Dual role for macrophages in vivo in pathogenesis and control of murine Salmonella enterica var. Typhimurium infections

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Salmonella spp. are regarded as facultative intracellular bacterial pathogens which are found inside macrophages (MΦ) after i.v. infection. It is generally assumed that MΦ restrict the replication of the bacteria during infection. In this study we examined the in vivo activities of MΦ during experimental S. typhimurium infections, using a selective liposome-based MΦ elimination technique. Unexpectedly, elimination of MΦ prior to infection with virulent S. typhimurium decreased morbidity and mortality, suggesting that MΦ mediate the pathology caused by S. typhimurium. Removal of MΦ during vaccination with attenuated S. typhimurium did not affect protection against challenge with virulent S. typhimurium, suggesting that MΦ are not required for the induction of protective immunity and that other cells must function as antigen-presenting cell to elicit T cell-mediated protection. However, MΦ appeared to be important effectors of protection against challenge infection since elimination of MΦ from vaccinated mice prior to challenge infection with virulent S. typhimurium significantly decreased protection. These results enhance our understanding of the control of S. typhimurium growth in vivo, and moreover suggest that MΦ play a major role in the pathology of virulent S. typhimurium infections. As such, these cells may present a novel target for therapeutic intervention.

Key words: Salmonella enterica / Pathogenesis / Innate resistance / Macrophage / Enteric infection

1 Introduction

Salmonella enterica are significant worldwide pathogens which cause localized and systemic diseases in many animal species. The World Health Organisation estimates that typhoid fever, the most important human salmonellosis, is responsible for 600 000 deaths from 16 million cases worldwide [1]. S. enterica var. typhimurium causes a systemic disease in mice which has been used to model the pathogenesis and immunology of human typhoid fever. Studies of oral S. enterica infections in humans and mice suggest that Salmonella spp. are facultative intracellular bacterial pathogens which invade the gastrointestinal mucosa through the specialized M cells in the Peyer’s patches, then spread into the draining mesenteric lymph nodes where they are thought to reside and multiply in MΦ [2, 3]. From the mesenteric lymph nodes, bacteria disseminate via the thoracic duct into the bloodstream, and are removed by the MΦ of the reticuloendothelial system (RES), mainly in the spleen, liver and bone marrow [2, 3]. Dogma states that Salmonella multiply in the RES MΦ and are seeded back into the bloodstream, possibly following apoptosis of these cells, until a T cell-mediated immune response begins to control infection through up-regulation of RES MΦ. In the absence of a T cell response, the infection is fatal to mice [4, 5].

This murine salmonellosis model holds that the MΦ-Salmonella interaction is central to the early control of S. typhimurium growth, and the subsequent induction of acquired immunity [6, 7]. Much of the evidence used to support this hypothesis comes from in vitro studies in

Abbreviations: CL-2MDP: Dichloromethylene diphosphonate C-frag: C-fragment of tetanus toxin DC: Dendritic cell(s) RES: Reticuloendothelial system NO: Nitric oxide

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which MΦ-like cell lines or bone marrow-derived MΦ were used [8–11]. These in vitro studies showed that S. typhimurium is able to survive and replicate in phagosomes of non-activated MΦ and, more recently, that this replication can lead to apoptosis [11]. In contrast, in vivo studies have suggested that S. typhimurium may reside inside multiple cell types including neutrophils, MΦ and/or hepatocytes; only one recent study has shown the exclusive localization of S. typhimurium in MΦ [12–14].

A role for the MΦ in innate, genetically determined resistance to infection [15], and subsequent acquired resistance to reinfection, was inferred from gene mapping studies which demonstrated that natural resistance is mediated by a locus on chromosome 1 called Nramp1, which coincidently confers resistance to diseases caused by other MΦ-associated pathogens, e.g. Mycobacterium [16]. The natural resistance-associated MΦ protein (Nramp1) is almost exclusively expressed by MΦ and susceptibility and resistance to murine S. typhimurium infection is determined by a mutation in Nramp1 which results in a single amino acid substitution [16, 17]. Mice expressing the resistant allele of Nramp1 can control the growth rate of S. typhimurium in vivo, allowing the development of acquired immune responses.

