Effects of *in vivo* administration of anti-IL-10 monoclonal antibody on the host defence mechanism against murine *Salmonella* infection

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SUMMARY

Interleukin-10 (IL-10)is a cytokine that regulates various macrophage functions. To elucidate the involvement of endogenous IL-10 in the early stage of murine salmonellosis, we examined the effect of anti-IL-10 monoclonal antibody (mAb) administration on the host defence mechanism against *Salmonella choleraesuis* infection. The *in vivo* administration of anti-IL-10 mAb significantly enhanced host resistance at the early stage of *Salmonella* infection, as assessed by bacterial growth in the peritoneal cavity and the liver. Enhanced levels of monokine mRNA, including IL-1α, tumour necrosis factor-α (TNF-α) and IL-12, were observed from day 1 after infection in the peritoneal macrophages in anti-IL-10 mAb-treated mice compared with those in control mAb-treated mice. Mice treated with anti-IL-10 mAb exhibited significantly higher levels of interferon-γ (IFN-γ) in the peritoneal exudates and major histocompatibility complex (MHC) class II expression on the peritoneal macrophages on days 3 and 5 after infection. Notably, *in vivo* anti-IL-10 mAb brought about an increment of γδ T cells in the peritoneal cavity at the early phase of infection, which was correlated with the expression of endogenous heat-shock protein 60 (HSP60), which is implicated as a potential ligand for γδ T cells, in the infected macrophages. Our results suggest that the neutralization of endogenous IL-10 accelerates some macrophage functions and, consequently, the activation of immunocompetent cells, including γδ T cells, at the early stage of infection, resulting in an enhanced host defence against *Salmonella* infection.

INTRODUCTION

The host defence against *Salmonella* species, like other intracellular pathogens, in mice consists of early natural resistance and the following pathogen-specific T-cell mediated immunity. During the early phase (about 1 week) after the inoculation of mice with a sublethal dose of *Salmonella typhimurium*, the bacteria grow exponentially in organs such as the liver and spleen.[1] The replication of bacteria within macrophages in this early stage of infection is under the control of the *Ity* gene,[2] which is thought to encode a macrophage-specific membrane transport function,[3] and of macrophage activation through interferon-γ (IFN-γ) released from several

cell types. Although T lymphocytes, including CD8^{+} T cells and the T-helper type-1 (Th1) subset of CD4^{+} T cells, represent the major source of IFN-γ, natural killer (NK) cells are possible IFN-γ producers at the early stage of infection, as shown in murine listeriosis and murine salmonellosis.[4–7] In addition, we have reported that γδ T cells, which appear at an early stage of infection with *Listeria monocytogenes* and *S. choleraesuis* avirulent strain 31N-1 in mice, responded to heat-shock protein (HSP) homologous to the mycobacterial 65 000 MW HSP and secreted IFN-γ.[8,9] It has also been demonstrated that the Ity locus is closely related to the expression of HSP by macrophages after infection with *S. choleraesuis*, which in turn stimulates the γδ T cells in the host during the early phase of salmonellosis.[10] It would thus appear that the γδ T cells that appear early during salmonellosis may play a role in protection through IFN-γ production at the early stage of *Salmonella* infection.

Interleukin-10 (IL-10), originally described as a cytokine synthesis inhibitory factor produced by the Th2 subset of CD4^{+} T lymphocytes, acts directly upon antigen-presenting cells (APC) and indirectly upon Th1 cells to inhibit cytokine production.[11,12] Recently, IL-10 has been shown to be a key cytokine that regulates macrophage functions including

cytokine production, major histocompatibility complex (MHC) class II expression, and nitric oxide (NO) production.[13-16] In addition, anti-IL-10 monoclonal antibody (mAb) treatment has been shown to enhance the resistance of mice to infection with intracellular parasites such as *Mycobacterium avium*,[17] *Candida albicans*[16] and *L. monocytogenes*.[18] On the other hand, anti-IL-10 mAb-treated mice exhibit an increased susceptibility to septic shock caused by Gram-negative bacteria, suggesting that IL-10 plays a protective role in endotoxic shock through the inhibition of excessive tumour necrosis factor-α (TNF-α) production.[19] Thus, IL-10 has various functional aspects in protection against various microbial infections.

In this study, to understand the involvement of IL-10 in the host defence mechanisms against infection with *Salmonella*, an intracellular Gram-negative rod with lipopolysaccharide (LPS), we examined the effect of anti-IL-10 mAb administration on the mechanisms against *S. choleraesuis* infection, including the cytokine profiles, macrophage functions and the induction of γδ T cells. Our results demonstrate that IL-10 neutralization induces a higher amount of IFN-γ production and shows a protective effect at the early phase of *S. choleraesuis* infection. This enhanced resistance brought about by blockade of endogenous IL-10 is closely associated with the increased induction of monokine mRNA, such as TNF-αand HSP, in the macrophages, and an increment of γδ T cells in anti-IL-10 mAb-treated mice. We discuss the regulatory roles of endogenous IL-10 in host defence against *Salmonella* infection.

