in different morphological areas in the lungs. Cells at the periphery of the sections were excluded. Based on these criteria, all stained cells and the total number of nucleated cells were counted in each field. Percentage of stained cells was calculated from averages of overall total of cells counted and recorded as percentage of all cells. Data was analysed by SPSS 9.0 statistical program 2000. The Mann–Whitney *U*-test was used to compare two independent groups.

Results

Expression of M.Ags, CD40, CD40L, IL-12, IFN-γ, TNF-α and TGF-β1 in lesions of infection

There were few Mϕs expressing M.Ags, CD40, CD40L, IL-12, IFN-γ, TNF-α and TGF-β1 during week 8. From week 12, however, variable numbers of positive cells were observed. Figures 1 and 2 show the percentages of M.Ags, CD40, CD40L, IL-12, IFN-γ, TNF-α and TGF-β1 during the two phases of infection.

M.Ags staining was strong and cytoplasmic and detected in MPDAs only (Fig. 1A). The percentage of M.Ag-positive Mϕs was low during early infection at week 8, increasing dramatically at week 12 and then maintained during both phases 1 and 2 with no significant differences between the two phases ($P = 0.396$). In normal looking infected lungs, M.Ags were occasionally detected in bronchial epithelial cells during phase 1 only.

CD40 staining was strong and expressed on membranes and cytoplasm. Staining was detected only in MPDAs (Fig. 1B). The staining varied from moderate during initial infection to very strong in late phase of infection. During phase 1, the percentage of CD40-positive cells was low. During phase 2, a statistically significant increase in the percentage of positive cells was observed ($P = 0.04$). In normal looking infected lungs, CD40-positive staining was detected in endothelial cells and alveolar macrophages (AMϕs) as well as in smooth muscle of the bronchial tree.

CD40L staining was variable in expression (Fig. 1C). The strength of staining varied from moderate to strong corresponding with duration of infection. The staining was moderate during early infection at week 8 and very strong at week 70. In MPDAs, during phase 1, there was a small percentage of CD40L-positive cells at week 8, gradually increasing to a peak at week 24. A gradual decrease was then observed but there was no statistical difference between the phases. In LPDAs (Fig. 1D), the percentage of CD40L-positive lymphocytes was less compared with that of positively stained Mϕs. During phase 1, CD40L-positive lymphocytes were detected at week 12. There was variable expression during phase 1 and 2 but with no statistical difference. In normal looking infected lungs, CD40L expression was strong during early infection and detected in AMϕs, bronchial smooth muscle and some epithelial cells.

IL-12 expression was mainly cytoplasmic and varied from moderate to very strong in the positive cells (Fig. 2A,B). In some Mϕs, however, anti-IL-12 p35a staining was membranous and granular. In MPDAs (Fig. 2A), during phase 1, the percentage of IL-12-positive cells was low compared with M.Ag- and CD40-positive cells at the same time points. The pattern shows few IL-12-positive cells during phase 1, but during phase 2, a significant increase was observed and maintained through the entire period ($P = 0.007$).

There was a low percentage of IL-12-positive cells in LPDAs (Fig. 2B) compared with those in the MPDAs. During phase 1, the percentage of positive cells increased from week 20 and was maintained during phase 2. In infected normal looking lungs, IL-12p35a was strongly positive in AMϕs with cytoplasmic and membranous granularity. Some bronchial epithelial cells were also positively stained.

IFN-γ was expressed diffusely (Fig. 2C,D). The intensity varied from weak to moderate and strong with duration of infection. There was a lower percentage of IFN-γ-positive cells compared with M.Ag- and CD40-positive cells in the same areas during both phases. In MPDA (Fig. 2C), the percentage of IFN-γ-positive cells increased during phase 1