The induction of acquired, predominantly T cell-mediated, immune responses is essential to the eventual clearance of S. typhimurium and recovery from infection [4, 5, 18]. Salmonella-derived peptides are efficiently presented by MΦ to T cells in vitro [19, 20], suggesting that MΦ might function as APC in vivo for the induction of protective T cell responses. However, most of these in vitro studies used T cell hybridoma proliferation to quantitate antigen presentation by MΦ or MΦ-like cell lines. It is now recognized that while T cell hybridomas can be stimulated by MΦ in vitro, dendritic cells (DC) are the principal APC involved in activation of naive T cells in vivo [21]. A primary role for MΦ as APC for the induction of acquired immune responses against S. typhimurium in vivo therefore remains unproven.

In this study we examined the role of MΦ in vivo in protection and induction of acquired immune responses against experimental S. typhimurium infections. We used a liposome-based MΦ depletion technique [22] to eliminate MΦ in vivo. This technique has been studied extensively and is based on the liposome-mediated intracellular delivery of the drug dichloromethylene diphosphonate (Cl2MDP). Accumulation of Cl2MDP in the cytosol disturbs the metabolism of the MΦ, leading to irreversible damage, ultimately resulting in their death and depletion from the tissues in the absence of an inflammatory response [22]. The selective depletion of MΦ has been confirmed in a large number of in vivo studies, both ultra-structurally and by loss of MΦ-specific markers [22]. Several studies have shown that the Cl2MDP-liposomes do neither affect the tissue architecture nor the presence and function of other phagocytic cells, such as blood monocytes, granulocytes or DC [23–25].

In the present study we compared Salmonella infections in MΦ-depleted mice and normal mice, and our results contribute to the current understanding of the importance of the in vivo MΦ–S. typhimurium interaction in the initial control of the infection, and for vaccine-mediated protection against Salmonella infections. Our studies suggest that MΦ play a dual role in S. typhimurium infections. We were able to show that MΦ are centrally involved in the morbidity and mortality caused by virulent S. typhimurium infections in naive animals, and as immune effectors which facilitate clearance of bacteria and recovery from infection in vaccinated mice.

2 Results

2.1 In vivo elimination of MΦ using a liposome-mediated depletion technique

In this study, we used a liposome-mediated MΦ depletion technique to eliminate spleen and liver MΦ in vivo. Mice were injected i.v. with 0.1 ml Cl2MDP-liposomes per 10 g body weight, either 2 days before immunization and/or 2 days before challenge with virulent S. typhimurium. This treatment resulted in the complete elimination of MΦ residing in the red pulp and marginal zone area of the spleen (Fig. 1) and in the liver (results not shown).

![Fig. 1. Effect of Cl2MDP-liposome administration on MΦ populations in the spleen. Shown are cryostat sections of spleens obtained from normal (A) and Cl2MDP-liposome-treated (B) mice stained for acid phosphatase. The Cl2MDP-liposomes were administered i.v. 2 days earlier, resulting in complete elimination of red pulp MΦ and marginal zone MΦ. RP = red pulp, WP = white pulp.](image-url)
2.2 Effect of in vivo MΦ depletion on challenge with virulent *S. typhimurium*

To determine the role of MΦ in controlling low dose wild-type infection by virulent *S. typhimurium* SL1344, normal and MΦ-depleted mice were infected i.v. with 100 CFU SL1344, and monitored for survival against time (Fig. 2). All normal mice and mice treated with PBS-liposomes 2 days before infection died within 6–7 days after infection, whereas mice treated once with Cl2MDP-liposomes survived the infection for a longer period, and died 8–9 days after infection. These results suggested that MΦ-depleted naive mice were more resistant to mortality as a result of *S. typhimurium* infection in comparison with normal mice. To maintain the depletion of MΦ from the tissues [26], mice were given 50 μl Cl2MDP liposomes on day 3 and day 8 after infection. Mice which were therefore continuously depleted of MΦ [26] survived up to 10 days after infection (Fig. 2).

