

Interleukin-4 (IL-4) and Interferon-gamma (IFN-gamma) in pregnant C57BL/6 Mice infected with *L. major* at different gestational periods

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Summary

Background and Objective:- To assess if gestational factors affect the resistance of C57BL/6 mice to *L. major* infection, this study determined the levels of IL-4 and IFN-gamma in popliteal lymph node cells of pregnant C57BL/6 mice infected with *L. major* at 16 hours, 5 days-, 10 days- and 15 days- post plug by PCR, ELISA and BIOASSAY.

Design/Setting:- Experimental.

Results:- Infected pregnant C57BL/6 mice developed larger cutaneous footpad lesions compared with non-pregnant C57BL/6 mice (that showed signs of resolution 7 - 10 weeks after infection). But, the lesions in infected pregnant C57BL/6 mice and infected non-pregnant C57BL/6 mice were not as large as in susceptible BALB/c mice. The mean litter weight was also reduced in pregnant infected C57BL/6 mice particularly in the groups infected at later stages of pregnancy (day 10- and day 15-post plug). The levels of both IL-4 and IFN-gamma increased with gestation in pregnant infected C57BL/6 mice compared with pregnant non-infected group, while only IL-4 was raised in pregnant infected mice compared with infected non pregnant mice.

Conclusions:- It may be concluded that increased IL-4 in pregnant infected C57BL/6 mice caused the transient susceptibility to *L. major* infection while reduced litter weight was associated with increased IFN-gamma. These effects were pronounced in C57BL/6 mice infected with *L. major* in late pregnancy.

Keywords: Cytokines, Pregnancy, *L. major*, Pregnant mice

Résumé

Introduction et objectif:- Evaluer si des gestation touchent la résistance de C57BL/6 souris au *L. major* infection majeure; l'objet de cette étude est de décider les taux IL-4 et IFN-gamma dans les cellules ganglion lymphatique popliteal de souris fécond C57BL/6 infecté avec *L. major* à 16 heures, 5 jours, 10 jours, et 15 jours post tampon par PCR, ELISA et BIOASSAY

Plan/Cadre:- Expérimental

Résultats:- Souris fécond infecté C57BL/6 a développé des lésions de pas sourds cutané très grand par rapport au souris non fécond C57BL/6 (qui a démontré des indications de résolution 7 - 10 semaines après l'infection). Mais, les lésions dans le souris fécond infecté C57BL/6 et souris non fécond infecté C57BL/6 n'était pas aussi grand par rapport au souris BALB/C susceptible. Le poids de portée moyenne était également en baisse chez le souris fécond infecté C57BL/6 en particulier dans les groupes infectés dans les derniers étapes de la grossesse (de 10ème et 15ème jour post tampon). Les taux de IL-4 et IFN-gamma les deux ont augmenté avec la gestation chez le souris fécond infecté C57BL/6 par rapport

au groupe de fécond non-infecté, tandis que IL-4 seulement était levé chez le souris fécond infecté par rapport au souris fécond non infecté.

Conclusion:- On pourrait conclure qu'une augmentation de IL-4 chez le souris fécond infecté C57BL/6 a provoqué la susceptibilité transitoire à l'infection grave tandis qu'un poids porté réduit était attribuable à une augmentation de gamma IFN. Ces effets étaient accusés chez le souris C57BL/6 infecté avec *L. major* pendant la grossesse tardive.

Introduction

It has been recognised that rheumatoid arthritis improve during pregnancy¹ while systemic lupus erythematosus, toxoplasmosis, leprosy, listeriosis, malaria and tuberculosis are aggravated during pregnancy². The dichotomy of these responses is mediated by subsets of Th cells secreting different patterns of cytokines³.

