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Suppression of T-helper type-1 immune response against *Listeria monocytogenes* by treatment of mice with goat antibodies to mouse IgD

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SUMMARY

Injection of goat anti-mouse IgD antibodies (GAM IgD) to mice has been shown to induce polyclonal IgG1 and IgE production by B cells and interleukin-4 (IL-4) production by goat Ig-specific T cells. Surface IgD cross-linking also activates B cells to function as antigen-presenting cells (APC). Although the GAM IgD treatment is a well-established system for analysis of B-cell dependent antigen presentation, the influence of GAM IgD treatment on the immune response to irrelevant antigens is not known. To address this issue, we analysed effects of GAM IgD treatment on (1) the mitogen response of freshly isolated T cells, and (2) the listerial antigen-specific response after immunization with viable *Listeria monocytogenes*, which induces $CD4^+$ interferon-γ (IFN-γ) producing protective T cells in normal mice. Spleen $CD4^+$ T cells from the GAM IgD-treated mice produced higher levels of IL-4 but lower levels of IFN-γ and IL-2 than those from the control mice when they were stimulated with concanavalin A (Con A) *in vitro*. When spleen T cells were stimulated with listerial antigen 10 days after a low dose (1/20 LD_{50}) of *L. monocytogenes* infection, $CD4^+$ T cells from the GAM IgD-treated mice showed increased IL-4 production and decreased IFN-γ and IL-2 production compared with those from the control *L. monocytogenes*-infected mice. Furthermore, the GAM IgD treatment resulted in a reduction of the survival rate after a high dose (1/2 LD_{50}) of *L. monocytogenes* infection. These results suggest that treatment of mice with GAM IgD suppresses the T-helper type-1 (Th1)-type T-cell response and induces a Th2-type response against irrelevant antigens, even when they are injected after GAM IgD treatment.

INTRODUCTION

T cells are important for optimal host defence against murine *Listeria monocytogenes* infection.[1–3] We have reported previously that $CD4^+$ T cells mediating both delayed-type hypersensitivity and protective immunity were generated in mice after immunization with viable *L. monocytogenes* (VLM).[4] Among several cytokines examined, it was found that the ability to produce interferon-γ (IFN-γ) was a characteristic of T cells mediating protective immunity,[5] and the importance of IFN-γ in protection against *L. monocytogenes* was proved by using IFN-γ receptor gene mutant mice.[6]

Murine $CD4^+$ T cells have been reported to develop into T-helper type-1 (Th1) or Th2 cells.[7] Th1 cells produce interleukin-2 (IL-2), IFN-γ and tumour necrosis factor-β (TNF-β) and cause a cell-mediated immune response. In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and provide helper activity for B cell Ig production. Furthermore, Th0 cells that produce both Th1- and Th2-type cytokines have been reported to be precursors of Th1 and Th2.[8] The development of Th1 and Th2 cells is regulated by cytokines IL-4, IL-10, IFN-γ and IL-12.[9–13]

It has been reported that injection of goat anti-mouse IgD antibodies (GAM IgD) induces polyclonal IgG1 and IgE production by B cells and IL-4 production by goat Ig-specific T cells.[14–17] The antigen-presenting cell (APC) function of B cells is also activated after surface IgD cross-linking.[18] It is possible that the immune response to irrelevant antigens is also modulated to a Th2 type in GAM IgD-treated mice through IL-4 production and alteration of B-cell APC function. To address this issue, we analysed the immune response of GAM IgD-treated mice against *L. monocytogenes*, which induce a Th1-type immune response in normal mice. Our results show that the Th1-type T-cell response against *L. monocytogenes* is suppressed in GAM IgD-treated mice after infection with *L. monocytogenes*.

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Abbreviations: APC, antigen-presenting cells; GAM IgD, goat anti-mouse IgD antibodies; HKLM, heat-killed *Listeria monocytogenes*; LD_{50}, 50% lethal dose; mAb, monoclonal antibody; PBS, phosphate-buffered saline; VLM, viable *L. monocytogenes*.

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MATERIALS AND METHODS

Microorganisms

Listeria monocytogenes, strain EGD, was used in all experiments. Bacterial virulence was maintained by serial passages in BALB/c mice. Fresh isolates were obtained from infected spleens, grown in tryptic soy broth (Difco Laboratories, Detroit, MI), washed repeatedly, resuspended in phosphate-buffered saline (PBS), and stored at −70° in small aliquots. Fifty per cent lethal dose (LD_{50}) of the bacterial preparation in normal BALB/c mice was 2×10^4 colony forming units (CFU). Heat-killed *L. monocytogenes* (HKLM) were prepared by heating VLM at 74° for 120 min. HKLM have an intact shape at light microscopy level but they are non-viable because they do not grow when spread on agar plates (data not shown).

