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# Kinetic Analysis of Phenotypes of Cells and Their Cytokine Expression in the Development of Colitis in Nude Mice with MAIDS Colitis

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***Summary.*** **LP-BM5 murine leukemia virus is known to induce murine acquired immunodeficiency syndrome (MAIDS). We have shown that adoptively transferred spleen cells of MAIDS mice can induce inflammatory bowel disease(IBD)-like colitis termed "MAIDS colitis" in nude mice. We also reported that the Mac-1$^+$ macrophages and CD4$^+$ T cells that produce IFN-$\gamma$ or IL-10 might play a crucial role in the pathogenesis of MAIDS colitis. To reveal the sequential profiles of the immune response of IBD animal models, we analyzed the kinetics of the phenotype of cells and their cytokine expression in the colon of MAIDS colitis mice. Four-week-old C57BL/6(B6) mice were inoculated intraperitoneally with LP-BM5 murine leukemia virus (MuLV); 8 weeks after infection, lymph node cells from the mice were transferred into B6 nude mice intraperitoneally to induce MAIDS colitis. Mice were killed every week after cell transfer, and histological and immunohistochemical analyses were performed on their colons. In addition, mRNAs of IFN-$\gamma$ and IL-10 were detected by RT-PCR, and the protein-expression of these cytokines was detected by double color-staining immunofulorescence analysis. Cellular infiltration into the lamina propria of the colon appeared at week 2, and the number of colon-infiltrating cells gradually increased in MAIDS colitis. These cells were mainly composed of as many CD4$^+$ T cells as Mac-1$^+$ ones. Epithelial cell hyperplasia was observed at week 4, but remarkable epithelial cell erosions appeared at week 6. At week 3, mRNA of IL10 expression increased and reached a peak; that of IFN-$\gamma$ remarkably increased at week 6. From early stages through the late ones, IL-10$^+$ CD4$^+$ T cells (Th2) and IFN-$\gamma^+$ ones (Th1) coexisted, though the former exceeded the latter. Throughout the development of colitis, Mac-1$^+$ cells expressed both IFN-$\gamma$ and IL-10, and the number of both cells was almost equal. In conclusion, the distinct phenotype of macrophages and their kinetics as well as those of CD4$^+$ Th cells should be considered in the development of MAIDS colitis and IBD.**

**Key words**—IFN-$\gamma$, IL-10, inflammatory bowel disease, murine AIDS, LP-BM5 murine leukemia virus.

## INTRODUCTION

Inflammatory bowel diseases (IBD), which are mainly composed of ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of the gut with an unknown etiology. A number of recent studies have provided a considerable -though not uniform-- body of evidence suggesting that the cytokine profiles in inflamed mucosa and serum differ between UC and CD[1]. It has been suggested that CD represents the Th1 response, whereas UC is associated with the Th2 response. The imbalance between Th1 and Th2 CD4$^+$ T cells may be responsible for the development of IBD. It is difficult to observe the early events leading to IBD because patients with IBD appear at clinics only when their disease has progressed and become symptomatic.

To reveal the pathogenesis of IBD, many animal models have been developed, including dextran sulfate sodium (DSS)-induced colitis[2], TNBS-induced colitis[3], interleukin (IL)-2-deficient mice[4], TGF-$\beta$1-

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deficient mice[5,6], IL-10 deficient mice[7], and SCID mice that have been received CD45RB$^{high}$ T cells[8]. Analyses of these mice have strongly suggested that a strong polarization of mucosal T cell responses along either the Th1 or Th2 pathway can result in IBD, and that among the many cytokines, IFN-$\gamma$ and IL-10 play a key role in the pathogenesis of IBD.