To further study the effect of MΦ depletion on protection of mice against infection with virulent *S. typhimurium* SL1344, the number of bacteria in spleens and livers of infected animals was determined at indicated time points after i.v. inoculation with 100 CFU SL1344. Macroscopic examination of SL1344-infected mice showed a significant enlargement of spleen (Fig. 3 A) and liver (hepatosplenomegaly) and widespread necrotic lesions in the organs in normal and PBS-liposome-treated mice on day 3 and 4 which worsened in time, whereas the organs of Cl2MDP-liposome-treated mice showed no signs of pathology until day 6 after infection (results not shown). In untreated infected mice, bacterial counts increased approximately tenfold per day, and reached to approximately $2.2 \times 10^8$ in the spleen (Fig. 3 B) and $2.6 \times 10^8$ in the liver (Fig. 3 C) 5 days after infection. The number of bacteria in mice treated with PBS-liposomes was equivalent to those found in untreated infected mice (results not shown). Treatment of mice with Cl2MDP-liposomes 2 days before infection with SL1344 resulted in a reduction of bacterial load in both the spleen and the liver. A significant ($p = 0.001$) 10- to 50-fold reduction in the number of SL1344 was observed in the spleen on day 4 after infection, and on day 5 the bacterial load was 100-fold lower compared with untreated infected mice. Similarly, treatment with Cl2MDP-liposomes before inoculation of SL1344 resulted in a reduced bacterial load in
the liver (Fig. 3 C). These experiments were performed at least six times, and in each experiment the in vivo growth rate of SL1344 was equivalent, and the level of growth of the bacteria in normal (non-depleted) mice was always much greater than the bacterial growth rate in MΦ-depleted mice. These results suggested that in vivo replication of S. typhimurium was reduced in the absence of MΦ. The growth rates of SL1344 and BRD509 incubated in vitro with up to 1 mg/ml Cl₂MDP was not affected (data not shown).

2.3 Effect of in vivo MΦ depletion on induction of acquired immune responses

To study the importance of in vivo MΦ-S. typhimurium interactions for the induction of acquired immune responses against expressed by S. typhimurium, we compared the generation of antigen-specific antibody responses in normal and MΦ-depleted mice after i.v. immunization with an attenuated, aromatic-depent mutant of S. typhimurium expressing the C-fragment of tetanus toxin (C-frag). Cl₂MDP-liposomes were injected 2 days before immunization and every 5 days thereafter to maintain MΦ depletion throughout the experiment. Treatment of mice with Cl₂MDP-liposomes prior to and during immunization with BRD509/C-frag neither altered the number of bacteria in the spleen or liver nor the bacterial clearance kinetics, and did not cause mortality among the vaccinated mice (Fig. 4). In addition, treatment of normal and MΦ-depleted mice with gentamicin during immunization with BRD509/C-frag did not affect bacterial load in the organs, suggesting that all recovered bacteria were residing inside cells (results not shown).

Immunized mice were bled weekly and serum antibodies against C-frag or S. typhimurium LPS were measured by ELISA. Fig. 5 shows that Cl₂MDP-liposome treatment before and during immunization with BRD509/C-frag did not affect the induction of antigen-specific antibodies, since both the kinetics of the response, and serum endpoint titers were equivalent in untreated and Cl₂MDP-liposome-treated mice. Moreover, both groups of mice showed similar isotype distribution in the LPS- and TT-specific humoral responses (results not shown).

To further determine the effect of the absence of MΦ on the induction of acquired immune responses, antigen-specific T cell proliferation was measured in normal and MΦ-depleted mice after i.v. immunization with either BRD509 or BRD509/C-frag. Splenic T cells of immunized mice were cultured for 4 days with recombinant C-frag. T cells obtained from naive mice or from mice immunized with BRD509 did not proliferate upon stimulation with C-frag, whereas T cells isolated from BRD509/C-frag-immunized mice showed antigen-dependent proliferation. As is evident from Fig. 6, Cl₂MDP-liposome treatment before and during immunization with BRD509/C-frag resulted in significantly increased T cell proliferative responses (\( p < 0.01 \)) towards C-frag in comparison with C-frag-specific T cell responses in untreated immunized mice. Nonspecific T cell proliferation induced with ConA was not affected by Cl₂MDP-liposome treatment (results not shown), sug-

![Fig. 4. MΦ depletion does not affect growth or clearance of attenuated S. typhimurium. PBS-treated mice (■) and Cl₂MDP-liposome-treated mice (□) were inoculated i.v. with 10⁵ CFU BRD509/C-frag. MΦ depletion was maintained throughout the experiment by injecting Cl₂MDP-liposomes i.v. every 5 days. The number of bacteria in spleen and liver homogenates was determined by viable counts on LB media. Presented are the mean ± SE of groups of five mice.](image)
Fig. 6. MΦ depletion increases T cell responses to *S. typhimurium*-associated antigens. Groups of five PBS-treated mice (■) and C\(_2\)MDP-liposome-treated mice (■) were immunized i.v. with 10\(^5\) CFU BRD509 or BRD509/C-frag. C\(_2\)MDP-liposomes were injected i.v. every 5 days to maintain MΦ depletion. T cell proliferation assays were performed 10 days after immunization. Shown is [\(^3\)H\)] thymidine incorporation of pooled splenic T cells cultured with 1 μg/ml C-frag.

suggesting that the increased C-frag-specific T cell proliferation in C\(_2\)MDP-liposome-treated mice was not due to an increased proliferative capacity of these T cells.