Animals

Female BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). Mice were used at 8–12 weeks of age. Mice were treated with 800 μg of GAM IgD (Nordic Immunological Laboratories, Tilburg, the Netherlands) or with PBS as control. In some experiments, mice were treated with diluted goat serum containing 800 μg protein or 800 μg goat anti-mouse IgA antibodies (Nordic Immunological Laboratory). Ten days after the treatment, mice were inoculated intraperitoneally (i.p.) with a high dose (1/2 LD_{50}) or a low dose (1/20 LD_{50}) of VLM.

Preparation of CD8-depleted spleen cells

Cell suspensions were prepared by squeezing spleens between two glass slides and repeated pipetting of cell suspensions in Hanks' balanced salt solution. $CD8^+$ cells were depleted by anti-Lyt-2.2 monoclonal antibody (mAb) (Meiji Institute of Health Science, Tokyo, Japan) and guinea-pig complement. The cells were finally suspended to yield 5×10^6 cells/ml in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) (complete medium).

Cytokine assay

The CD8-depleted spleen cell suspension was incubated with concanavalin (Con A) (5 μg/ml), HKLM (2×10^8 dead bacteria/ml) or medium, and cytokine concentrations in the supernatants were determined after 48 hr culture.

IFN-γ was detected by a two-site enzyme-linked immunosorbent assay (ELISA) using hamster anti-mouse IFN-γ mAb (Genzyme, Boston, MA) and rabbit anti-mouse IFN-γ serum (a gift from Central Research Institute, Daiichi Seiyaku Co. Ltd, Tokyo, Japan). Microplates (EIA/RIA plate 3591; Costar, Cambridge, MA) were coated with 100 μl of 1·5 μg/ml anti-IFN-γ mAb in coating buffer of 0·1 M sodium phosphate buffer (pH 7·2) for 12 hr at 4°, followed by blocking with 100 μl of 0·5% bovine serum albumin (BSA) for 30 min at room temperature. Samples and mouse recombinant IFN-γ standard ($4{\cdot}5 \times 10^6$ U/mg; Genzyme) were diluted in 0·05% PBS–Tween-20, and then added to anti-IFN-γ-coated plates and incubated for 90 min at room temperature. After the plates were washed, 50 μl of 100-fold diluted rabbit anti-mouse IFN-γ serum in PBS–Tween-20 was added to the plates. After 90 min, the plates were washed and incubated with 50 μl of a 1 : 800 dilution of goat anti-rabbit IgG/horseradish peroxidase-conjugated IgG (Tago Inc., Burlingame, CA) in PBS–Tween-20 for 90 min. The plates were washed three times with PBS–Tween-20, and 100 μl of substrate consisting of orthophenylenediamine (0·4 mg/ml) and 0·003% H_2O_2 in citrate buffer was added. The reaction was terminated by adding 2·5 M H_2SO_4 and absorbance was measured at a wavelength of 492 nm and a reference of 620 nm on an ELISA reader (EAR 400 FW; SLT-Lab. Instruments, Salzburg, Austria).

IL-4 was detected by a two-site ELISA using rat anti-mouse IL-4 mAb BVB4–1D11 and biotinylated BVD6–24G2 (PharMingen, San Diego, CA). Microplates were coated with BVB4–1D11, loaded with samples or mouse rIL-4 standard (4×10^7 U/mg, Genzyme) and reacted with biotinylated BVD6–24G2 as described for the IFN-γ ELISA. Then 100 μl of 1/1000 diluted streptavidin-β-galactosidase (Gibco BRL) was added to the plates, incubated for 60 min, washed, and 100 μl of 0·2 mM 4-methyl-umbelliferyl-β-D-galactoside was added. The plates were incubated for 60 min at 37° and then 100 μl of 0·1 M glycin–NaOH was added to stop the reaction. Fluorescence intensity was measured with a MTP-32 microplate reader (Corona Electric, Ibaragi, Japan).