MATERIALS AND METHODS

Animals and micro-organisms

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Eight- to 10-week-old mice were used for the experiments. *Salmonella choleraesuis* strain 31N-1, which is a derivative cured of its 50 kb virulent plasmid,[20] was used throughout the experiments. The approximate intraperitoneal 50% lethal dose (LD_{50}) of this strain for BALB/c mice was 5×10^6 colony-forming units (CFU). Fresh isolates were obtained from infected spleen, grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37° for 4 hr, harvested, washed, resuspended in phosphate-buffered saline (PBS), and stored at −70° in small aliquots.

Antibodies and reagents

The hybridoma line SXC-1, secreting rat IgM anti-mouse IL-10,[21,22] was used. The hybridoma line WFL4F12.3, secreting control rat IgM, was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The mAbs were obtained by growing hybridoma cells in serum-free medium SFM 101 (Nissui, Tokyo, Japan) and collecting supenatants, which were concentrated and purified by 50% ammonium sulphate precipitation. The purity of preparations was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the concentration of antibodies was determined by the Lowry method. The mAbs diluted to 1 mg/ml in PBS were stored at −70° until use. Fluorescein (FITC)-conjugated anti-CD3 mAb (145-2C11), phycoerythrin (PE)-conjugated anti-T-cell receptor (TCR)αβ (H57–579) mAb, PE-conjugated anti-TCR γδ (GL3) mAb and FITC-conjugated anti-I-A^d mAb were purchased from PharMingen (San Diego, CA). PE-conjugated anti-Thy-1.2 mAb, FITC-

conjugated anti-Ly-5 (B220) mAb and PE-conjugated anti-Mac-1 mAb (M1/70) were purchased from Caltag Laboratories Inc.(South San Francisco, CA). Streptavidin-conjugated Red613 was purchased from Gibco BRL (Gaithersburg, MD). The anti-FcγRII/III-specific mAb (2.4G2) was obtained from ATCC. The mouse mAb ML-30 (IgG1), which was originally raised against mycobacterial HSP 65 (epitope 286–297)[23] and found to be cross-reactive with human and murine HSP 60 molecule,[24] was kindly provided by Dr J. Ivanyi (Royal Postgraduate Medical School, London, UK).

Kinetics of bacterial growth after intraperitoneal infection with S. choleraesuis

Mice were given an intraperitoneal (i.p.) injection of 200 μg anti-IL-10 mAb SXC-1 in 200 μl 2 hr before an i.p. inoculation with 5×10^4 viable *S. choleraesuis* 31N-1 in 200 μl of PBS. As a control, mice were injected with same amount of mAb WFL4F12.3 or the same volume of PBS in the same manner. At the indicated times after infection, mice were killed by cervical dislocation. For enumeration of viable bacteria in the peritoneal cavity, peritoneal exudates were obtained by peritoneal lavage with 4 ml of Hanks' balanced saline solution (HBSS), and serial dilutions of exudate samples were plated in tryptic soy agar. The numbers of bacteria in the liver were counted by plating serial dilutions of organ homogenates on tryptic soy agar. The CFU per organ was estimated after 24 hr of culture at 37°.

Peritoneal exudate cells

Mice were killed at the indicated periods after infection. The peritoneal exudate cells (PEC) were harvested by peritoneal lavage with ice-cold HBSS containing 5 U/ml of heparin. The cells were collected by centrifugation at 110 *g* for 10 min, washed twice with HBSS, and resuspended at a concentration of 10^6/ml in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES. Cells were plated and allowed to adhere for 2 hr at 37° in a humidified atmosphere of 95% air and 5% CO_2. Non-adherent cells were washed, counted and used as mononuclear cells. Adherent cells that were collected by scraping with a rubber policeman were also washed and counted. Adherent cells constituted more than 95% macrophages as determined by esterase staining.

Kinetics of PEC populations

The kinetics of PEC populations were determined on cells spun on slides stained with May–Grünwald Giemsa and by flow cytometry (FCM) at various times during infection. For two-colour analysis, cells were stained with FITC–anti-CD3 mAb, PE–anti-TCRαβ and PE–anti-TCRγδ mAb, or with PE–anti-Thy-1.2 mAb, FITC–anti-B220 mAb and biotin–anti-Ly-1 mAb, followed by streptavidin-conjugated Red613. The cells were analysed using a FACScan™ (Becton Dickinson, San Jose, CA), and live cells were gated by forward and side scattering. To determine the subpopulatin percentage, total counts were integrated in a selected area of control plot.