2.4 Effect of *in vivo* MΦ depletion on induction of protective immunity against virulent *S. typhimurium*

Finally, we examined whether *in vivo* MΦ depletion during vaccination with BRD509 would affect induction of protective immunity against challenge with virulent *S. typhimurium*. Mice were depleted of MΦ either during vaccination with BRD509/C-frag and/or during challenge with virulent SL1344. Protection was measured by counting the number of SL1344 bacteria in spleen and liver 4 days after challenge (Fig. 7). Normal, BRD509-vaccinated mice were highly resistant to challenge with SL1344, as was evident from the 10\(^5\)-fold lower number of bacteria in their tissues compared with normal, non-vaccinated mice (p < 0.01). Treatment of BRD509-vaccinated mice with C\(_2\)MDP-liposomes during immunization did not significantly alter protection against challenge with SL1344, and the bacterial load of these mice was similar to that of normal BRD509-vaccinated mice (p = 0.2). In contrast, elimination of MΦ in BRD509-vaccinated mice at the time of challenge with SL1344 significantly reduced protection, as evidenced by a reduced clearance of the challenge inoculum from both the liver and the spleen (p < 0.01). Mice treated with C\(_2\)MDP-liposomes both during vaccination with BRD509 and at challenge with SL1344 also showed reduced capacity to clear the challenge inoculum compared with normal BRD509-vaccinated mice (p < 0.05). However, the number of SL1344 in spleen and liver of these mice was equivalent to the number of bacteria in BRD509-vaccinated mice which were depleted of MΦ at time of challenge only. These results suggested that the presence of MΦ was not essential for the induction of protective immunity during vaccination with BRD509, but that MΦ were involved in clearance of virulent *S. typhimurium* in vaccinated mice.

To further study the role of MΦ as effectors of protection against virulent *S. typhimurium* infection, we determined survival of mice after challenge with SL1344. Fig. 8A shows the effect of C\(_2\)MDP-liposome or PBS-liposome treatment during vaccination with BRD509 on survival of a challenge with SL1344. The vaccinated mice were able to control and survive the low-dose infection with virulent SL1344, and only one out of five normal vaccinated mice died, whereas all C\(_2\)MDP-liposome-treated mice lived. In contrast, injection of C\(_2\)MDP-liposomes in BRD509 vaccinated mice before challenge with SL1344
did affect protection and significantly increased mortality compared with normal or PBS-liposome-treated mice (Fig. 8 B). These results confirmed our observations that elimination of MΦ during immunization did not affect protection against virulent S. typhimurium, whereas removal of MΦ from immunized mice before challenge with virulent S. typhimurium significantly reduced protection.

### 3 Discussion

In this study we investigated the importance of the in vivo MΦ-S. typhimurium interaction in the immunobiology of murine salmonellosis. Most of the previous studies investigating the interaction between MΦ and Salmonella used MΦ-like cell lines or bone marrow-derived MΦ for in vitro studies under conditions which do not necessarily reflect the in vivo situation [8–11]. Here, we used a highly specific technique to eliminate MΦ in vivo, which does not affect the in vivo function or presence of any other phagocytic cells such as granulocytes or DC [22–25], and obtained relevant information regarding the involvement of MΦ in murine defense against S. typhimurium infection.

Several early reports pointed to a role for MΦ in natural resistance to Salmonella infections. Hormaeche [7] used radiation chimeras and showed that early resistance was mediated by bone marrow-derived, radiation-resistant cells, most likely MΦ. In other reports it was demonstrated that innate resistance to S. typhimurium was decreased in silica-treated mice, presumably due to the destruction of MΦ in these mice [27]. In this study we showed that in naive mice, in vivo elimination of MΦ before and during infection with virulent S. typhimurium (SL1344) resulted in significantly reduced bacterial loads in spleen and liver for at least 6 days after infection, suggesting that during the initial stages of infection, S. typhimurium may reside in several cell types but replicates inside MΦ. Notably, bacterial numbers in MΦ-depleted mice increased from day 6 after infection despite the continuous depletion of MΦ, suggesting that in the absence of MΦ S. typhimurium is able to find an alternative niche for replication.