Maternal immune response in pregnancy is biased toward humoral and away from cell mediated immune responses and inflammation.² Pregnant mice produce high antibody and diminished delayed hypersensitivity responses (DHS) against paternal MHC and other foreign antigens⁴. Although Th 1 and Th 2 related cytokines are detected in the placenta/uterus during pregnancy⁵, the placental expression of Th 2 cytokines (IL-4, -5, -10) is relatively higher, and that of IFN-gamma lower, as compared with polyclonal spleen-cell responses⁶. Th 1-specific cytokines are harmful to pregnancy, whereas Th 2 cytokines (particularly IL-10) are protective^{5,6}. IFN-gamma, TNF-beta and IL-2 induce foetal loss, pre-implantation block and preterm labour.^{5,6} In contrast to the deleterious effects of inflammatory cytokines, some Th 2 cytokines aid foetal survival. IL-10 and TGF-beta suppress cell mediated immune responses³ thereby preventing spontaneous resorptions and abortions⁷.

The course of infection of *L. major* in mice can be mild or fatal depending on the genetic background of the animal. BALB/c mice develop a progressive infection whereas C57BL/6 mice develop a minimal self-healing lesion at the site of parasite inoculation⁸. Resistance or susceptibility to *L. major* is associated with the type of CD4 + T cell-mediated immune response mounted by the mouse. Susceptibility of BALB/c to *L. major* is caused by the development of a Th 2 response, driven by an early high level of IL-4 production⁸ which blocks activation of macrophages and up-regulation of gene expression mediated by IFN-gamma, especially the gene for interferon regulatory factor 1 (IRF-1).

Increased Th 2 cytokine production during pregnancy of C57BL/6 mice may cause a compromise of resistance to *L. major* or result in a poor pregnancy outcome (foetal loss or low birth weight). Studies by Krishnan et al^{10,11} showed that

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pregnancy impairs resistance of C57bl/6 mice to *L. major* and that *L. major* infection in pregnant C57BL/6 mice increased implantation failure and foetal resorptions. In these studies, C57BL/6 mice were infected with *L. major* before pregnancy and on the 1st day of pregnancy. However, the studies did not address the possible effects of *L. major* infection at mid- or late- pregnancy.

The present study determines the level of IL-4 and IFN-gamma in the popliteal lymph nodes of C57BL/6 mice infected at 16 hours post plug and 5 days post plug (both representing early gestation), day 10 post plug (representing middle gestation) and day 15 post plug (representing late gestation). The hypothesis is that, the mutual effects of pregnancy and *L. major* infection may vary with gestation as a result of variations in Th 1 and Th 2 cytokines at different stages of gestation.

Methodology

Mice and mating: One male and 2 female 6 - 8 weeks old mice were housed in the same cage overnight for mating and the presence of vaginal plugs in the females was checked on the following morning. The day on which the plug was observed was considered as day 0 of pregnancy. Pregnant females were removed from the mating cages and housed in separate cages.

Infection of mice: Mice were injected in the footpad with 3×10^6 stationary phase promastigotes suspended in 0.05ml of incomplete DMEM at 16 hours on day 5, day 10 and day 15 post plug. Age matched groups of control (non-pregnant female C57BL/6 and BALB/c) mice were also infected with the same number of parasites.

Experimental design: A set of mice were infected on 16 hours post plug (Group A), on day 5 post plug (Group B), on day 10 post plug (Group C) and day 15 (Group D). In each of Groups A and B, a total of 12 mice were used for the study. Also, in each of Groups C and D, a total of 9 mice were used. In Groups A and B, 3 mice each were sacrificed at 16 hours post infection, 10 days post infection and on the day of delivery/parturition. In Group C, 3 mice were sacrificed 16 hours post infection, and 10 days post infection (i.e. on the day of parturition). In Group D, 3 mice were sacrificed at 16 hours post infection and 5 days post infection (i.e. on the day of delivery). Three mice in each of the groups were reserved for assessment of the course of long term *L. major* infection. Each of the above mentioned groups of mice were matched by a group of same age controls (pregnant non-infected C57BL/6 mice, non-pregnant non-infected C57BL/6 and BALB/c mice, infected non-pregnant C57BL/6 mice and BALB/c mice).

Assessment of infection: The progression of cutaneous infection was assessed by measuring the thickness of the infected footpad weekly with a metric caliper. The lesion size was calculated by subtracting the value of the uninfected contra-lateral footpad from that of the infected ones.