FCM analysis of class II MHC expression

One million PEC was incubated first with 2.4G2 (anti-FcγR mAb), then stained with FITC-conjugated anti-I-A^d mAb and PE-conjugated anti-Mac-1 mAb. They were analysed using a

FACScan™, and live cells were gated by forward and side scattering. The analysis gate was set on Mac-1$^+$ cells, and the expression of I-A^d was presented as a single histogram.

TNF-α, IFN-γ and IL-10 assays

TNF-α, IFN-γ and IL-10 activities were assayed in the samples of sera and peritoneal exudates. The peritoneal exudates were obtained from mice at the indicated times post-infection by the i.p. injection of 600 µl PBS containing 5 U/ml heparin, and subsequent withdrawal. The TNF-α, IFN-γ and IL-10 activities were determined by ELISA using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Factor-Test™TNF-α; Genzyme, Cambridge, MA), a mouse IFN-γ ELISA kit (INTERTEST™-γ; Genzyme) and a murine IL-10 ELISA kit (Endogen, Boston, MA), with a sensitivity of 100 pg/ml, 125 pg/ml and 0·14 U/ml, respectively.

RNA extraction and semi-quantitative polymerase chain reaction analysis

Total RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method[25] from 1×10^7 adherent PEC, which were obtained from PEC pooled from five mice at various times after infection. Two micrograms of RNA was reverse transcribed (RT) with SuperScript™II RT (BRL, Gaithersburg, MD) and 1·5 µg of random primers (BRL) in 20 µl of reaction buffer, according to manufacturer's instructions. The synthesized first strand of cDNA was diluted to a total volume of 50 µl with distilled water. An aliquot of first strand cDNA was amplified by means of polymerase chain reaction (PCR) using 40 pmol of each primer with 2·5 U of Ampli Taq (Perkin-Elmer Cetus, Norwalk, CT) in a total volume of 50 µl of reaction buffer consisting of 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·01% gelatin and 200 µM dNTP. PCR cycles were run for 1 min at 94°, followed by 1 min at 57° and 30 seconds at 72°. Before the first cycle, denaturation for 7 min at 94° was included, and after 19–30 cycles extension was prolonged for 4 min at 72°. Five microlitres of PCR products was removed after 20, 23 and 26 cycles, electrophoresed on a 1·8 % agarose gel and transferred to Gene Screen Plus (New England Nuclear, Boston, MA). Southern blot analyses were performed with the cytokine-specific oligonucleotide probes, which were labelled with [γ-^{32}P]ATP using a MEGA-LABEL 5′ labelling kit (Takara Shuzo Co. Ltd, Kyoto, Japan), according to the manufacturer's instructions. After hybridization for 18 hr at 65° in 1% SDS, 1 M NaCl,10% dextran sulphate and 100 µg/ml heat-denatured salmon sperm DNA, the filter were washed in 2 × SSC (0·3 M NaCl, 0·03 M sodium citrate) and 1% SDS and exposed to X-ray film. The films were analysed by interpretive densitometer Master Scan™ (CSPI, Billerica, MA). The primers used were as follows: IL-1α sense: 5′-CTCTAGAGCAC-CATGCTACAGAC-3′, antisense: 5′-TGGAATCCAGGG-GAAACACTG-3′; IL-10 sense: 5′-TACCTGGTAGAAGT-GATGCC-3′, antisense: 5′- CATCATGTATGCTTCTATGC-3′; IL-12 sense: 5′-CGTGCTCATGGCTGGTGCAAAG-3′, antisense: 5′-CTTCATCTGCAAGTTCTTGGGC-3′; TNF-α sense: 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′, antisense: 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′; β-actin sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, antisense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; inducible nitric oxide synthase (iNOS-sense): 5′TGGGAATGGAGACTGT-

CCCAG-3′, antisense: 5′-GGGATCTGAATGTGATGTTTG-3′. The oligonucleotide probes were as follows: IL-1α: 5′-AATGATGTAAGAATACCCAG-3′; IL-10: 5′-GGTCTTC-AGCTTCTCACCCA-3′; IL-12: 5′-TCTGTCTGCAGAGA-AGGTCACA-3′; TNF-α: 5′-CCAGGTCACTGTCCCAGCAT-3′; β-actin: 5′-TTCTGCATCCTGTCAGCAAT-3′; iNOS: 5′-CTGCGCCTTTGCTCATGACA-3′.

Western blot assays

Ten microlitre per lane from 200 µl of lysates containing 2×10^6 cells were separated by means of SDS–PAGE with a 12·5% gel according to the modified procedure of Laemmli.[26] Western blot assay was performed according to the methods of Burnette[27] with a modification. After electrophoresis, the gel was immediately transferred to a membrane (Immobilon™ PVDF; Millipore, Bedford, MA) at a constant voltage (20 V) for 30 min in prechilled electrode buffer (192 mM glycine, 25 mM Tris base, 20% methanol, 0·03% SDS). The membrane was then blocked with 1% bovine serum albumin (BSA)–PBS for 2 hr at 25°, and incubated at 4° overnight in diluted ML30, anti-HSP 60 mAb, in 0·1% BSA–PBS. After washing with 0·05% Tween-20–PBS (washing buffer), the membrane was incubated at 25° for 1 hr in diluted horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Fab′)$_2$ fragment (Cappel, Durham, NC). Finally, after washing, the antigen was detected using ECL™ Western blotting detection reagents (Amersham Int., Amersham, UK).