The cellular location of S. typhimurium in vivo has been studied by several groups, and evidence has been presented that the bacteria may reside and/or replicate in MΦ, neutrophils and/or hepatocytes [12–14]. In a recent in vivo study using confocal microscopy, Richter-Dahlfors et al. showed that S. typhimurium resides in MΦ in the liver [14]. In addition, Gulig et al. showed that MΦ serve as the primary host cell for Spv-mediated intracellular replication [28]. Our results confirm the previous findings and provide further evidence that virulent S. typhimurium replicates in MΦ in vivo.

In this study we showed that elimination of MΦ before infection with virulent S. typhimurium delayed the appearance of necrotic foci in the liver and the onset of disease, and extended the mean survival time. In addition, the absence of hepatosplenomegaly in MΦ-depleted mice until the time point at which S. typhimurium replication increased (i.e., day 6 after infection) indicated an impaired recruitment of inflammatory cells in MΦ-depleted animals (data not shown). Taken together, these results suggest that MΦ not only provide a niche for virulent Salmonella to grow, but are also centrally involved in the pathogenesis of murine salmonellosis. Clinical symptoms of human S. typhi infection include enteric fever and organ failure as a result of extensive damage to these organs, reportedly due to the host inflammatory response triggered by LPS. Recently, Khan et al. [29] constructed a Salmonella mutant defective in secondary acylation of the lipid A domain of the LPS molecule, reducing its ability to induce cytokines and inducible nitric oxide synthase in MΦ. When injected into normal mice, the LPS-mutant Salmonella replicated to very high levels in the liver and spleen, but most of the animals survived and cleared the infection, suggesting
that mortality after infection with S. typhimurium is dependent on the toxicity of LPS. Collectively, these and our findings, and the previously reported fact that LPS-induced cytokine secretion and LPS-related biological responses are reduced in MΦ-depleted mice [30], make it tempting to speculate that, during the early stages of S. typhimurium infection, bacterial (cell wall-associated) LPS stimulates the production of inflammatory mediators from MΦ resulting in pathology and onset of disease. On the other hand, the MΦ inflammatory response is crucial to control S. typhimurium infections, since the MΦ-depleted mice were not able to control the infection. Indeed, it has been shown that mice which have a genetic defect in the lps gene and which are therefore hyporesponsive to the biological effects of LPS, are not able to control S. typhimurium infections [31].

Finally, the results of our study showed that MΦ are not involved in the induction of protective immunity, since elimination of MΦ during immunization with attenuated S. typhimurium did not affect protection against a challenge with virulent S. typhimurium. Importantly, elimination of MΦ at the time of challenge did partially affect protection against virulent S. typhimurium, implying that MΦ are an important effector cell of the host protective response. Elimination of MΦ from immunized mice resulted in an increased bacterial burden in spleen and liver 4 days after challenge, and augmented susceptibility and mortality. Depletion of MΦ both during immunization and at the time of challenge did not further affect the protection of the mice. These results are in contrast with our observations in naive mice infected with virulent S. typhimurium, where MΦ elimination resulted in a lower bacterial load and increased survival, and prove that in immune mice, MΦ play a major role in early control of bacterial growth in the liver and spleen, and are the main effector cell restricting bacterial replication in immunized mice. However, immune MΦ-depleted mice were still more resistant (40 % survival, Figs. 7 and 8) to challenge infection with virulent S. typhimurium than naive MΦ-depleted mice (0 % survival, Figs. 2 and 3), suggesting that other immune effector mechanisms help to protect immunized mice against virulent challenge infection. Results of preliminary studies in our laboratory investigating the effect of transfer of immune serum into naive normal and MΦ-depleted mice before infection with virulent S. typhimurium have suggested that serum factors might form an additional component mediating protection. Furthermore, our results also suggest that, although antigen-specific acquired T cell responses are essential for complete recovery from virulent Salmonella infections [5, 18], these responses by themselves are not sufficient to control Salmonella infections in MΦ-depleted mice.
immune mice, however, bactericidal activities of MΦ may be enhanced by IFN-γ derived form activated antigen-specific T cells, resulting in a more rapid clearance of bacterial infections, and on the other hand, activated MΦ may secrete cytokines such as IL-12 which may enhance T cell activation and secretion of IFN-γ. The importance of such cytokine-related interactions between MΦ and immune T cells was recently shown in humans which lacked the β-chain of the IL-12 receptor [36]. These individuals were deficient in signaling through the IL-12 receptor and in the production of IFN-γ, and were unable to control mycobacterial and Salmonella infections.