Pregnancy outcome: The number and weight of the litters

were documented at birth. The litters were weighed individually using a sensitive weighing scale and the mean weight was recorded. Mortality rate of the litter was determined by dividing the number of litters that died within 1 week post birth with the total number of delivered pups.

Cytokine measurement: Lymph-node cells from individual mice were used to assay for the levels of IFN-gamma mRNA and IL4 mRNA by PCR. The concentration of IFN-gamma and IL-4 in the supernatant from the culture of lymph node cells was determined by ELISA and BIOASSAY¹² respectively. Draining popliteal lymph node cells were pooled from infected mice and homogenized. After repeated washing, one million cells were aliquoted for RNA extraction and the rest were resuspended at 5×10^6 cells/ml in complete DMEM with 5% foetal calf serum. Cells were cultured at 200 μ l (96 well plate) or 1ml (24 well plate) and re-stimulated with UV-irradiated stationary *L. major* at 1×10^6 /ml or Con. A (2.5 μ g/ml). Supernatant were harvested after 48 hours (Con A) or 72 hours (UV *L. major*) and frozen at -70°C until assayed for cytokine levels. Lymph node cells from non-infected mice were treated in the same way except that lymph node included axillary, popliteal, suscapsular and inguinal. Axillary, popliteal, suscapsular and inguinal lymph nodes were used in the control mice because non-infected lymph nodes are usually not well developed to harvest many cells.

Quantitation of cytokines mRNA by competitive PCR: Total RNA was extracted from the cells using chloroform-isopropanol method and suspended in RNAase free water. cDNA was synthesised from diluted RNA by incubation and treatment with 1s' strand mix, dTT and primer working buffer. PCR was performed on this cDNA in 5 μ l volume containing dNTP, sterile water, PCR buffer, 5 primer, 3 primer, Taq polymerase and competitor. Thereafter, 15 μ l of the reaction product was analysed on a 1% agarose gel in TBE X1 buffer containing 0.1mg/ml ethidium bromide. Quantitation of cDNA was done by calculating how much of the competitor fragment will be required to achieve equal amount of products¹³.

ELISA assay: This was performed on the supernatant of 48 - 72 hours re-stimulated (with *L. major* or Concanavalin A) lymph node cells. 96 wells microtiter plate was coated with capture antibody overnight at 4°C, blocked with PBS-1% BSA, washed with PBS-Tween and supernatant/rIFN-gamma (neat and 1: 2 serial dilution) were added. After incubation, the wells were washed with PBS-Tween, followed by addition of detection antibody, streptavidin horse radish peroxidase and OPD. The reaction was stopped by addition of 4N HCl and the optical density was read at 492nm wave length. The concentration of cytokines in the supernatants was calculated from the graph of the standard. All samples were tested in duplicates.

BIOASSAY: The standard and samples were serially diluted 1: 3 with complete DMEM buffer in a 96 well microtiter plates at the final volume of 100 μ l. One hundred μ l/well of properly washed 5×10^4 CTL-44 cell suspension/ml was added, incubated for 30 - 36 hours in carbon-dioxide incubator at

37°C. Tritiated thymidine was then added at 0.5µCi/well. The cells were harvested on glass fiber filters and incorporated thymidine was counted in a beta-counter. The concentration of sample IL-4 was determined from the standard graph. All samples were tested in duplicates.

Results

Course of *L. major* infection in pregnant C57BL/6 mice:

Figure 1 demonstrates the progression of footpad lesions

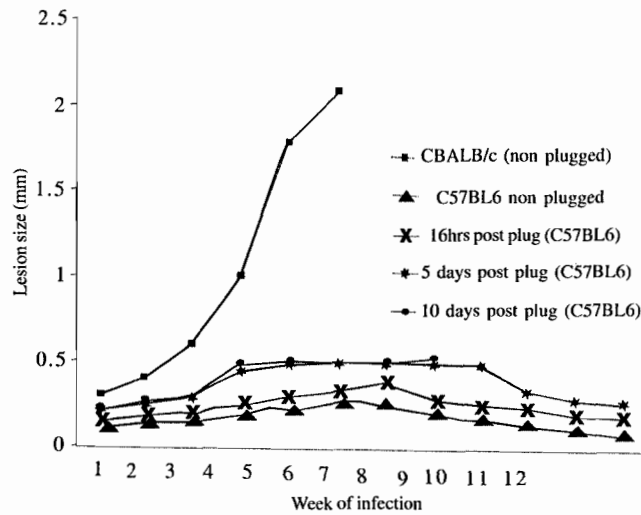


Fig. 1 Course of *L. major* infection in C57BL/6 mice (pregnant and non pregnant) and BALB/c mice (non pregnant)