Statistical analysis

Data were analysed by Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of anti-IL-10 mAb on the kinetics of the bacterial growth in the peritoneal cavity and liver after an i.p. infection with *S. choleraesuis*

We examined whether or not the *in vivo* effect of IL-10 neutralization is protective against infection with *S. choleraesuis*, which is a Gram-negative, facultative intracellular rod, that often causes endotoxic shock. A sublethal dose (5×10^4) of viable *S. choleraesuis* was inoculated i.p. in mice 2 hr after an i.p. injection of 200 µg of anti-IL-10 mAb, or the same amount of control mAb, and the kinetics of bacterial growth in the peritoneal cavity and the liver were examined on 3, 6 and 10 days after infection. As shown in Fig. 1, the anti-IL-10 mAb treatment provided significant protection against *S. choleraesuis* as assessed by bacterial growth in the peritoneal cavity and the liver. The influence of the mAb was evident on 3 days after infection in the liver, and maximally expressed on day 6 in both sites. These results suggest that neutralization of endogenous IL-10 augments the host defence against *Salmonella* from the early phase of infection.

Effect of anti-IL-10 mAb on IFN-γ and iNOS levels and MHC class II expression *in vivo* after an i.p. infection with *S. choleraesuis*

IFN-γ is reportedly a critical cytokine for host defence against *Salmonella* infection.[28–30] Therefore, we next compared the
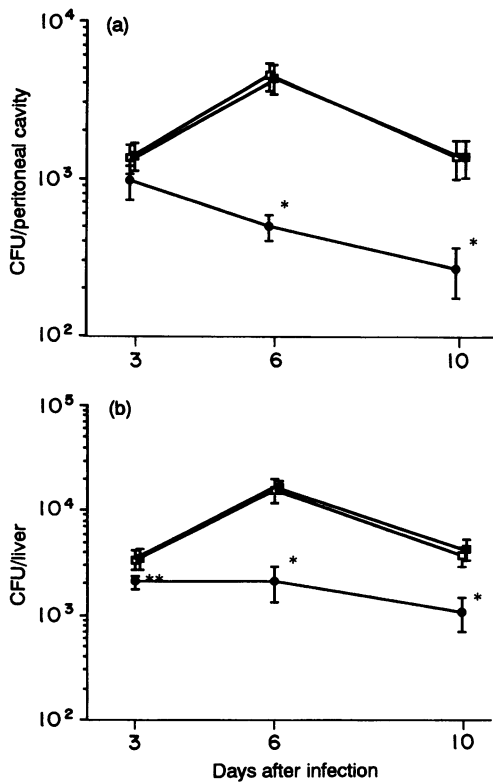
Figure 1. Effect of anti-IL-10 mAb on bacterial growth in the peritoneal cavity (a) and the liver (b). *Salmonella choleraesuis* (5 × 10⁴) was inoculated i.p. into mice 2 hr after an i.p. injection of 200 μg anti-IL-10 mAb (closed circles), WFL4F12.3 (open squares) or the same volume of PBS (closed squares). Data were obtained from three separate experiments and are expressed as the mean ± SD for six mice at each time point. $*P < 0.001$ versus control; $**P < 0.01$ versus control.
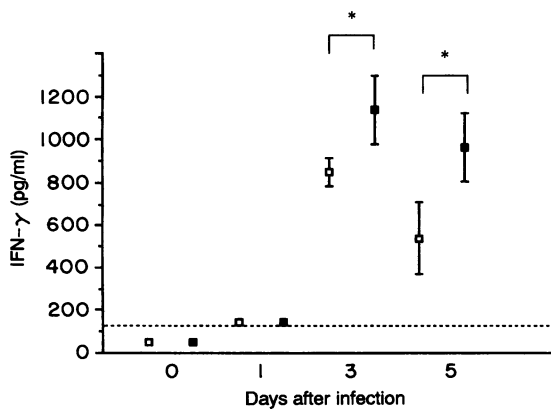

Figure 2. Effect of anti-IL-10 mAb on the IFN-γ production by PEC infected with *S. choleraesuis*. PBS containing 5 U/ml heparin was injected into the peritoneal cavity of anti-IL-10 mAb-treated (closed squares) or control mAb-treated (open squares) mice at the indicated time points following infection. The collection was assayed for IFN-γ activity using ELISA. Data are presented as the mean ± SD for five mice. $*P < 0.01$.