The LP-BM5 murine leukemia virus (MuLV) is a retrovirus that is known to induce murine acquired immunodeficiency syndrome (MAIDS)[9]. We have previously reported that Sjögren's syndrome (SjS)-like systemic exocrinopathy was induced in the virus-infected mice[10]. In addition, nude mice inoculated with lymph node cells from mice with MAIDS developed IBD-like colitis, which we termed 'MAIDS colitis' as well as SjS-like exocrinopathy[11]. Histopathologically, MAIDS colitis mimics UC with an infiltration of inflammatory cells into the mucosal and submucosal layers of the colon, mucosal erosion, and hyperplasia of epithelial cells. In the colon of MAIDS colitis, Th2 type CD4$^+$T cells predominate over Th1 type CD4$^+$T cells, and IFN-$\gamma$ or IL-10 positive macrophages can also be detected, especially beneath the eroded epithelial lesions of the colon[12]. Mac-1$^+$ macrophages producing IFN-$\gamma$ or IL-10 might play a crucial role in the pathogenesis of MAIDS colitis in combination with CD4$^+$ T cells[12]. In previous reports, however, our investigation of the colitis lesions was limited to a rather later stage, and we did not analyze the lesions sequentially.

In this study we analyzed the kinetics of histopathology and cytokine production in the colon of MAIDS colitis mice using RT-PCR and the double-color immunofluorescence (IF) technique in order to reveal the sequential profiles of the immune response in the early events leading to IBD.

## MATERIALS AND METHODS

### Animals

Female C57BL/6 (B6) mice were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). The B6 nude mice were provided by Dr. Norimitsu Sato of the Animal Center, Niigata University School of Medicine. All mice were maintained at the same animal center under specific pathogen-free conditions. All animal experiments were performed according to the "Guide for Animal Experiments" of the Niigata University School of Medicine.

### Virus

LP-BM5 MuLV was prepared from the supernatant of cloned G6 cells infected with the retrovirus. A twenty-four-hour culture supernatant of G6 cells contained approximately $5\times10^4$ plaque-forming units per milliliter of ecotropic virus, as determined by XC plaque assay. Aliquotes containing the virus were stored at $-80$℃ until use. For the infection, 4-week-old B6 mice were inoculated intraperitoneally with 0.3 ml of the stock solution of LP-BM5 MuLV.

### Induction of MAIDS and MAIDS colitis

Four-week-old B6 female mice were injected intraperitoneally with 0.3 ml of LP-BM5 MuLV virus stock solution. Induction of MAIDS was confirmed when the mice developed splenomegaly and generalized lymphadenopathy. Eight weeks after the viral inoculation, mice with MAIDS were killed by cervical dislocation under ether anesthesia, and their lymph nodes were collected. The lymph nodes were pressed and passed through a steel mesh, and the cell suspension was transferred intravenously to 10- to 13-week-old female B6 nude mice at a dose of $5\times10^7$ lymph node cells per head. Symptoms of colitis such as diarrhea and anal bleeding were observed 4 weeks after cell transfer, and all the mice died within 6 weeks after cell transfer. Five to 6 weeks after the cell transfer, the mice were killed and their colons removed for further analysis. Age- and sex-matched B6 nude mice that had received $5\times10^7$ lymph node cells from B6 mice and untreated B6 nude mice were used as controls. Four mice were analyzed for each group, and all the experiments were repeated three or four times.

### Monoclonal antibodies

For IF studies, the following monoclonal antibodies were used: anti-CD4 (clone GK1.5, IgG2b), anti-CD8 (clone 53-6.7, IgG2a), anti-B220 (clone RA3-6B2, IgG2a), anti-Mac-1 (clone M-70.15, IgG2b), anti-mouse INF-$\gamma$ (clone XMG1.2), and anti-mouse IL-10 (clone JES5-16E3).

### Detection of LP-BM5 MuLV by PCR

The PCR method used for detection of the virus has been reported previously[15]. In brief, the oligonucleotides used as PCR primers were 5'-CCTCTT-CCTTTATCGACACT-3' and 5'-ATTAGGGGGGG-AATAGCTCG-3', which are present in the p15 and p12 regions of the gag gene, respectively. The emplate DNAs were extracted from the frozen blocks of colon, spleen, and lymph nodes and subjected to 30 cycles of amplification. The PCR products (237 bp)

were analyzed by Southern blot hybridization with the internal probe 5'-TGTCAAAGGGACCAGTT-AAG-3'.

