Chapter III

Progression of autoimmune-mediated hepatic lesions in a murine graft-versus-host reaction by neutralizing IL-10.

1. Introduction

We have demonstrated that parental T cells which were injected into major histocompatibility complex (MHC) class II-disparate F1 hybrid mice induced graft-versus-host reaction (GVHR). This model shows lymphocyte infiltration in the portal area, pericholangitis around the interlobular bile ducts [1-5]. Three important mechanisms have been reported by analyzing MHC class II-disparate GVHR. 1) Donor $CD4^+$ T cells recognize MHC class II disparity [3] and retain their alloreactivity towards MHC class II antigens for a long time [6]. 2) Recipient-derived T cells invade at the site of the hepatic lesions, suggesting that the tolerance of self-reactive T cells is abrogated by GVHR [7]. 3) Recipient mice produce autoantibodies of a recipient origin [8]. From these findings, it is suggested that the autoimmunity play a part in the formation of MHC class II-different GVHR. Namely, GVHR with MHC class II disparity induced by $CD4^+$ T cells could show some pathological situation of autoimmune-related liver diseases.

We have analyzed hepatic lesions using murine GVHR in B6 T cells-injected MHC class II disparate (bm12 x B6) F1 mice [4,5,9]. To elucidate the relationship between the formation of hepatic lesions and the cytokine profile, we have examined the expression level of cytokine mRNA of liver-infiltrating $CD4^+$ T cells. As mentioned above, these cells are suggested to originate from the recipient. In our previous study, early production of interferon (IFN)-γ and delayed production of interleukin (IL)-10 was observed, which might play an important role in the formation of GVHR hepatic lesions [9]. However, this GVHR did not progress into fibrosis or cirrhosis, that is, it was self- limited [1]. To clarify the progression mechanism of the autoimmune liver diseases, we administered concanavalin A (Con A) into autoimmune GVHR, because it has been demonstrated recently that Con A induced T cell-mediated hepatic lesions [10]. In our previous study, Con A deteriorated undergoing autoimmune-mediated hepatic lesions induced by GVHR; however, it did not cause fibrosis or cirrhosis. IFN-γ was suggested to play an important role for the deterioration in the GVHR-added Con A model. On the other hand, the expression level of IL-10 in GVHR-added Con A mice was similar to that in GVHR mice [11]. Although IL-10 might be produced in liver by a reactive mechanism, its role in the GVHR hepatic lesions is not known exactly.

IL-10 is known as a multipotent cytokine and possesses anti-inflammatory and immunosuppressive activity both *in vitro* and *in vivo* [12]. IL-10 inhibits the ability of antigen-presenting cells to stimulate cytokine production by T helper (Th)1 cells [13]. The ability of endogenous and exogenous IL-10 to prevent the autoimmune diseases [14,15] and to decrease hepatotoxicity [16-22] has been shown in several animal models. Interestingly, IL-10 has been reported to ameliorate the liver inflammation of human HCV infection [23].

Therefore, we hypothesized that the production of IL-10 by liver-infiltrating $CD4^+$ T cells might suppress the progression of the hepatic lesions induced by GVHR. However, some studies have shown that IL-10 was unable to suppress an immune response *in vivo* [24-26]. Similarly, IL-10 was not able to prevent [27], and in some situations even exacerbated [28], GVHR. The discrepancy between these *in vivo* effects of IL-10 on animal models may be depend on the dosage [29] and timing of IL-10 administration, and/or due to the fact that IL-10 also stimulates activated $CD8^+$ T cells [30]. The aim of the present study is to clarify whether blocking of IL-10 deteriorate autoimmune-mediated hepatic lesions induced by GVHR, and to elucidate the change of the Th1 / Th2 cytokines in the liver under the neutralization of IL-10. We administered anti-L-10 monoclonal antibodies (mAbs) into GVHR mice to determine the participation of IL-10 in the progression of GVHR hepatic lesions.

2. Materials and Methods

Animals

C57BL/6(B6)[*H-2K*^{b}, *I-A*^{b}, *H-2D*^{b}] mice were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). B6.*C-H-2*^{bm12}(bm12)[*H-2K*^{b}, *I-A*^{bm12}, *H-2D*^{b}] mice with mutation at *I-A* region, originated from the Jackson Laboratory (Bar Harbor, ME, USA), were used to obtain (bm12 x B6) F1 hybrid mice. All animals received human care in accordance with the guidelines of the University of Tsukuba for the care of laboratory animals.