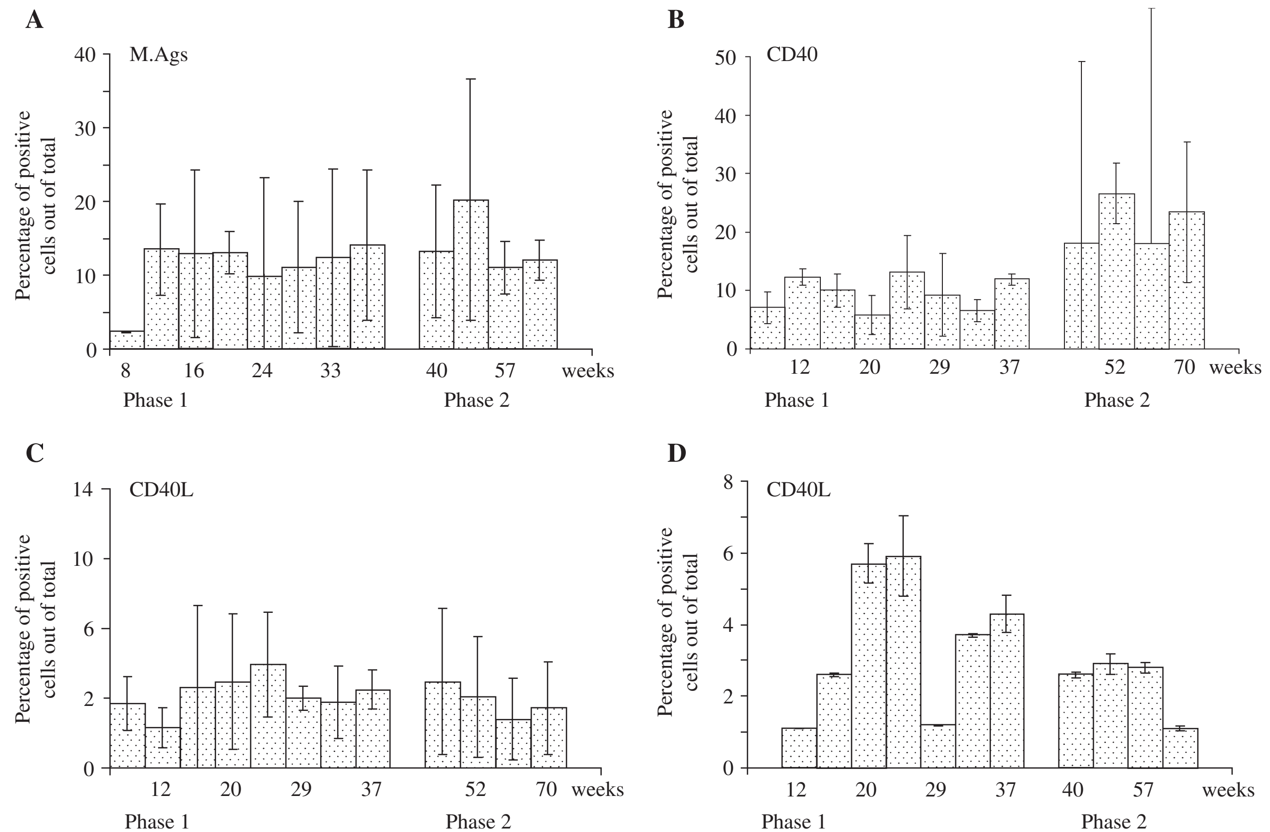

Figure 1 Distribution of infected cells positively stained for mycobacterial antigens (M.Ags) and anti-CD40, -CD40 ligand in lung lesions of B6D2F1 hybrid mice with slowly progressive primary tuberculosis analysed with immunohistochemistry. Total of 33 mice were killed, three mice per week. Weeks 8–37 and 40–70 represent phases 1 and 2 of infection, respectively. The bars represent percentages of positively stained cells out of the total, and error bars represent standard errors of the means (SEM). Percentages of positive cells in macrophage-predominant aggregates (MPDAs) are shown in A–D. With progress of infection, CD40-positive cells increased in MPDAs during phase 2. The distribution of infected cells stained with anti-CD40, CD40L (154) in MPDAs and lymphocyte-predominant areas (LPDAs) is shown in C and D. There was no difference in the percentages of CD40L-positive cells in both MPDAs and LPDAs with progress of infection.

and declined at week 37. There was, however, no difference between phases 1 and 2 ($P > 0.2$). In LPDA (Fig. 2D), the distribution of IFN-γ-positive cells was low and stable as compared with those in MPDAs. During phase 2, the percentage of positive cells was increased compared with phase 1 ($P = 0.027$). In normal looking infected lungs, IFN-γ expression was weak and diffuse in AMϕs and smooth muscle.