IL-2 was detected using a CTLL-2 cell line that proliferates in response to IL-2. CTLL-2 cells (5×10^3 cells/well) were cultured with samples or human rIL-2 ($1{\cdot}2 \times 10^7$ U/mg; kindly provided by Takeda Chemical Industries Ltd, Osaka, Japan) for 24 hr. During the last 4 hr of culture, 37 kBq of [^{3}H]thymidine was added. The cultures were harvested and the incorporated radioactivity was counted in a liquid scintillation counter.

Proliferation assay

To analyse the proliferative response, the CD8-depleted spleen cell suspension was incubated with Con A or HKLM for 48 hr, pulsed with 37 kBq [^{3}H]thymidine for a final 8 hr, and harvested. Incorporation of [^{3}H]thymidine was counted with a scintillation counter.

Statistics

Statistical analysis was performed using Student's *t*-test. A *P*-value of $< 0{\cdot}05$ was considered to be significant.

RESULTS

Cytokine production of $CD4^+$ T cells from GAM IgD-treated mice after mitogen stimulation

To know the effects of GAM IgD treatment on cytokine production by $CD4^+$ T cells, we first analysed cytokine production of the T cells from the GAM IgD-treated mice after mitogen stimulation. The CD8-depleted spleen cells from the GAM IgD-treated mice produced a higher level of IL-4 with a simultaneous reduction in the production of IFN-γ and IL-2 after stimulation with Con A compared to that from the PBS-treated control mice (Fig. 1). Although the titres of the cytokines were small, independent analyses showed nearly the same cytokine titre. In contrast, the proliferative response of the CD8-depleted spleen cells to Con A stimulation was not affected by the GAM IgD treatment (Fig. 2). These results suggest that the $CD4^+$ T-cell response shifts polyclonally to a

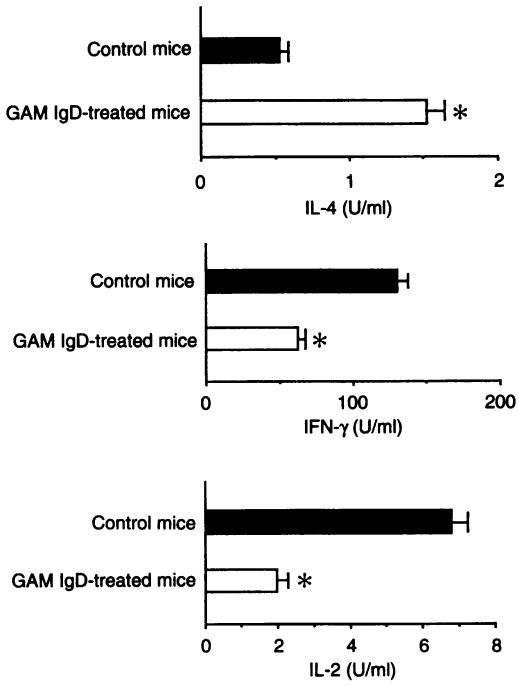

Figure 1. Analysis on IL-4, IFN-γ and IL-2 production by the T cells of the GAM IgD-treated or the control mice after Con A stimulation. The CD8-depleted spleen cells from the control or the GAM IgD-treated BALB/c mice were stimulated with Con A *in vitro* for 48 hr. The culture supernatants were then collected and cytokines were assayed. Data are expressed as mean ± SD of three measurements. $*P < 0{\cdot}05$ compared to the control.

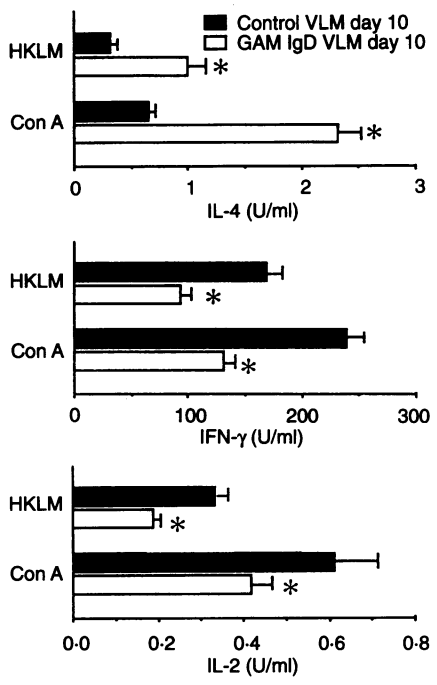

Figure 3. Analysis of IL-4, IFN-γ and IL-2 production by the T cells from the GAM IgD-treated mice infected with a low dose of *L. monocytogenes*. The control or the GAM IgD-treated mice were inoculated i.p. with 1/20 LD_{50} of VLM. Ten days after VLM inoculation, the CD8-depleted spleen cells were prepared and stimulated with HKLM or Con A *in vitro* for 48 hr. Cytokines in the supernatants were assayed. Data are expressed as mean ± SD of three measurements. $*P < 0{\cdot}05$ compared to control.