### Semiquantitative RT-PCR to detect cytokine mRNAs

Total RNA was extracted from colon specimens with Trizol (Gibco BRL) according to the standard protocol. The final product was air-dried, dissolved in DEPC-treated water, and stored at −80℃. Sample RNA levels were quantitated by reading the absorbance at 260 nm. First strand complementary DNA (cDNA) was synthesized using a SuperScript Preamplification System (Gibco BRL). Five micrograms of RNA was reverse-transcribed at 42℃ for 50 min in 20 μl buffer containing 20 mM Tris-HCl, 2.5 mM MgCl2, 1 mM each dATP, dCTP, dGTP, and dTTP, 0.5 μg oligo (dT), and 50 U SuperScript II. Reactions were stopped by heat inactivation at 90℃ for 5 min and chilled on ice. Subsequently 3 ul of the cDNA mixture was amplified by PCR in 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.4 μM each 5' and 3' primers, and 2.5 U AmpliTaq Gold. The following mouse sequences were used as primers: 1) IFN-γ: 5' primer, 5'-TAC TGC CAC GGC ACA GTC ATT GAA-3'; 3' primer, 5'-TAC TGC CAC GGC ACA GTC ATT GAA-3'; 2) IL-10: 5' primer, 5'-CCA GTT TTA CCT GGT AGA AGT GAT-3'; 3' primer, 5'-TGT CTA GGT CCT GGA GTC CAG CAG-3'; 3) β-actin: 5' primer, 5'-TGT GAT GGT GGG AAT GG TCA G-3'; 3' primer, 5'-TTT GAT GTC ACG CAC GAT TTC C-3'. After the initial denaturing step (95℃ for 9 min), the mixture was subjected to a round of denaturation (95℃ for 30 sec), annealing (60℃), and extension (72℃ for 1 min). Each sample was subjected to 30 cycles. The optimal cycle numbers were determined in a preliminary trial to be in the linear phase of amplification. The PCR products were separated by electrophoresis on a 1.0% agarose gel with ethidium bromide staining. The band intensities were determined by image analysis using a Macintosh computer and Densitometry (Atto, Tokyo, Japan) software program.

### Histopathological examination

Tissue samples were taken from the colon and rectum, fixed in 10% buffered formalin, and then embedded in paraffin wax blocks. Sections 4 μm thick were made in the usual way and stained with hematoxylin and eosin. The stained sections were then examined by light microscopy.

To assess the degree of colitis, the number of inflammatory cells in a high power field (×400) were counted under a microscope. Cell numbers at three different points in the lamina propria of the colon of each mouse were counted and the data from three mice of each group were compared statistically.

### IF staining procedure

Frozen sections of colon tissue were prepared in a cryostat and stained with several fluorescent dye-conjugated anti-mouse antibodies as described above. The sections were observed by fluorescence microscopy.

### Double IF staining procedure

For the simultaneous demonstration of cell surface antigens and cytokines, aceton-fixed frozen sections were incubated sequentially with biotinylated anti-cell surface antigen antibody and then with Alexa-594 (Molecular Probe Inc.)-conjugated avidin as the first step. As the second step, the sections were incubated with fluorescein (FITC)-conjugated anti-cytokine (manuscript describing details of the methods in preparation). The sections were observed by fluorescence microscopy. Controls for the double staining were prepared by omitting the primary antibodies in the first or second step. To assess the distinct cytokine expression, the numbers of $CD4^+$ and $Mac\text{-}1^+$ cells that were double-positive for IFN-γ or IL-10 were counted under a microscope in a high power field (×400). Cell numbers at different three points in the lamina propria of the colon of each mouse were counted and the data from three mice of each group were compared statistically.