TNF-α expression was cytoplasmic and varied from very strong during initial infection to moderate and/or strong during late phases of infection (Fig. 2E). Low numbers of TNF-α-positive cells were observed compared with M.Ags and CD40. During phase 2, a significant decrease in the percentage of TNF-α-positive cells was observed as compared with phase 1 ($P = 0.011$). No TNF-α-positive cells were observed in LPDAs. In infected normal looking lungs, TNF-α-positive cells were occasionally detected in AMϕs and endothelial cells as well as in smooth muscle cells.

TGF-β1 expression was only observed in MPDAs (Fig. 2F). Anti-TGF-β1 staining was granular, cytoplasmic and membranous varying from moderate to very strong in positive cells. Stronger signals were observed during the middle half of phase 2. The percentage of positive cells was initially low and then increased gradually during phase 1, whereas during phase 2, a significant increase was observed as compared with phase 1 ($P = 0.011$). In infected normal looking lungs, TGF-β1 was strongly positive in AMϕs. Some bronchial epithelial cells were also positively stained.

Correlation between CD40, CD40L, IL-12 and mycobacterial antigens

Figure 3A-D shows the distribution of M.Ags, CD40, CD40L and IL-12 during infection with *M. tuberculosis* in slowly progressive disease. In MPDAs, M.Ag-positive cells were distributed in areas that correspond with the same areas staining with CD40-positive cells. M.Ag-positive cells were distributed in the pale staining areas, which varied in size and seem to increase with duration of infection. Strong CD40-positive cells were similarly seen in the same areas; however, there were more M.Ag-positive cells compared with CD40-positive cells in the lesions. Nonetheless, the patterns of staining with anti-CD40