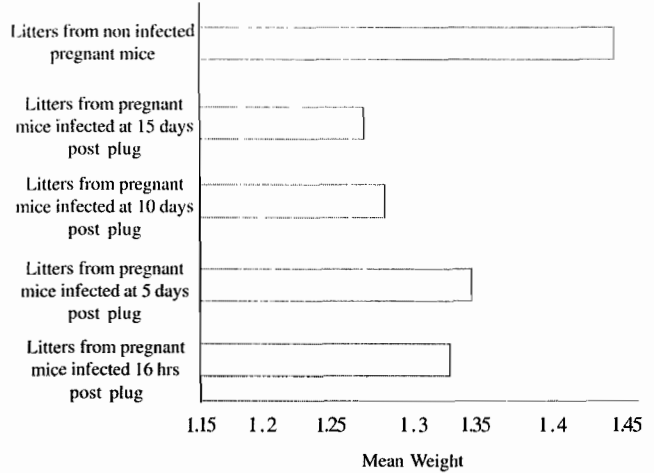


Fig. 2 Mean weight (grammes) of litters from C57BL/6 mice infected with *L. major* at different gestations.

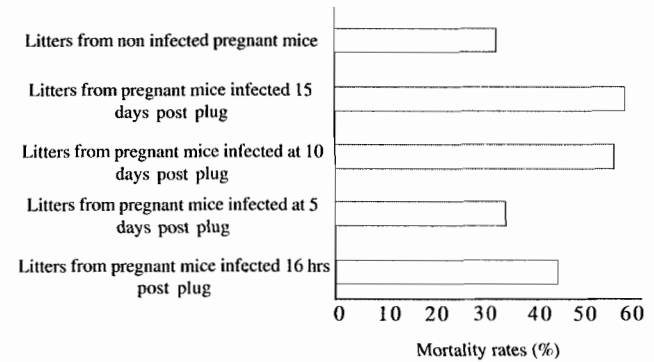


Fig. 3 Mortality rates (%) of litters from C57BL/6 mice infected with *L. major* at different gestations

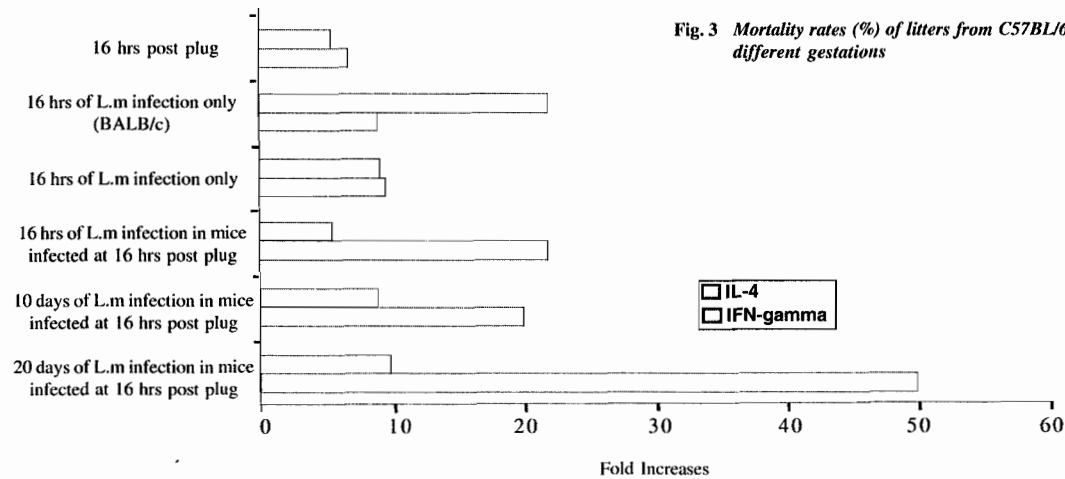


Fig. 4a Fold increases in levels of IFN-gamma mRNA and IL-4 mRNA in C57BL/6 mice infected with *L. major* at 16 hrs post plug.