### Statistical analysis

Data are expressed as means + SE. Statistical analyses were performed using the unpaired Student's *t* test or the nonparametric Mann-Whitney test. Differences were considered significant at $P<0.05$.

## RESULTS

### Kinetic analysis of histopathology of MAIDS colitis

As shown in Fig. 1, mononuclear cellular infiltration into the lamina propria and submucosal layer of the colon was observed at week 2 after the induction of colitis. The number of these colon-infiltrating cells increased gradually (Fig. 1). Epithelial cell hyperplasia of the colonic crypts was also observed at

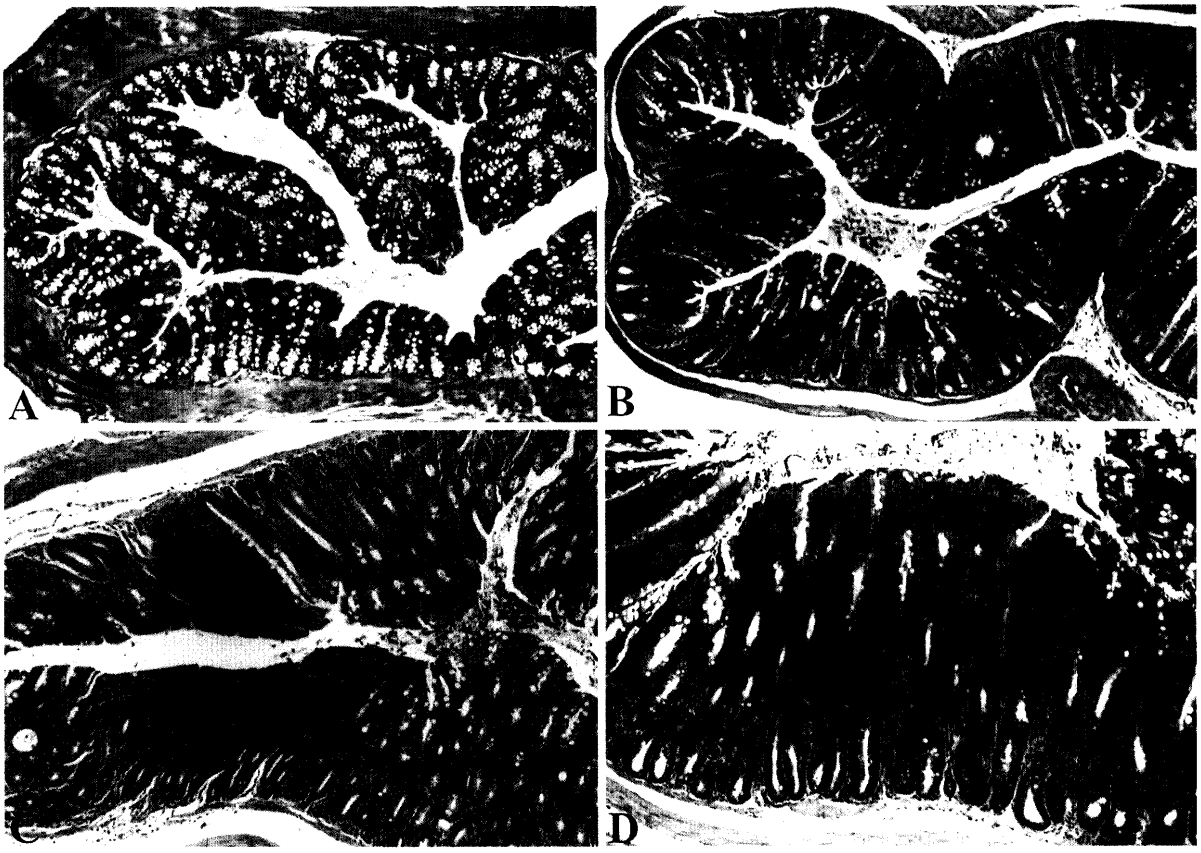


**Fig. 1.** Histopathology of the development of MAIDS colitis. (**A**) MAIDS colitis at day 0, (**B**) 2 weeks after cell transfer, (**C**) 5 weeks after cell transfer, (**D**) 6 weeks after cell transfer. (Hematoxyline-eosin; original magnification ×20)