levels of IFN-γ production in the peritoneal exudates from mice given anti-IL-10 mAb and control mAb. The former produced a significantly larger amount of IFN-γ in the peritoneal exudates on days 3 and 5 after infection than control mice (Fig. 2). As evidence for enhanced production of IFN-γ by anti-IL-10 mAb-treated mice, FCM analysis revealed that MHC class II expression by the peritoneal macrophages was increased in the anti-IL-10 mAb-treated, compared with control, mice on days 3 and 5 after infection (Fig. 3). In addition, mRNA of iNOS, which is strictly dependent upon IFN-γ production,[31] in the macrophages of anti-IL-10 mAb-treated mice were expressed at higher levels than in control mice from 1 to 5 days after infection (Fig. 4). These results suggest that IFN-γ functions in a microbicidal mechanism that is enhanced via the neutralization of endogenous IL-10. TNF-α and IL-10 activities in the sera and peritoneal exudates were
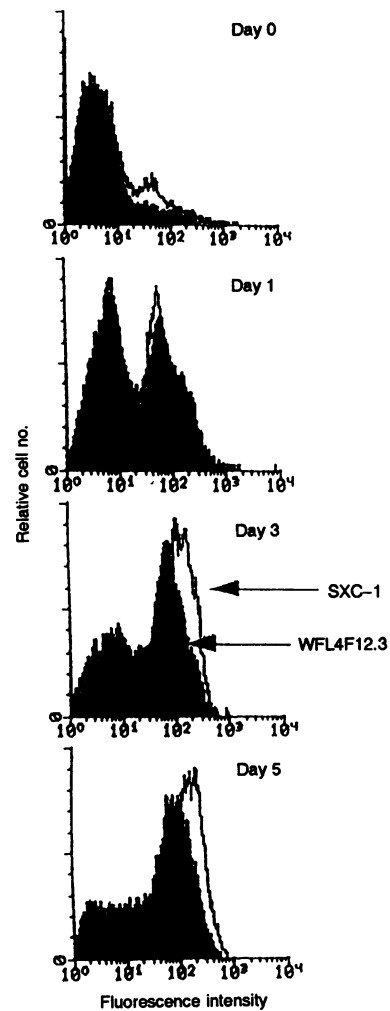

Figure 3. Effect of anti-IL-10 mAb on MHC class II expression on the peritoneal macrophages from mice infected with *S. choleraesuis*. PEC were obtained from anti-IL-10 mAb- or control mAb-treated mice at the indicated time points post-infection. The cells were incubated first with 2.4G2 (anti-FcγR mAb), then stained with FITC-conjugated anti-I-A^d mAb and PE-conjugated anti-Mac-1 mAb. The analysis gate was set on Mac-1$^+$ cells, and a single histogram on expression of I-A^d is presented.
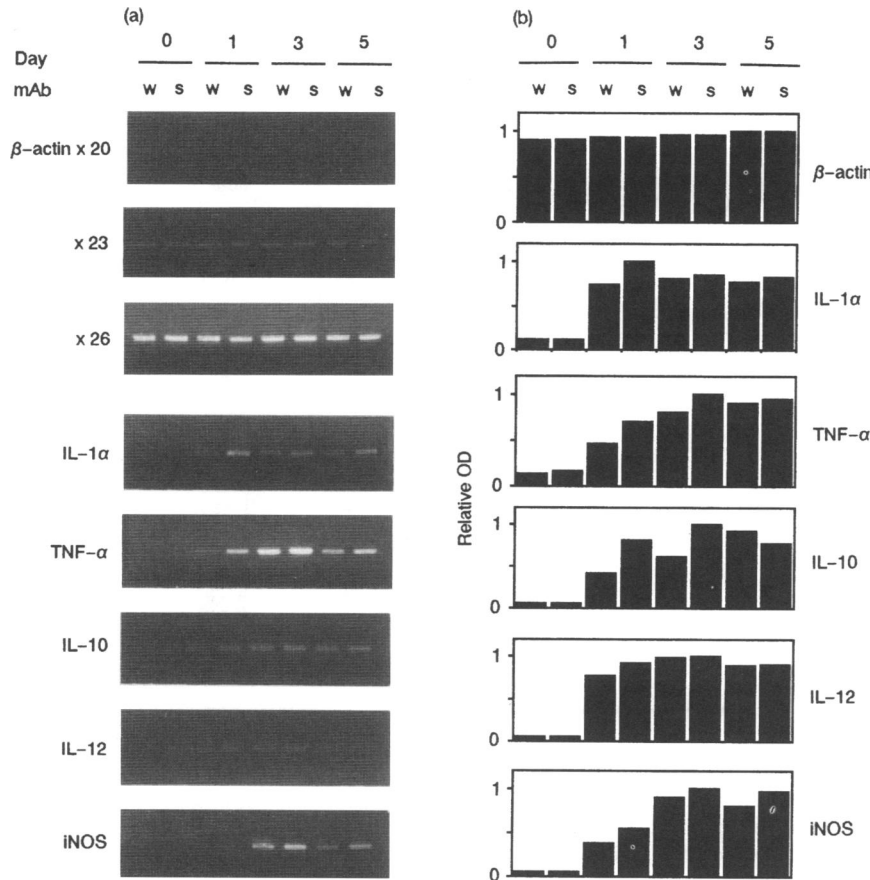
Figure 4. Effects of anti-IL-10 mAb on the expression of cytokine-specific mRNA by macrophages from mice infected with *S. choleraesuis*. Messenger RNA was isolated from macrophages obtained from anti-IL-10 mAb- or control mAb-treated mice at the indicated time points post-infection. The expression of β-actin, each cytokine and iNOS was determined by PCR-assisted mRNA amplification (a). Southern transfers of PCR products were hybridized with ^{32}P-labelled internal probes and the exposed X-ray films were analysed quantitatively using a densitometer (Master Scan^{TM}). The signal intensity is presented as the relative value to the maximal OD of each cytokine (b). W, WFL4F12.3; S, SXC-1.