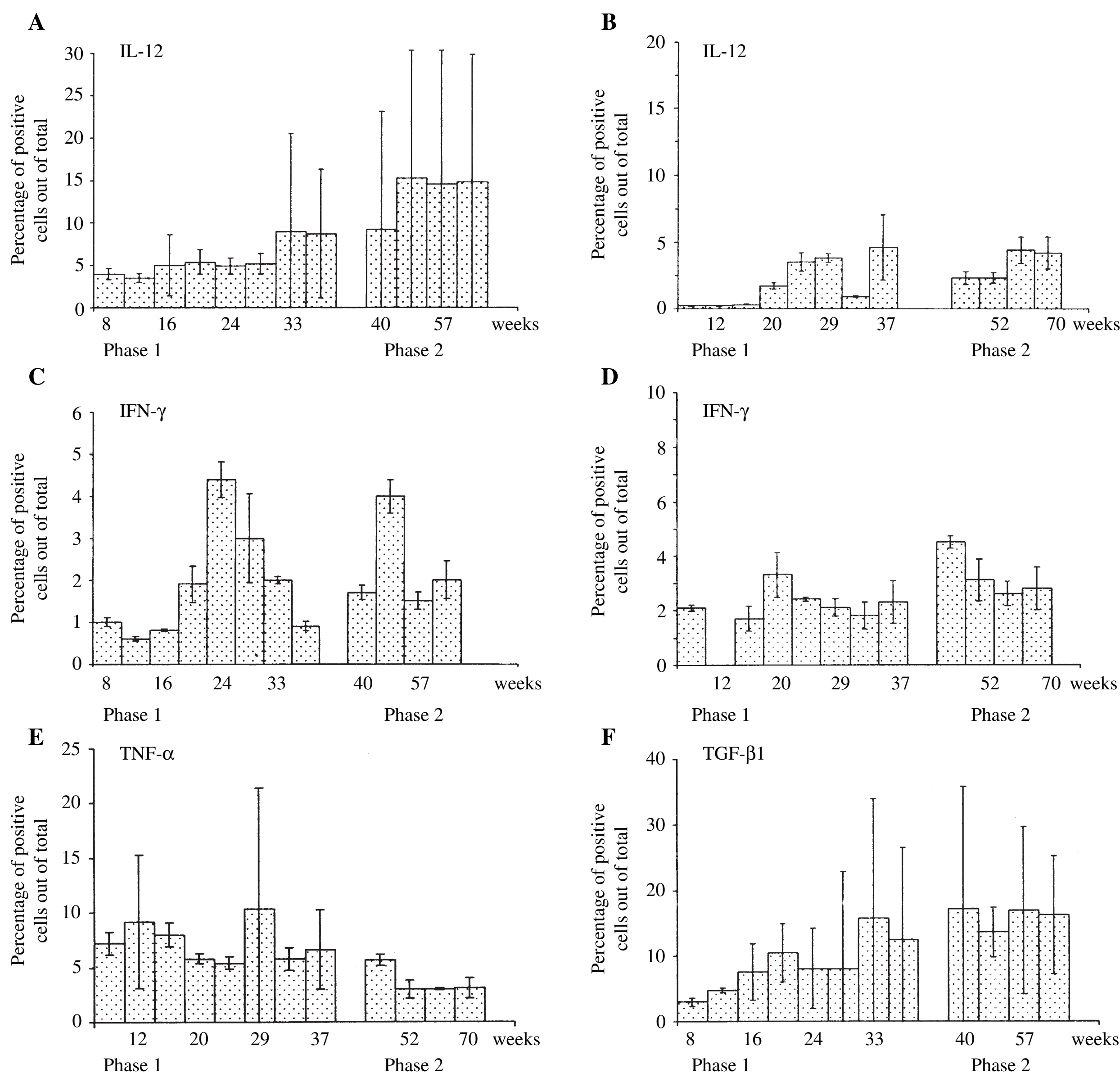

Figure 2 Distribution of infected cells positively stained for interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and transforming growth factor-β1 (TGF-β1) in lung lesions of B6D2F1 hybrid mice with slowly progressive primary tuberculosis analysed with immunohistochemistry. Total of 33 mice were killed, three mice per week. Weeks 8–37 and 40–70 represent phases 1 and 2 of infection, respectively. The bars represent percentages of positively stained cells out of the total, and error bars represent standard errors of the means (SEM). In macrophage-predominant aggregates (MPDAs), with progress of infection, IL-12- (A), TGF-β1 (F)-positive cells increased during phase 2. The percentage of IFN-γ-positive cells (C) was maintained; however, a reduction in percentages of TNF-α-positive cells was observed (E). In lymphocyte-predominant areas (LPDAs) (B, D), there was an increase in IFN-γ-positive cells during phase 2 as compared to phase 1 (D), while percentage of IL-12-positive lymphocytes was maintained (B).

and M.Ags positively correlated through the phases (Fig 1A,B). The areas of cells positive for anti-CD40L colocalize with areas of cells positive for M.Ags, IL-12 and CD40 (Figs 3A,D and B,C). In 26 of 33 serial sections that were 5–15 μm thick, CD40L-positive cells were distributed in the same areas containing CD40 and M.Ag-positive cells. The pattern of expression of CD40, CD40L, M.Ags and IL-12 was similar during early phase 1 (Figs 1A-C and Fig. 2A), but higher values of CD40 were observed during phase 2. In lymphocyte aggregates, the percentages were low for both CD40L and IL-12 and patterns were different (Fig. 1D and 2B).