Taken together, our results are important for the understanding of the host response to S. typhimurium, and provide further insight into the relevance of in vivo MΦ-S. typhimurium interactions for the immunobiology of murine salmonellosis. We suggest that the interaction between MΦ and S. typhimurium is significant for the pathology of infection and much less important in the induction of protective immunity. The experiments described here were performed in ItyS mice, which are known to harbor a single point mutation in the Nramp1 gene, resulting in a significant reduction in innate resistance against S. typhimurium infection [16]. In ItyS mice, MΦ express a functional Nramp1 gene, and removal of MΦ may therefore adversely affect the innate resistance of naïve mice to virulent S. typhimurium infections. It is not known whether humans express alleles of Nramp1, but promoter polymorphisms, which may yield phenotypes similar to the murine ItyH and ItyR, have been observed [37]. However, further understanding of the mechanism behind MΦ-mediated pathology of S. typhimurium infections in susceptible individuals would be of great benefit for the development of therapeutic strategies. Such new treatments, used in combination with antibiotic treatment, should focus on transient inhibition of early release of cytokines and/or other inflammatory mediators by MΦ which are mainly responsible for pathology associated with Salmonella infections, thereby limiting the damage in infected tissues and sepsiticaemia, and potentially reducing the clinical symptoms of typhoid fever.

4 Materials and methods

4.1 Bacteria

In this study we used the aroA aroD mutant of S. typhimurium SL1344 (BRD509, a kind gift of Prof. G. Dougan, Imperial College, London, GB) expressing C-frag from the pTET-tac4 expression construct (BRD509/C-frag) [38], and virulent S. typhimurium SL1344 [39].

4.2 In vivo MΦ depletion

MΦ can be depleted in vivo using CI1,MDP-loaded liposomes [22–25]. We have previously established that CI1,MDP does not affect the growth of BRD509 or SL1344 in vitro (results not shown). Liposomes were prepared as previously described [22]; CI1,MDP was a kind gift of Boehringer Mannheim (Mannheim, Germany). In some experiments, mice were treated with PBS-loaded liposomes as a control. Successful elimination of MΦ from spleen and liver was confirmed by immunohistology.

4.3 Immunization protocol

BRD509, BRD509/C-frag and SL1344 were grown overnight in stationary Luria-Bertani (LB) broth cultures (supplemented with antibiotics) at 37 °C and were diluted with PBS. Normal and CI1,MDP-liposome-treated 6- to 8-week-old female BALB/c mice (ItyS) were inoculated i.v. with 10⁵ CFU BRD509/C-frag or BRD509 in 0.2 ml PBS. When indicated, mice were challenged i.v. with 100 CFU virulent SL1344 (10 x LD₅₀) in 0.2 ml PBS.

4.4 Viable counts of S. typhimurium in organs

Groups of five mice were killed at indicated time points after infection, and aseptically removed spleens and livers were homogenized in sterile bags (Starstedt, Ingle Farm, SA, Australia) containing 5 ml of sterile PBS, using a Stomacher 80 homogenizer (Seward Medical, London, GB). The number of viable S. typhimurium in tissue homogenates was determined by viable count on LB agar plates containing the appropriate antibiotics.

4.5 Detection of antigen-specific antibodies in serum by ELISA

Serum antibodies against C-frag or LPS were detected by ELISA on tetanus toxin-coated (2 Lf/ml, CSL Ltd, Melbourne, Australia) or S. typhimurium LPS-coated (10 μg/ml, Sigma Chemical Co., MO) microtiter plates (NUNC, Denmark) respectively, as described in detail previously [40]. Serum titers are presented as the highest dilution with an absorbance of 0.10 above normal mouse serum.

4.6 Lymphocyte proliferation assay

T cell proliferation assays were performed 10 days after immunization in 96-well flat-bottom plates (NUNC, Denmark) in triplicate cultures. Single-cell suspensions prepared from spleens of three mice were enriched for T cells by passing through nylon wool columns. Splenic T cells (3 x 10⁵/well) were cultured with serial dilutions of recombinant C-frag and 3 x 10⁵ irradiated (3000 rad) naive splenocytes as
stimulators in RPMI 1640 (Gibco-BRL Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM glutamine, antibiotics and 2-ME. The cells were cultured for 96 h and the last 16 h pulsed with [3H]thymidine. Cells harvested on fiberglass filters and [3H]thymidine incorporation was measured.

4.7 Statistical analysis

The non-parametric Mann-Whitney U-test was used to determine statistically significant differences between groups of mice.

References


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