below the detectable level in both anti-IL-10 mAb-treated and control mice during infection in our *in vivo* system.

Effect of anti-IL-10 mAb on expression of monokine mRNA by the peritoneal adherent cells after an i.p. infection with *S. choleraesuis*

IL-10 is reported to be a potent macrophage-deactivating cytokine *in vitro* and to inhibit the production of proinflammatory cytokines including TNF-α by lipopolysaccharide (LPS)-activated monocytes and macrophages.[12,14] To investigate the underlying mechanism of the enhanced host defence against *Salmonella* infection in anti-IL-10 mAb-treated mice, we focused upon the effects of anti-IL-10 mAb treatment on monokine mRNA expression during an i.p. infection with *S. choleraesuis*. We compared cytokine mRNA expression in the adherent PEC from anti-IL-10 mAb-treated and control mice using semi-quantitative RT-PCR analysis. The total RNA extracted from each sample were reverse transcribed and amplified with cytokine-specific primers, as described in the Materials and Methods. We confirmed that the amounts of β-actin-specific PCR products were equal among samples from 20

to 26 cycles (Fig. 4). The levels of cytokine-specific PCR products are presented in Fig. 4 after 29 or 31 cycles for IL-1α and TNF-α, and IL-10 and IL-12, respectively. Southern transfers of PCR products were hybridized with ^{32}P-labelled internal probes and the exposed X-ray films were quantitatively analysed using a densitometer (Master Scan^{TM}). The signal intensity is presented as the value relative to the maximal optical density (OD) of each cytokine (Fig. 4). It shows that all of IL-1α, TNF-α, IL-10 and IL-12 mRNA expression was evident from 1 to 5 days post-infection in control mice, and that the levels of those in anti-IL-10 mAb-treated mice were higher than those in control mice. These results suggest that anti-IL-10 mAb compelled macrophages to induce more cytokines, which have been shown to up-regulate IFN-γ production by inflammatory cells including NK cells,[32] resulting in enhanced resistance against *Salmonella* infection.

Effect of anti-IL-10 mAb on $\gamma\delta$ T cells after an i.p. infection with *S. choleraesuis*

To analyse further the effects of neutralizing endogenous IL-10 on the cellular responses to *Salmonella* infection, we examined
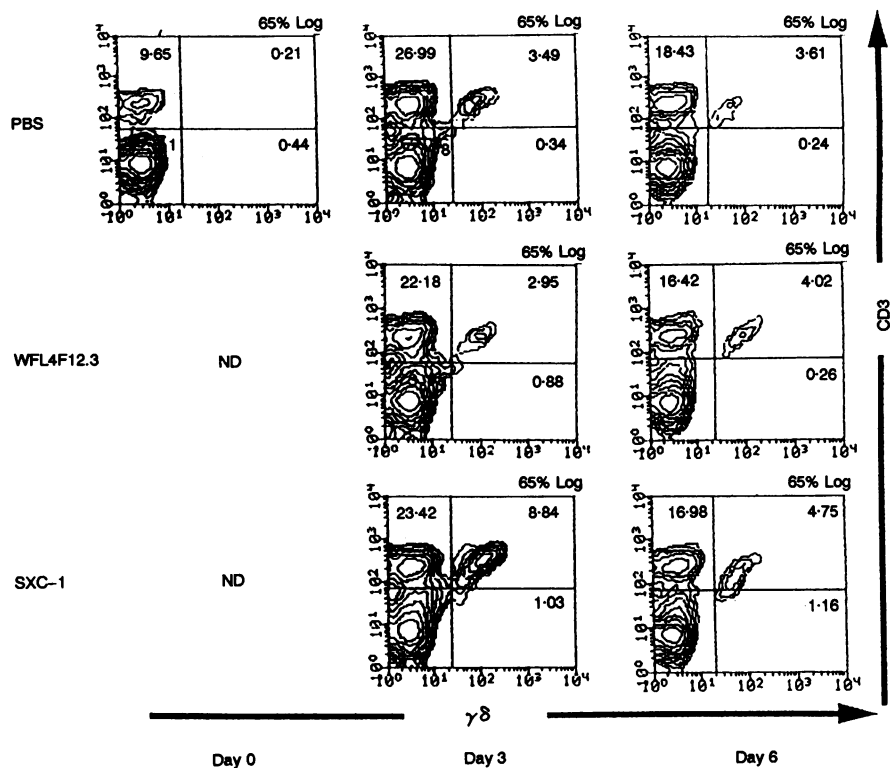
Figure 5. Effect of anti-IL-10 mAb administration on the appearance of $\gamma\delta$ T cells in the peritoneal cavity after i.p. infection with *S. choleraesuis*. Mice were inoculated i.p. with 5×10^4 *S. choleraesuis* 2 hr after an i.p. injection with 200 μg of mAb WFL4F12.3, SXC-1 or the same volume of PBS. Non-adherent peritoneal cells were stained with FITC-conjugated anti-CD3 mAb and PE-conjugated anti-TCR$\gamma\delta$ mAb. ND, not done.