Discussion

We report for the first time, *in situ* distribution and expression of CD40/CD40L in murine lungs with slowly progressive primary tuberculosis infection. Our findings,

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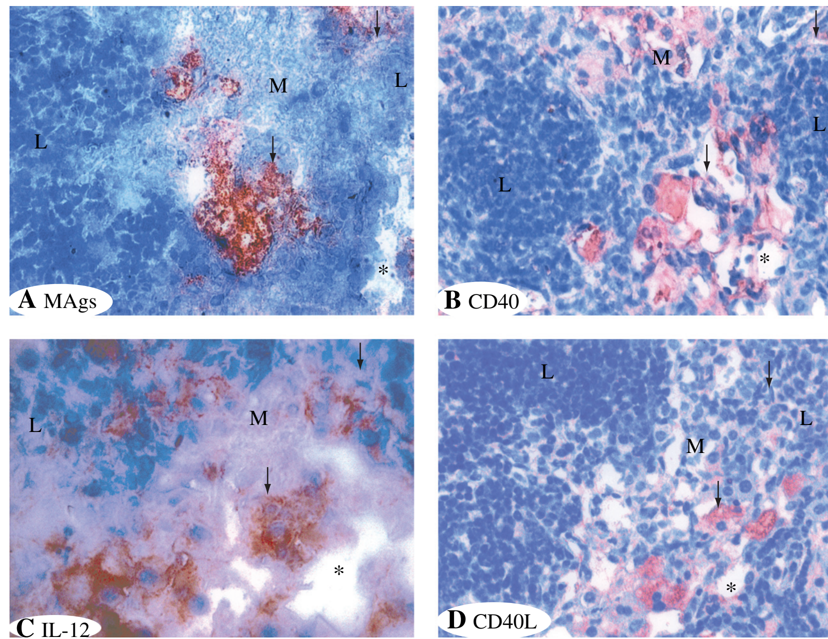

Figure 3 Expression of mycobacterial antigen- (M.Ags) (×400), CD40- (×400), interleukin-12- (IL-12) (×600) and CD40L (2 ×400)-positive cells in lung lesions of mice with slowly progressive primary tuberculosis at week 20 after infection detected by immunohistochemistry. Arrows represent infected, positive stained cells with anti-BCG, -CD0, -IL-12 and -CD40L, respectively. The asterisks denote landmarks in lesions for colocalization of stained areas. The pale areas represent large vacuolated cells in macrophage-predominant areas and basophilic areas represent lymphocytes in lymphocyte-predominant areas. Sections are not serial and range from 10–25 μm apart, respectively.

showing that CD40 and CD40L were expressed during H37Rv *M. tuberculosis* infection of a slowly progressive primary disease, contradict previous results from human and other *in vitro* studies stating that CD40L was of no importance in *M. tuberculosis* infection for the development of CMI [23, 24]. Although CD40 is shown to be a B-cell marker, we observed increased expression of CD40 in Mϕs in MPDAs. CD40 is widely distributed in various cell types including Mϕs, B cells, lymphocytes, dendritic and other cells [19, 26, 27]. During early infection, very few CD40-positive cells were observed in the LPDAs. Previous results from the same model showed that some of the cells in LPDAs were not positive with the T-cell marker CD3, CD4 or CD8 [2]. We suggest that the CD40-positive cells in the LPDAs could be B cells.

Our results show that CD40 and CD40L were coexpressed in some Mϕs. However, there was a low percentage of CD40L-positive cells in lymphocyte aggregates, although constitutive expression is reported in normal lymphocytes [28]. The suppression of CD40L expression in LPDAs might be due to the influence of *M. tuberculosis* infection. This may constitute a possible way by which *M. tuberculosis* evades CMI killing inside infected Mϕs. However, it is not known whether CD40L expressed in Mϕs aggregates may be another source of ligand for CD40 other than that on lymphocytes. A soluble source of CD40 (sCD40L) has indeed been reported [21, 28].