Th2 type after GAM IgD treatment. However, it is still possible that pre-stimulated goat Ig-specific Th2 cells responded predominantly to Con A and suppressed IFN-γ and IL-2 production.

Modulation of helper T-cell response against *L. monocytogenes* by GAM IgD treatment

We next analysed the T-cell response against listerial antigen after a low dose of *L. monocytogenes* (1/20 LD_{50}) infection in the mice treated with GAM IgD and control mice. All the GAM IgD-treated mice and the control mice survived (data not shown). Ten days after the low dose infection, the CD8-depleted spleen cells were stimulated with HKLM or Con A for 48 hr, and the supernatants were assayed. The CD8-depleted spleen cells from the control *L. monocytogenes*-infected mice produced a large amount of IFN-γ and IL-2 and a small amount of IL-4 after HKLM stimulation, suggesting *L. monocytogenes* infection preferentially induced a listerial antigen-specific Th1-type response (Fig. 3). In contrast, the CD8-depleted spleen cells from the *L. monocytogenes*-infected GAM IgD-treated mice produced lower levels of IFN-γ and IL-2, and higher levels of IL-4 than those from the control mice when stimulated with HKLM. Cytokine profiles after Con A stimulation were nearly the same as those after HKLM stimulation in both the control and the GAM IgD-treated groups (Fig. 3). We further analysed the goat serum-treated mice to rule out the possibility that the GAM IgD treatment modulated the T-cell response through a non-specific effect of the massive injection of xenogeneic protein rather than through specific binding of the GAM IgD to IgD. As shown in Fig. 4, the goat serum-treated *L. monocytogenes*-infected mice produced similar levels of IL-4 and IFN-γ as the PBS control mice (Fig. 3) after HKLM or Con A stimulation. Furthermore the GAM IgD-treated mice produced higher levels of IL-4 and lower levels of IFN-γ even when they were compared with the goat serum-treated mice (Fig. 4), although we could not detect a significant difference in IL-2 production after HKLM stimulation. The T cells from the mice treated with goat

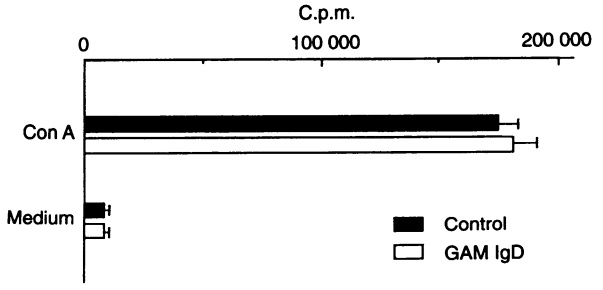

Figure 2. Proliferative response of the CD8-depleted spleen cells from the control or the GAM IgD-treated mice to Con A stimulation. The CD8-depleted spleen cells from the control or the GAM IgD-treated mice were stimulated with Con A *in vitro* for 48 hr. [^{3}H]thymidine was added to the culture 8 hr before harvest and incorporation of [^{3}H]thymidine was analysed. Data are expressed as mean ± SD of three measurements.