the kinetics of PEC from anti-IL-10 mAb-treated and control mAb-treated mice after an i.p. inoculation with 5×10^4 *S. choleraesuis*. Judging from the absolute number of PEC and the morphological characteristics of cells on slides stained with May–Grünwald Giemsa, there were no significant differences in the number of monocytes/macrophages, polymorphonuclear cells and lymphocytes between anti-IL-10 mAb and control mAb groups, on days 3, 6, and 10 after inoculation (data not shown). FCM analysis revealed that $\gamma\delta$ T cells significantly increased in number and proportion in the peritoneal cavity on day 3 in anti-IL-10 mAb-treated mice ($8.5 \pm 2.2\%$ versus $3.2 \pm 1.3\%$, $P < 0.01$) (Fig. 5), whereas there were no significant differences in the appearance of NK cells (Thy-1.2$^+$ CD3$^-$ cells), $\alpha\beta$ T cells or B cells between anti-IL-10 mAb- and control mAb-treated mice. Thus, the neutralization of endogenous IL-10 may preferentially induce $\gamma\delta$ T cells at inflamed sites after *Salmonella* infection.

Effect of anti-IL-10 mAb on the expression of endogenous HSP in peritoneal adherent cells

We have previously shown that $\gamma\delta$ T cells proliferate in response to the peritoneal adherent cells in mice infected with *S. choleraesuis* 31N-1, which express a high level of endogenous HSP homologous to the mycobacterial 65 000 MW HSP, and that the different appearance of $\gamma\delta$ T cells during salmonellosis between *Ity^s* and *Ity^r* mice may be due to different expression of HSP on infected macrophages.[9,10] To determine whether an

increase in the number of $\gamma\delta$ T cells in the peritoneal cavity of anti-IL-10 mAb-treated mice was related to a higher level of HSP 60 in the infected peritoneal macrophages, we examined the effects of anti-IL-10 mAb on HSP 60 induction in the peritoneal adherent cells by Western blot analysis using ML30 mAb, which reacts with mycobacterial and murine HSP 60 but not with the salmonella analogue of this protein.[33] As shown in Fig. 6, the peritoneal macrophages from anti-IL-10 mAb-treated mice at 3 days post-infection expressed much more
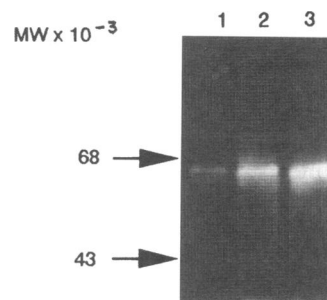

Figure 6. Effect of blocking endogenous IL-10 on HSP expression in the peritoneal adherent cells from mice infected with *S. choleraesuis*. Cell lysates obtained from naive mice (lane 1), control mAb- (lane 2) or anti-IL-10 mAb- (lane 3) treated mice at 3 days post-infection were run under reducing conditions in a SDS–PAGE system, electroblotted, and stained with the anti-HSP 60 mAb ML30.

endogenous HSP homologous to the mycobacterial 65 000 MW HSP. These results suggest that endogenous IL-10 regulates the induction of endogenous HSP 60 and that an increased level of HSP expression on the macrophages infected with *S. choleraesuis* may in turn stimulate $\gamma\delta$ T cells in anti-IL-10 mAb-treated mice.