Although cognate interaction between CD40 and its ligand CD40 (154) has multiple functions in humoral and cell-mediated immunity [20], the role in the latter is not yet clear. We evaluated cytokines involved in CD40 signalling [29–31] and compared their expression with M.Ags. We have shown that IL-12, IFN-γ and CD40 expression was increased during late infection (phase 2), whereas CD40L-expressing cells were maintained at about 10% (Fig. 1B). However, large and vacuolated Mϕs became predominant during late infection, suggesting an increase in secretory capacity and shift in the immune response. M.Ags thus seem to partake in the shift providing continuous stimulus and eliciting cytokine release and regulation. The changes observed correlates with clinical observations described earlier during phases 1 and 2 [1, 3]. Thus, the sustained release of CD40L and increase in CD40 with progression of infection in concert with increased IL-12 suggests the importance of CD40L signalling in antigen-driven T-cell-mediated activation of Mϕs during *M. tuberculosis* infection in mice. We, therefore, concur with previous studies, which showed that CD40–CD40L interaction plays a critical role in the immunity against intracellular pathogens through upregulation of IL-12 [31–36].

Our data show an inverse relationship in the expression of TNF-α and TGF-β1 during early and late infection. The expression of TNF-α during early infection was proportional to that of M.Ags, whereas reduction was observed during late infection. Conversely, TGF-β1 was increased with the progress of infection. We have shown that high M.Ag-positive Mϕs were maintained during late infection. TNF-α acts as a proinflammatory cytokine playing an important role in granuloma formation and immune protection during early phase of infection, while TGF-β1 in low concentrations has also been shown to be proinflammatory [37]. Results from our model show that there was an increase in the expression of TGF-β1-positive cells and decreased expression of TNF-α in the infected cells with progression of infection, whereas high percentage

of M.Ag-positive cells was maintained in the lesions. This finding suggests that the persistence of high amounts of M.Ags despite microbicidal activities of these cytokines may be responsible for sustained recruitment of inflammatory cells and perpetuation of infection. In high concentrations, TGF-β1 has been shown to be inhibitory to the proinflammatory effects of TNF-α and also cause the deactivation of Mϕs [10, 37–41], thus perpetuating the infection.

M. tuberculosis has been shown to successfully modulate the components of the apoptotic pathways [3, 4, 42, 43]. The downstream events leading to apoptosis or resistance of M.Ags-containing Mϕs to apoptosis are complex and as yet, unclear. Some studies suggest that CD40 ligation induces the expression and/or activation of both ionomycin and anti-immunoglobulin M (IgM) resulting in the activation of cystein protease-32 (CPP32) [44] and directly implicates CD40 in apoptosis. Other studies suggest that CD40 signalling may be in synergy with increased expression of Bcl-xL resulting in increased resistance of the same cells to apoptosis [45]. Despite numerous claims of CD40 involvement in apoptosis, elucidating its direct role remains a problem. Cross-linking CD40 on B cells has been suggested to prevent apoptosis in B cells [46], and this might apply on Mϕs as well. CD40 might also be linked via some mechanism to tyrosine kinase (PTK) cascade and be directly involved in apoptosis [47]. Our current results show that both CD40 and CD40L were expressed and distributed in lesions of *M. tuberculosis* infection. This suggests that M.Ags can induce and modulate their expression. In the same model, we have previously shown that *M. tuberculosis* infection was associated with increased expression of Bcl-2 in M.Ag-containing cells and Bax was reduced in the same cells [4]. Further results from our laboratory have shown that *M. tuberculosis* induces the upregulation of FasL and reduces the expression of Fas on the infected Mϕs, thus sparing these M.Ag-containing cells from CMI-induced apoptosis [3]. Together, these studies indicate that CD40–CD40L signalling has an anti-apoptotic role and this phenomenon is increasingly becoming associated with *M. tuberculosis* survival [45, 48].

We conclude that changes in cognate interaction and expression of CD40–CD40L and cytokines during progress of disease were associated with *M. tuberculosis* infection. Although host mechanisms operate to resolve infection, *M. tuberculosis* modulates cytokine (IL-12p35a, TNF-α, IFN-γ and TGF-β1) release and CD40 –CD40L expression in infected cells. This may be important in the understanding of how Mϕs containing *M. tuberculosis* become refractory to CMI killing. We suggest further *in vitro* studies to confirm the role of CD40 signalling in *M. tuberculosis*-induced apoptosis.

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