Treatment of mice

The female F1 mice between 12 and 14 weeks of age received donor B6 T cells for the induction of GVHR as previously reported [31]. Briefly, spleen cell suspension was treated with 0.83% ammonium chloride-Tris buffer to remove red blood cells, followed by passage through a nylon wool column for purification of T cells. Sex-matched 1-2 x 10^7 B6 T cells were injected into recipients via the tail vein. Either monoclonal anti-mouse IL-10 antibodies (JES052A5, Genzyme / Techne, Cambridge, MA, USA) or control monoclonal antibodies (mAbs, Rat IgG1 Isotype Control, 43414.11 Genzyme / Techne, Cambridge, MA, USA) were dissolved in pathogen-free saline, and injected intraperitoneally at a dose of 500μg/body 4h before the cell transfer. Various doses (250, 500, 1000μg/body) of antibodies and the timing of administration were tried, and the condition which showed the maximum histological change caused by minimum dose was chosen. We divided the mice into four groups as follows: Group 1, normal control mice (NML); Group 2, GVHR mice; Group 3, GVHR mice with control mAbs; Group 4, GVHR mice with anti-IL-10 mAbs. Recipient mice were sacrificed at 14 days after the cell transfer or at the same age as untreated mice. We performed liver perfusion to eliminate blood contained in liver by the injection of 2 ml of phosphate-buffered saline (PBS) via the portal vein. After the perfusion, the liver and spleen were immediately removed.

Assessment of the Occurrence of GVHR

The spleen was weighed separately to calculate the spleen index by the following formula:

Spleen index = spleen weight (mg)/body weight (g).
The occurrence of GVHR in the cell-transferred group was confirmed by the elevated spleen indices as compared with that of non-cell-transferred mice [4,5].

Assay of Serum Transaminase

At the time of sacrifice, about 1ml of blood was drained through the heart, and serum aspartate aminotransferase (AST), and serum alanine aminotransferase (ALT) were measured by the JSCC (Japan Society of Clinical Chemistry) consensus method.

Histological analysis

After the mice were killed, small blocks of the left lobe of the liver were excised from each mouse and fixed in 10% buffered formalin. Paraffin section were made and stained with hematoxylin-eosin and Masson's Trichrome by using standard methods for histological examination.

Image analysis of portal cellular infiltration area

We evaluated the area of infiltrated cells of the portal area in the liver. Five portal areas which had similar-sized bile ducts were randomly chosen from each specimen, and the area (μm^2) of infiltrated cells were measured by using NIH Image (National Institutes of Health, MD, USA). The area of vessels and bile ducts were excluded from the infiltrated area for image analysis.

Preparation of lymphocytes from the liver

Hepatic mononuclear cells were prepared essentially as previously described [32,33]. Briefly, the liver was passed through a 200-gauge stainless steel mesh and suspended in a minimal essential medium supplemented with 5% fetal calf serum. Liver-infiltrating lymphocytes were prepared using 35% Percoll (Pharmacica Biotech, Tokyo, Japan) solution and a red blood cells lysis solution.

RNA expression levels of IFN-γ and IL-4 by real-time polymerase chain reaction

Total RNA was isolated from liver-infiltrating lymphocytes from each cell-transferred mouse using Isogen (Nippon Gene, Osaka, Japan) following the

manufacturer's instructions. Four to five-pooled untreated mice were used for one-time RNA extraction. RNase-free DNase I (Nippon Gene, Osaka, Japan) was utilized to avoid contamination of genomic DNA during the total RNA extraction. RNA (2 μg per reaction) was reverse transcribed to cDNA using a SuperScript First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA, USA). A 2.5μl cDNA template was used for each PCR. Real–time PCR [34,35] was conducted using the ABI Prism 7700 Sequence Detection System (AB Applied Biosystems, Foster City, CA, USA) following the Taqman Universal PCR Master Mix Protocol (AB Applied Biosystems, Foster City, CA, USA). Primers and probes used for the analysis of cytokine expression are as in the study by Overbergh *et al.* (GAPDH, IFN-γ) [36] and Castels *et al.* (IL-4) [37]. IFN-γ and IL-4 genes were normalized to a housekeeping gene (glyceraldehydes-3-phosphate dehydrogenase) before fold changes were calculated to account for variations between different samples. The threshold cycle and standard curve method were used for calculating the relative amount of the target RNA as described for AB. Each PCR amplification was performed under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, followed by a total of 45 two-temperature cycles (15 seconds at 95°C and 1 minute at 60°C). Six cell-transferred mice from each of groups 2 and 4, 3 mice from group 3 and 6 samples consisting of 4-5 pooled untreated mice for each sample were used for the relative quantification.

Statistical analysis

Results are expressed as mean ± SD in each group. A Kruskal-Wallis test was applied to the spleen index and quantitative analysis of cytokine mRNA. The serum transaminase data were analyzed using unpaired Student's *t* test. The portal infiltration area was assessed by a repeated measure ANOVA. Multiple comparison test was performed by Scheffe. A level of $P < 0.05$ was judged to be significant.