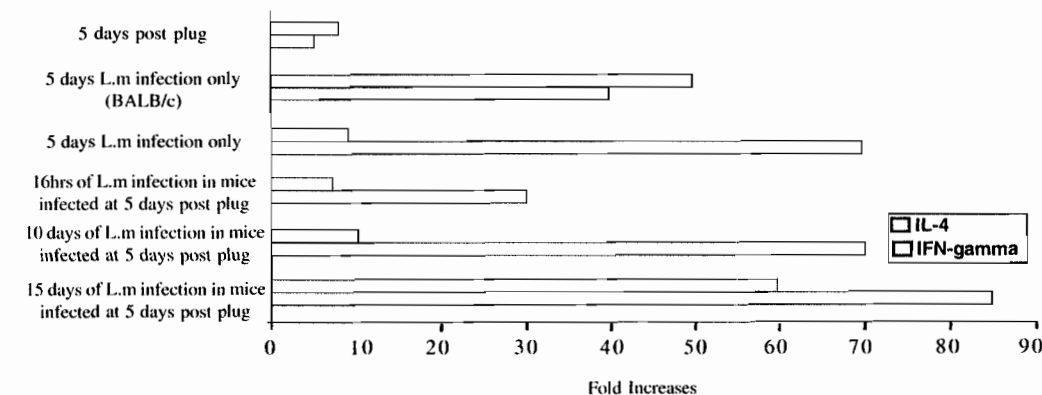


Fig. 4b Fold increases in levels of IFN-gamma mRNA and IL-4 mRNA in C57BL/6 mice infected with *L. major* at 16 hrs post plug

(thickness of the infected footpad minus that of the uninfected footpad) as measured by metric caliper. The footpad lesion in non-pregnant infected BALB/c mice was significantly higher than that of non-pregnant infected C57BL/6 mice from 2nd week of infection onward. The lesion resolved in non-pregnant infected C57BL/6 after 6th week as opposed to the unresolved lesion in BALB/c. However, in the pregnant infected C57BL/6 mice, lesion sizes showed significant increase when compared with non-

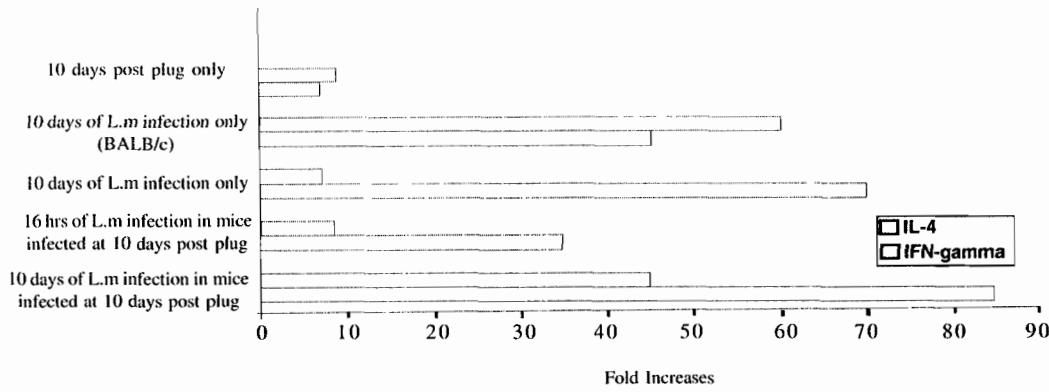


Fig. 4c Fold increases in levels of IFN-gamma mRNA and IL-4 mRNA in C57BL/6 mice infected with *L.major* at 10 days post plug