week 4 (Fig.1). Erosions of colonic epithelial cells and crypt abscess were observed at week 6 after cell transfer. No pathological lesions were observed in the colon of mice at day 0. We next evaluated statistically the number of colon-infiltrating cells in MAIDS colitis. The number of cells infiltrating the lamina propria of the colon increased sequentially after cell transfer (Fig. 2).

### Kinetic analysis of detection of mRNAs of IFN-$\gamma$ and IL-10 in MAIDS colitis

To reveal the sequential changes of the immune response in the colon of MAIDS colitis mice, we analyzed the expression of mRNA of IFN-$\gamma$ and IL-10 by the RT-PCR method. We chose IFN-$\gamma$ representative for the Th1 response and IL-10 representative for the Th2 response. The expression level of mRNA of IFN-$\gamma$ increased by 10 times over that of day 0 by 3 weeks after cell transfer, and its level significantly rose to over 60 times higher by 5 weeks

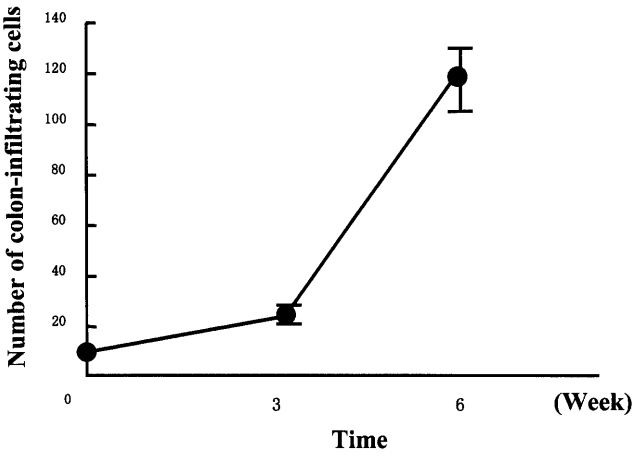


**Fig. 2.** The kinetics of the numbers of colon-infiltrating cells in MAIDS colitis. The numbers of colon-infiltrating cells were counted under a microscope at high power magnification for three different areas per mouse and the data of each group were measured with three mice from each group and compared with each other.

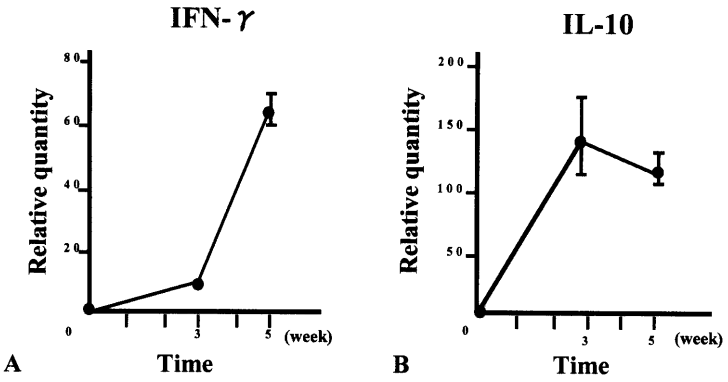


**Fig. 3.** The kinetics of mRNA expression in the colon of MAIDS colitis. Each amount was normalized to the level of GAPDH and the final relative values were expressed relative to the calibrators; normalized amount on day 0. Representative findings from three independent experiments.