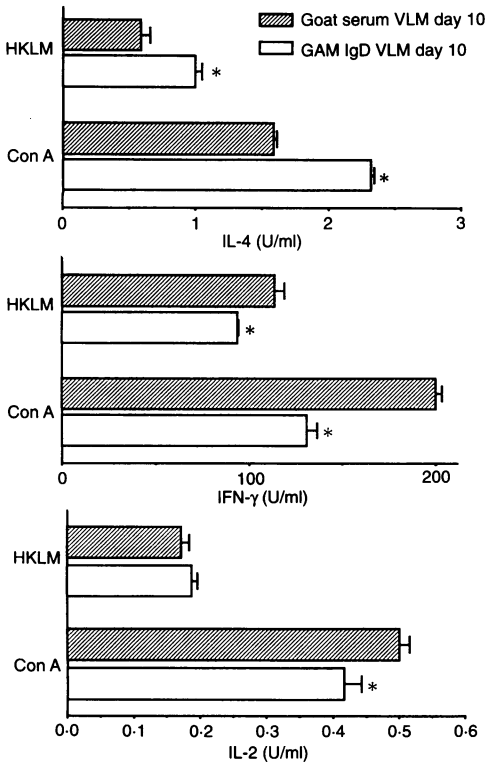

Figure 4. Analysis of IL-4, IFN-γ and IL-2 production by the T cells from the goat serum-treated or the GAM IgD-treated mice with a low dose of *L. monocytogenes* infection. The goat serum-treated or the GAM IgD-treated mice were inoculated i.p. with 1/20 LD_{50} of VLM. Ten days after VLM inoculation, the CD8-depleted spleen cells were prepared and stimulated with HKLM or Con A *in vitro* for 48 hr. Cytokines in the supernatants were assayed. Data are expressed as mean ± SD of three measurements. *$P < 0.05$ compared to control.

anti-mouse IgA antibodies also showed similar levels of IFN-γ production as the PBS-treated control mice and the goat serum-treated mice after Con A stimulation (data not shown), which again rules out a non-specific xenogeneic effect of GAM IgD. These results suggest that GAM IgD treatment induces a Th2-type response against stimuli that induce a Th1-type response in normal conditions.

Suppressive effect of GAM IgD treatment on the anti-listerial defence mechanism

We next analysed the effects of GAM IgD treatment on the anti-listerial defence mechanism. Figure 5 shows the survival rate of the GAM IgD-treated mice and the PBS-treated control mice after a high dose (1/2 LD_{50}) of *L. monocytogenes* infection. The survival rate was 66% in the GAM IgD-treated mice, while 93% of the control mice survived. To know the cause of the difference in survival between the control mice and the GAM IgD-treated mice after *Listeria* infection, we analysed the cytokine production of the CD8-depleted spleen cells after stimulation with HKLM or Con A. Figure 6 shows that IFN-γ production by the cells from the GAM IgD-treated mice was lower than the cells from the control mice when stimulated with HKLM or Con A. The CD8-depleted spleen cells of the GAM IgD-treated mice also showed lower levels of IL-2 production

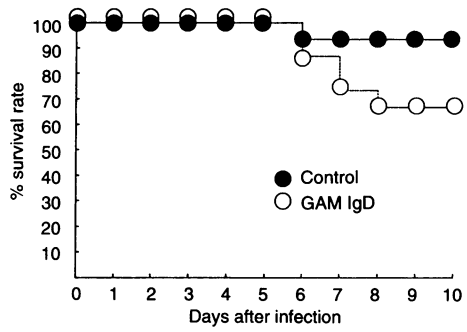

Figure 5. Survival rate of the control or the GAM IgD-treated mice after inoculation of a high dose of *L. monocytogenes*. Fifteen control and GAM IgD-treated mice were inoculated i.p. with a high dose (1/2 LD_{50}) of *L. monocytogenes* and the survival rate was observed.

than those of the control mice when they were stimulated with HKLM, while Con A stimulation resulted in higher IL-2 production by the cells of the GAM IgD-treated mice than those of the control mice. Interestingly, IL-4 was produced at low levels by the CD8-depleted spleen cells from both the GAM IgD-treated mice and the control mice after the high dose infection.

DISCUSSION

GAM IgD treatment of mice is known to induce polyclonal

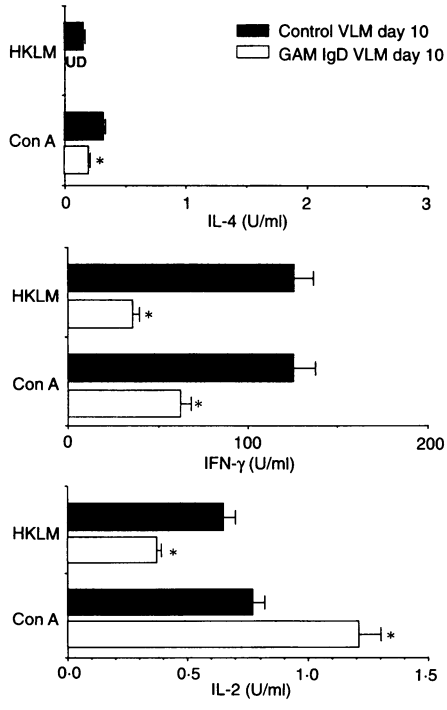

Figure 6. Analysis of IL-4, IFN-γ and IL-2 production by the T cells from the GAM IgD-treated mice infected with a high dose of *L. monocytogenes*. The control or the GAM IgD-treated mice were inoculated i.p. with 1/2 LD_{50} of VLM. Ten days after VLM inoculation, the CD8-depleted spleen cells were prepared and stimulated with HKLM or Con A *in vitro* for 48 hr. Cytokines in the supernatants were assayed. Data are expressed as mean ± SD of three measurements. *$P < 0.05$ compared to control. UD, undetectable level.