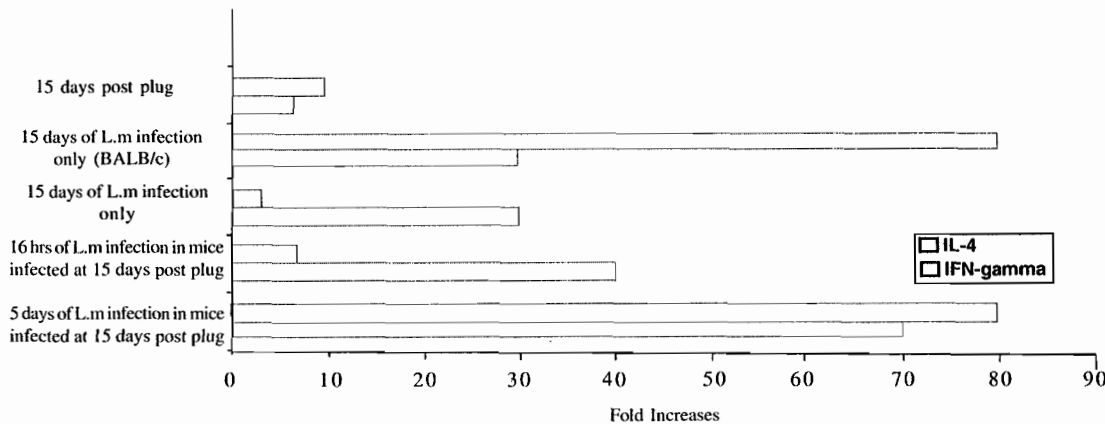


Fig. 4d Fold increases in levels of IFN-gamma mRNA and IL-4 mRNA in C57BL/6 mice infected with *L.major* at 15 days post plug

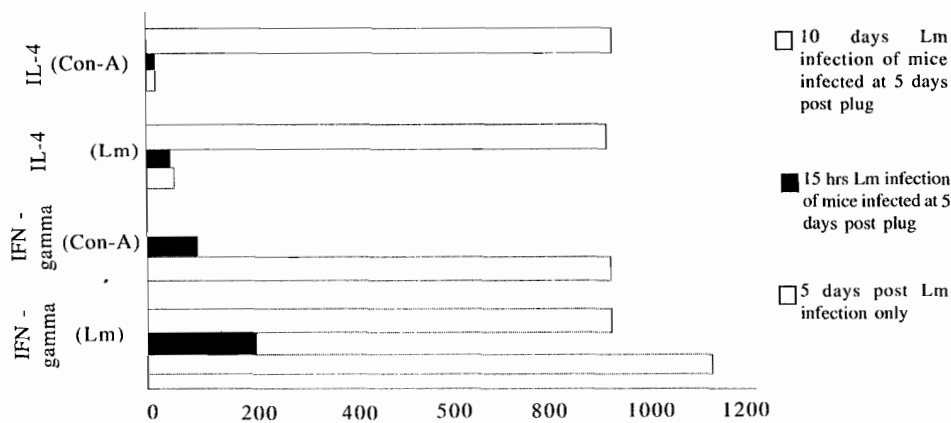


Fig. 5a IL-4 (pg/ml) and IFN-gamma (IU/ml) in the supernatant of lymph node cells from C57BL/6 mice infected with *L. major* at 5 days post plug

pregnant infected C57BL/6 from 3rd week of infection onward, particularly in mice infected at day 5, day 10 and day 15 post plug. The lesions resolved totally after the 7th week in C57BL/6 mice infected at 16 hours post plug 8th week in C57BL/6 mice infected at day 5 post plug but much later in C57BL/6 mice infected at day 10 or day 15 post plug. Though the lesions finally resolved in these pregnant infected C57BL/6 mice, the sizes of the lesion were higher compared with non-pregnant infected C57BL/6 controls.

gamma and IL-4 were raised compared with pregnant non-infected C57BL/6 mice. But only IL-4 was raised in pregnant infected mice when compared with infected non-pregnant mice. These changes were more pronounced with increased duration of infection and pregnancy.