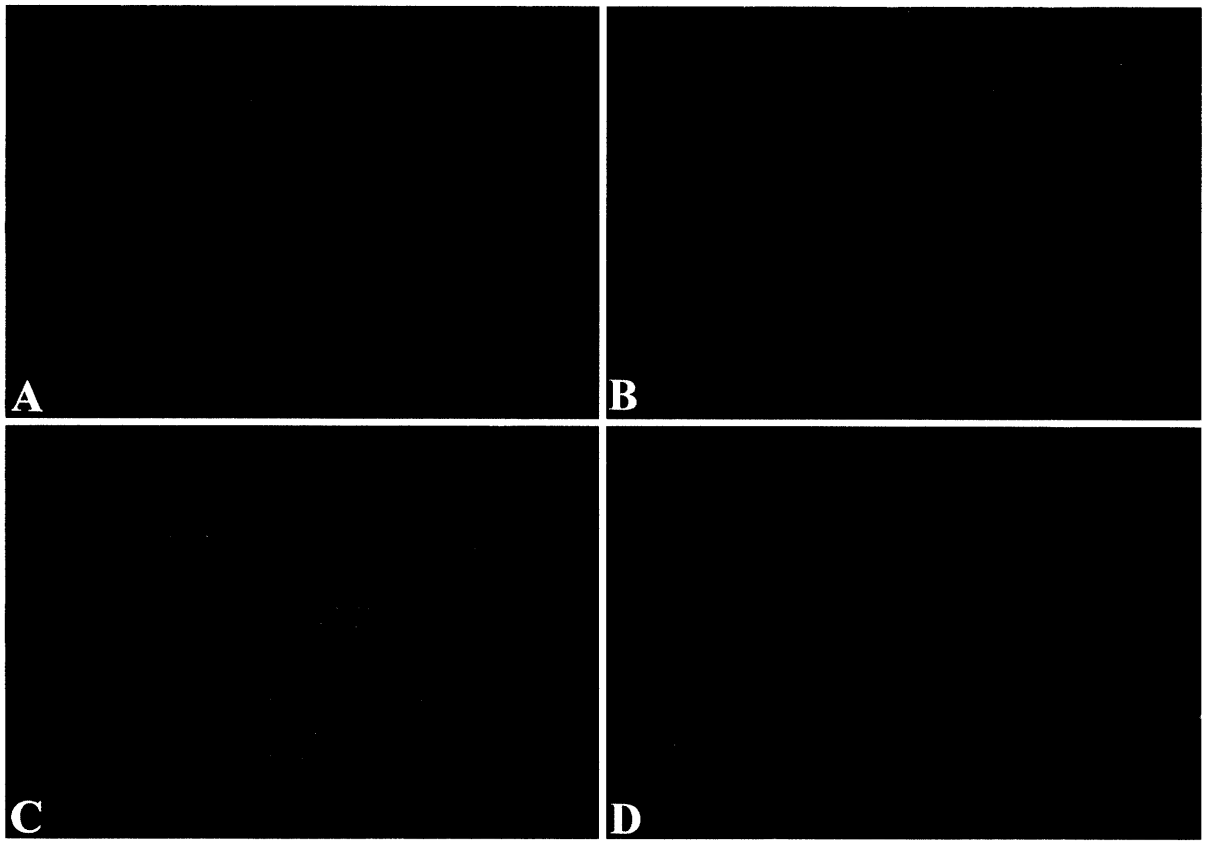


**Fig. 4.** $CD4^+$ T and $Mac\text{-}1^+$ cells and their expression of IFN-γ or IL-10 in the colon of MAIDS colitis mice at 6 weeks after cell transfer. $CD4^+$ T cells were stained with biotinylated anti-CD4 mAb and Alexa 594-conjugated avidin (**A** and **B**); $Mac\text{-}1^+$ cells were stained with biotinylated anti-Mac-1 mAb and Alexa-594-conjutated avidin (**C** and **D**). IFN-γ was detected by FITC-conjugated anti-IFN-γ mAb (**A** and **C**), and IL-10 was detected by FITC-conjugated anti-IL-10 mAb (**B** and **D**).