IgG1 and IgE production and a goat Ig-specific Th2 response.[14–17] In this report we analysed the effects of GAM IgD on the Th response against *L. monocytogenes*, which induces IFN-γ production in normal mice. GAM IgD treatment of mice before a low dose of *L. monocytogenes* infection enhanced IL-4 production and suppressed IFN-γ and IL-2 production by $CD4^+$ T cells in *in vitro* HKLM stimulation. These results imply that GAM IgD treatment suppresses the Th1-type response against irrelevant antigens. The mechanism of the Th response deviation induced by GAM IgD can be explained in several ways.

It is possible that goat Ig-specific Th2 cells induced by GAM IgD treatment produce IL-4 and suppress differentiation of naive T cells into Th1. Svetic *et al.*[19] showed that high levels of IL-4 gene expression were found in $CD4^+$ spleen cells from GAM IgD-treated mice. Spontaneous IL-4 production[17] and Con A-induced IL-4 production (Fig. 1) increased after the GAM IgD treatment. As IL-4 is known to promote development of naive T cells to Th2 type,[9,11] it is possible that the GAM IgD-induced IL-4 suppresses a Th1-type anti-listerial immune response in the GAM IgD-treated mice after *L. monocytogenes* infection. However, it is difficult to explain the suppression of the Th1 response by IL-4 alone because IFN-γ production was also suppressed in the GAM IgD-treated high-dose *L. monocytogenes*-infected mice, which showed low levels of IL-4 production (Fig. 6).

IL-10 is another cytokine that is important in the suppression of the Th1-type response. An increase in IL-10 gene expression was observed in the spleen $CD4^+$ T cells from the GAM IgD-treated mice.[19] IL-10 has been reported to inhibit IFN-γ production of Th1 cells after antigen stimulation by suppressing macrophage functions such as monokine production (IL-1, IL-6, IL-12 and TNF-α) and MHC class II expression.[20–23] We have also reported that IL-10 suppresses induction of listerial antigen-specific IFN-γ-producing T cells *in vitro*.[24] It is possible that IL-10 produced after GAM IgD treatment also participates in suppression of the Th1-type immune response against listerial antigen.

A recent report by Kricek *et al.*[25] demonstrated that the GAM IgD treatment not only induces expression of IL-4 gene, but also IL-12 p35 gene. Furthermore it has been shown that IFN-γ gene expression does not decrease within 1 week after GAM IgD treatment.[19,25] From these observations, Kricek *et al.*[25] suggested that the GAM IgD treatment induced a strong Th2 response but that the deviation of the immune response to Th2 was not strict because the treatment also induced IL-12 expression, which resulted in IFN-γ induction. These observations may explain why IFN-γ production by $CD4^+$ T cells was not completely suppressed in the GAM IgD-treated mice after Con A or HKLM stimulation in the present experiments.

It has been reported that B cells activated by IgD cross-linking can efficiently present antigen to naive T cells and induce a strong T-cell-dependent IgG1 response (possibly through a Th2 response) when the antigen is internalized efficiently by B cells.[18] Therefore it is possible that up-regulation of the B cell APC function induced by GAM IgD treatment causes a deviation of the T-cell response to a Th2 type. If B cells can present listerial antigen to T cells, the B cells of the GAM IgD-treated mice may induce a listerial antigen-specific Th2 response. In concert with IL-10-mediated suppression of macrophage APC function, dominance of B cells as APC may accelerate an immune deviation to Th2 after stimulation of naive T cells.

The GAM IgD-treated mice showed a reduced protection against *L. monocytogenes* infection although the reduction was made when a high dose (1/2 LD_{50}) of *L. monocytogenes* was inoculated. Analysis of the cytokine production of the T cells from the surviving mice showed that the T cells from the GAM IgD-treated mice produced reduced levels of IFN-γ compared to those from the control mice. The decrease in IFN-γ production may cause a decrease in survival rate of the GAM IgD-treated mice. In contrast, IL-4 production was at a low level in both the T cells from the GAM IgD-treated mice and the control mice after high dose-infection, which is different from the data of the low dose infection. We need further information to clarify this discrepancy.

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