Discussion

The results of this study showed that pregnancy diminishes the resistance of C57BL/6 mice to *L. major* infection. More so, *L. major* infection leads to a reduction in the litter weight at birth. These two observations were

***L. major* infection decreased litter weight and increased litter mortality:**

Litter weight was taken at birth with a weighing scale. Though the mean weight of litters from infected C57BL/6 mice was lower than those of the controls, only mice infected at day 10 and day 15 post plug showed slightly significant reduction (Figure 2). The number of litters (8.0 ± 1.5) produced by infected C57BL/6 mice were similar to those of controls (8.3 ± 1.9) but highest mortality rates were observed in

the litters from group of mice infected at day 10 and day 15 post plug (Figure 3).

***L. major* infection increased the levels of both IFN-gamma and IL-4 in lymph nodes of pregnant C57BL/6 mice.**

Figure 4a - 5c show the expression and production of IL-4 and IFN-gamma by lymph node cells of mice in infected and non-infected mice. In all pregnant infected C57BL/6 mice, the levels of IFN-

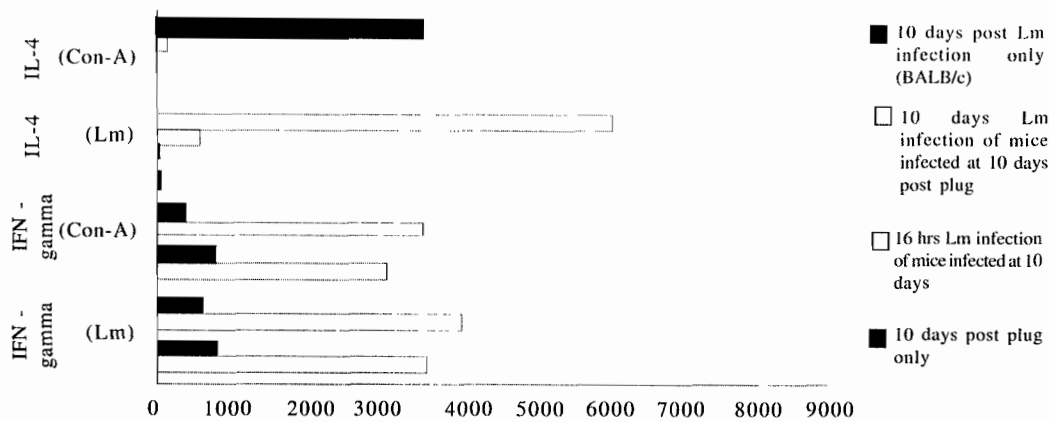


Fig. 5b IL-4 (pg/ml) and IFN-gamma (IU/ml) in the supernatant of lymph node cells from C57BL/6 mice infected with *L. major* at 10 days post plug

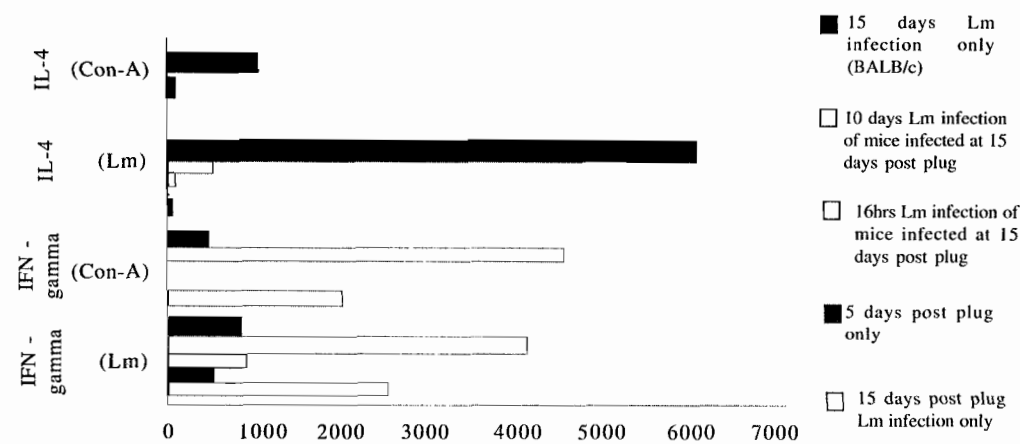


Fig. 5c IL-4 (pg/ml) and IFN-gamma (IU/ml) in the supernatant of lymph node cells from C57BL/6 mice infected with *L. major* at 15 days post plug