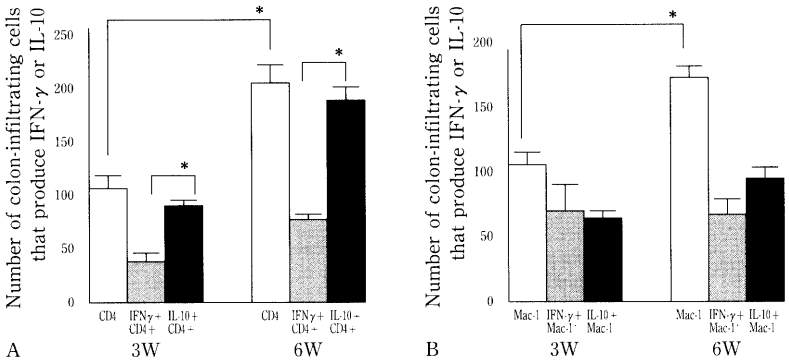


**Fig. 5.** Kinetics of CD4$^+$ T and Mac-1$^+$ cells that produce IFN-$\gamma$ or IL-10 in the colon of MAIDS colitis. *: $p<0.01$(Student's $t$ test)

(Fig. 3). The expression of mRNA of IL-10 significantly increased and reached a peak by week 3, and kept its level through week 5 after the induction of colitis (Fig. 3).

### Kinetic immunofluorescent analysis of MAIDS colitis

At week 3 and week 6 after the induction of colitis, IF studies demonstrated that CD4$^+$ T and Mac-1$^+$ cells infiltrated diffusely within the lamina propria of the colon of MAIDS colitis mice. The number of these cells increased from week 3 through week 6 (Data not shown). CD8$^+$ T cells were located mainly within the epithelial cell layer of the colon and a few B220$^+$ cells were observed in some parts of the mucosa of the colon, forming small clusters in the lower part of the lamina propria. These cell surface marker positive cells were rarely observed at day 0.

IFN-$\gamma$-positive cells and IL-10-positive cells were scattered within the lamina propria of the MAIDS colitis. The number of these cells increased from week 3 through week 6. The number of cells double positive for IFN-$\gamma$ was dominant in Mac-1$^+$ cells compared with CD4$^+$ T cells. Neither of these cytokines was expressed at day 0.

To determine the phenotypes of the cells producing these cytokines, we performed double-color IF using both anti-cytokine Abs and anti-cell surface marker Abs. As shown in Fig. 4, CD4$^+$ T cells expressed IFN-$\gamma$ or IL-10 in the colon of MAIDS colitis. The number of CD4$^+$ T cells double-positive for IL-10 exceeded that for IFN-$\gamma$ (Fig. 4A and B). Mac-1$^+$ cells also expressed IFN-$\gamma$ or IL-10 in the colon. The number of Mac-1$^+$ cells double-positive for IL-10 and that for IFN-$\gamma$ was almost equal (Fig. 4C and D). Neither IFN-$\gamma$ nor IL-10 was detected on CD8$^+$ or B220$^+$ cells (data not shown).

Next we compared the number of CD4$^+$ T and Mac-1$^+$ cells that produced IFN-$\gamma$ or IL-10 in the colon of MAIDS colitis between that at week 3 and that at week 6 after cell transfer. Through the course of colitis, the number of Th2 type cells predominated over that of the Th1 type (Fig. 5A), and there were as many IFN-$\gamma^+$ Mac-1$^+$ cells as IL-10 $^+$ Mac-1$^+$ ones (Fig. 5B).