DISCUSSION

This current study has revealed that a blockade of endogenous IL-10 confers enhanced resistance to *Salmonella* infection, particularly in the early phase. We observed the early and profound induction of monokine mRNA expression, such as that of TNF-α and IL-1, in the peritoneal macrophages derived from mice treated with anti-IL-10 mAb. Notably, more increased levels of IFN-γ production and $\gamma\delta$ T-cell accumulation were detected in the peritoneal cavity of anti-IL-10 mAb-treated mice compared with control mice at the early phase of infection. In addition, HSP 60 expression by the peritoneal macrophages was shown to be enhanced by neutralization of endogenous IL-10. It may be possible to speculate that an increased number of $\gamma\delta$ T cells contributes to the elevation of IFN-γ in anti-IL-10 mAb-treated mice at the early phase of *Salmonella* infection, although we do not deny the possible contribution of NK cells. The activation of $\gamma\delta$ T cells following the neutralization of endogenous IL-10 may be at least partially involved in the enhanced resistance to *Salmonella* infection seen in anti-IL-10 mAb-treated mice.

The effects of anti-IL-10 mAb on the host defence mechanisms against microbial infection have been demonstrated by several groups. Romani *et al.*[16] reported that the neutralization of IL-10 up-regulated NO production and protected susceptible mice from challenge with *Candida albicans*. Similar observations have been made recently with *Mycobacterium avium*-infected mice, showing that IL-10 neutralization augments mouse resistance to systemic *Mycobacterium avium* infection by enhancing the mycobacteriostatic activity of macrophages.[17] Both groups concluded that macrophage activation following the neutralization of endogenous IL-10 is responsible for the enhanced resistance to the bacterial infection. The present study extends their findings to include the host responses to infection with the LPS-containing Gram-negative intracellular bacterium, *S. choleraesuis*. As an underlying mechanism of the effect of IL-10 neutralization on the host defence against *Salmonella* infection, we obtained evidence that higher levels of monokine mRNA, including TNF-α and IL-1-α, were induced in the peritoneal macrophages of mice given anti-IL-10 mAb from 1 day after i.p. infection, compared with those given control mAb. These monokines seemingly activate macrophages via the autocrine pathway.[34-38]

One notable finding in this study is that the induction of $\gamma\delta$ T cells during i.p. infection with *S. choleraesuis* is augmented by the administration of anti-IL-10 mAb. We and others have demonstrated that the $\gamma\delta$ T cells play a significant role in primary resistance to infection with intracellular pathogens, including *Salmonella*.[8,39,40] We reported that $\gamma\delta$ T cells, which appeared at the early stage of infection with *L. monocytogenes*, responded to the 65 000 MW mycobacterial HSP and produced IFN-γ.[8] We also reported that infection with an avirulent strain of *S. choleraesuis* induced a larger amount of endogenous

HSP 60 in infected macrophages, which in turn increased the number of $\gamma\delta$ T cells *in vivo*.[9] Furthermore, we demonstrated that the different appearance in number of $\gamma\delta$ T cells was due to different levels of HSP induction in the macrophages of *Ity*^{s} and *Ity*^{r} mouse strains in response to *S. choleraesuis*.[10] The Western blot analysis revealed that expression of HSP 60 in the peritoneal macrophages infected with *Salmonella* was apparently augmented by the neutralization of endogenous IL-10 *in vivo*. As suggested by our previous studies, the increased level of HSP expression may cause an increase of $\gamma\delta$ T cells in the peritoneal cavity at the early stage of *Salmonella* infection in anti-IL-10 mAb-treated mice, which may be at least partly responsible for the elevated IFN-γ production at the early stage of infection in these mice. Although there still remains the possibility that the elevated level of IFN-γ seen in anti-IL-10 mAb-treated mice may be caused by NK cells stimulated by IL-12, IL-1 and TNF-α, all of which were augmented by the treatment of anti-IL-10 mAb *in vivo*, we could not detect any significant differences in the increment of NK cells during infection between mice given anti-IL-10 or control mAb.

Another notable finding in our study on the biological function of IL-10 is that the neutralization of endogenous IL-10 augmented endogenous HSP expression in macrophages after infection with *S. choleraesuis in vivo*. To our knowledge, this is the first evidence of a regulatory role of IL-10 in the expression of HSP by macrophages. Inflammation caused by infection with various pathogens has been closely linked to HSP synthesis in inflammatory cells such as monocytes/macrophages.[41] After the phagocytosis of bacteria, macrophages produce toxic oxygen metabolites, protease, lipid metabolites and monokines such as TNF-α, all of which can induce HSP in the macrophages.[42] Further examinations are needed to elucidate the regulatory role of IL-10 on HSP induction of *Salmonella*-infected macrophages.

In conclusion, our data provide evidence that endogenous IL-10 is vitally involved in the host defence against *Salmonella* infection from the early phase of infection. The enhanced resistance brought about by neutralization of endogenous IL-10 is closely associated with increased monokine production and HSP 60 expression by the macrophages and an increment of γ/δ T cells. These may be partly responsible for the up-regulation of IFN-γ production in anti-IL-10 mAb-treated mice during salmonellosis.

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