pronounced when mice were infected at the middle - or late - gestation. This may be related to increase in IFN-gamma and IL-4 in pregnant infected C57BL/6 mice. Our results partially contrast those of Krishnan et al^{10,11} where diminished IFN-gamma, increased IL-4, IL-5 and IL-10 were obtained. This might be due to differences in methodology. Krishnan et al (1996a and 1996b) determined cytokine levels in the supernatants of lymph node cells, placenta cells and spleen cells obtained from C57BL/6 mice infected with *L. major* before mating and immediately after conception.

In pregnant non-infected C57BL/6 mice, IFN-gamma level was slightly raised at 16 hours post plug (early pregnancy), thus maintaining the ability to resist *L. major* infection while increased IL-4 levels at day 10 and day 15 post plug predisposes C57BL/6 mice to severe *L. major* infection. The detection of high level of IL-4 in non-pregnant infected BALB/c mice and high level of IFN-gamma in non-pregnant infected C57BL/6 mice supports the well established paradigm of Th 1/Th 2 polarisation of immune response in these two different strains of inbred mice.

In all groups of pregnant infected C57BL/6 mice, both IL-4 and IFN-gamma were significantly raised when compared with pregnant non-infected C57BL/6 mice. In mice infected with *L. major* at 16 hours post plug, only IFN-gamma showed significant increase while the level of IL-4 was similar to infected non-pregnant group. This explains the ability of C57BL/6 mice to resolve *L. major* infection earlier than other groups of mice infected later in pregnancy. In mice infected

at day 5, day 10 and day 15 post plug, both IFN-gamma and IL-4 were raised (particularly at delivery) when compared with infected non-pregnant mice. This explains larger footpad lesion sizes and lower mean litter weights in these groups of mice.

Th 2 cytokines (IL-4, IL-10 and TGF-beta) aid gestation¹⁴, inhibit macrophage activation¹⁵ and aggravate *L. major* infection¹⁶. Th 2 cytokines downregulate Th 1 effector functions by inhibiting the production of inflammatory cytokines such as IFN-gamma and TNF-alpha³ which mediates the

clearance of *L. major* through activated macrophages and possibly through CD8 + T-cells⁹. IFN-gamma activates the inducible nitric oxide synthase in macrophages leading to production of reactive nitrogen radicals that is toxic to *L. major*¹⁷. Reactive nitrogen radicals also maintain the quiescent status of *L. major* infection¹⁸. IL-4 has been shown to hamper IFN-gamma-induced macrophage activation¹⁹ and suppress up-regulation of gene expression mediated by IFN-gamma²⁰. Thus, the presence of IL-4 (a potential inhibitor of IFN-gamma) in mice infected at late pregnancy might have reduced macrophage production of reactive nitrogen radicals or disrupted the ability of IFN-gamma to maintain *L. major* at a quiescent status, therefore affecting the ability to resolve *L. major* infection. The present study indicates that pregnancy do not totally abrogate the curative Th 1 response in normally resistant C57BL/6 mice. Pregnant C57BL/6 mice showed diminished resistance to *L. major*, the footpad lesion resolved 1 - 3 weeks after the resolution of footpad lesion in non-pregnant infected C57BL/6 mice. Moreover, none of the mice developed the progressive infection characteristic of highly susceptible BALB/c. This could be due to the fact that pregnant infected mice produced concurrently raised levels of IFN-gamma and IL-4.

Increased production of IFN-gamma by C57BL/6 mice in response to *L. major* infection appears to disturb pregnancy by reducing the birth weight of litters. Krishnan et al¹¹ have shown that the Th 1 response (against *L. major*) increases the risk of pre- and post- implantation pregnancy loss.

Positive correlations have also been reported between murine foetal resorption²¹, decreased birth weight²² and the expression of IFN-gamma. Low birth weight infants are at higher risk to die early in life²³, therefore raised IFN-gamma in pregnant infected mice could be responsible for higher mortality rate of litters produced by pregnant mice infected at day 10 and day 15 post plug.

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