## DISCUSSION

From the data presented here, we can determine three phases in the development of MAIDS colitis as follows: phase I, non inflammation phase; phase II, cellular infiltration into the colon and colonic epithelial hyperplasia phase; phase III, tissue destruction phase. The mucosal immune system can be divided into inductive sites and effector regions. The former is known as gut-associated lymphoreticular tissues (GALT) including the Peyer's patch and colonic patches. The latter is the lamina propria, diffuse collections of lymphocytes and plasma cells. In the TNBS-induced colitis model, Dohi et al. recently reported that the Th2-type responses are associated with colonic patch enlargement and inflammation of the mucosal layer[13]. It is supposed that the hapten given by a rectal enema induced the specific immune response in the inductive site of GALT, and thereafter recruited immunized cells into effecter regions of lamina propria of the colon to induce colitis. On the other hand, in our MAIDS colitis, no remarkable enlargement of the colonic patch was observed. We have shown that the adoptive transfer of Mac-1$^+$ fraction of MAIDS lymph node cells can induce colitis but not SjS-like exocrinopathy in recipient

nude mice[14]. In contrast, the transfer of the CD4$^+$ T cell fraction of MAIDS lymph node cells can induce SjS-like exocrinopathy but not MAIDS colitis[14]. In the development of MAIDS colitis, we assume that adoptively transferred Mac-1$^+$ cells of MAIDS migrate into the colon after the interval of "phase I" and recruit other immune cells such as T and B cells into effecter regions to induce overt colitis during "phase II". The initial event of the inflammation of MAIDS colitis is not induced by a rectal stimulus as TNBS-induced colitis. This might explain the lack of any marked enlargement of the colonic patch of MAIDS colitis. The mechanism by which these Mac-1$^+$ cells of MAIDS mice migrate into the colon of recipient nude mice remains unknown, though some specific chemokines and their receptors might play a role. We should therefore clarify characterizations such as the cytokine and chemokine expression of these cells in a future study.

IBD animal models that have been established so far are divided into two categories such as Th1 and Th2 models from their skewed cytokine production pattern of mucosal CD4$^+$ T helper cells in the colon[1]. In our MAIDS colitis, although Th2 type CD4$^+$ T cells predominated over Th1 types throughout the course of the disease, both Th1 and Th2 type T cells coexisted in the colon. Such a coexistence of Th1 and Th2 responses during colitis is also reported in TNBS-induced colitis by Dohi et al.[13], in DSS-induced colitis[15], and in IL-2 deficient mice[16]. Interestingly, Dohi et al. suspected that, in TNBS-induce colitis, the Th2 immune response is important for the development of UC-like lesions such as epithelial erosions and epithelial hyperplasia and Th1 for CD-like lesions[13]. Thus in MAIDS colitis, the predominating Th2 immune response might explain the cause of UC-like lesions.

Apart from the inductive role of the MAIDS colitis, Mac-1$^+$ cells of MAIDS mice seem to the primary effectors of the tissue destruction in MAIDS colitis. Our present RT-PCR study revealed that the expression of IFN-$\gamma$ remarkably increased at later stages and tissue destruction appeared only at a late phase. We assume that these Mac-1$^+$ cells expressing IFN-$\gamma$ might be responsible for tissue destruction during "phase III" of MAIDS colitis. It is suggested that TNF-$\alpha$ production by both T cells and non-T cells is important in the development of intestinal inflammation[1]. Macrophages are considered to produce tumor-necrosis factor-$\alpha$ (TNF-$\alpha$) in TNBS-colitis[17]. We did not analyze TNF-$\alpha$ expression in MAIDS colitis, and therefore the expression of other cytokines including TNF-$\alpha$ should be clarified in a future study.

Mac-1$^+$ cells comprised macrophages, granulocytes, and NK cells. As reported previously[12], macrophages recognized by anti- Mac-3 mAbs were the major population in the colon of MAIDS colitis. In contrast, Gr-1$^+$ neutrophils and NK1.1$^+$ NK cells were less numerous. To induce MAIDS colitis, we have used nude mice as recipients. It is known that NK cells and granulocytes increase in nude mice, which lack conventional T cells. We should better analyze these NK cells and granulocytes as well as T cells and macrophages for analyzing the developmental mechanism of MAIDS colitis.

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