3. Results

Evaluation of changes in the spleen index

We examined the induction of GVHR using the spleen index that was calculated by the formula previously indicated [4,5]. As shown in Table 3-1, the average of the spleen index was 2.9 in group 1(NML), 15.9 in group 2 (GVHR), 15.4 in group 3 (GVHR+Control mAbs), and 24.5 in group 4 (GVHR+Anti-IL-10). In the mice from groups 2 and 3 the average spleen indies were elevated, with no significant difference between the groups. However, in group 4 mice the spleen index was significantly increased compared with that of groups 2 and 3($P<0.01$). The body weight of anti-IL-10 mAbs treated mice did not differ markedly from those of the other groups (data not shown), indicating that the major effect of anti-IL-10 mAbs was to promote the expansion of cellular elements within the spleen of GVHR mice.

Biochemical analysis of liver injury

In GVHR mice the increase of serum transaminase was not recognized as previously reported [11]. In GVHR+Anti-IL-10 mice (n = 6) AST was 106 ± 35 and ALT was 40 ± 17, compared with 101 ± 34 and 28 ± 4 in normal mice (n = 6), respectively (mean ± SD). There was no significant difference between these mice.

Morphological changes in GVHR hepatic lesions by anti-IL-10 mAbs

On histological examination, a moderate degree of mononuclear cell infiltration was observed in the portal area and around the central veins in the hepatic lobule in group 2 (GVHR) at day 14 as previously described [1] (Fig. 3-1A). There was no difference between groups 2 and 3 (GVHR+Control mAbs, Fig. 3-1B, 3-1D) with regard to the extent of the portal cellular infiltration. On the other hand, the portal area of the mice treated with anti-IL-10 mAbs (group 4) was expanded with the mononuclear cell infiltration (Fig. 3-1C, 3-1E) compared to groups 2 and 3. However, there was no change in the cellular phenotype of portal infiltration consisting of lymphocytes, plasma cells, and histiocytes. Focal intraepithelial lymphocyte infiltration was observed in all of the cell-transferred mice. Moreover, in group 4 (GVHR+Anti-IL-10) the loss of continuity of the bile duct wall, one of the features of non-suppurative

destructive cholangitis (NSDC), was observed (Fig. 3-1F).

Evaluation of the extent of portal cellular infiltration area using image analysis

Next, we measured the area (μm^2) of infiltrated cells using NIH Image. For image analysis five portal areas of each specimen with similar-sized bile ducts were randomly chosen. As shown in Fig. 3-2, the measured cellular infiltrated portal area was 73270 ± 39525 μm^2 in group 2 (GVHR), 62550 ± 45959 μm^2 in group 3 (GVHR+Control mAbs), and 218314 ± 103897 μm^2 (mean ± S.D.) in group 4 (GVHR+Anti-IL-10). There was no significant difference between groups 2 and 3, whereas group 4 mice showed a 3-fold increase in the mean of the cellular infiltrated portal area compared with other groups ($P<0.0001$).

Cytokine profile of liver-infiltrating lymphocytes

To elucidate the change of the Th1 / Th2 cytokines in the liver under neutralizing IL-10, we performed real-time PCR analysis. The relative amount of the target RNA from each group was measured followed by normalization using GAPDH as an internal standard. As shown in Fig. 3-3A, the mean IFN-γ expression of group 2 (GVHR mice) was significantly higher than that of group 1 (NML), whereas concerning the steady state of IL-4 mRNA level (Fig. 3-3B), there was no significant difference between groups 1 and 2. These results suggest that IFN-γ plays an important role in the formation of GVHR hepatic lesions. IFN-γ and IL-4 mRNA levels of group 3 (GVHR + Control mAbs) did not show significant difference compared with those of group 2. The expressions of both the steady state of IFN-γ and IL-4 mRNA levels were significantly increased by neutralizing IL-10 (group 4) compared with group 3 mice.

4. Discussion

We have demonstrated that parental T cells that were injected into MHC class II-disparate F1 hybrid mice induced autoimmune GVHR. In this GVHR it is suggested that donor $CD4^+$ T cells recognize MHC class II disparity [3] and retain their alloreactivity towards MHC class II antigens for a long time [6]. These cells then cause the infiltration of recipient inflammatory cells in the liver, suggesting that the tolerance of self-reactive T cells is abrogated by GVHR [7]. Moreover recipient mice produce autoantibodies of a recipient origin [8]. From these findings, it is suggested that the hepatic inflammation in MHC class II-different GVHR may be enhanced by an autoimmune-mediated mechanism. Analyzing the cytokines, especially produced from $CD4^+$ T cells, is important in order to elucidate the pathogenic mechanism of this GVHR. We have reported that early production of IFN-γ and delayed production of IL-10 from liver-infiltrating $CD4^+$ T cells might play an important role in the formation of GVHR hepatic lesions [9]. Moreover, Con A deteriorated GVHR hepatic lesions and IFN-γ was suggested to play an important role for the deterioration. On the other hand, the expression level of IL-10 mRNA in GVHR-added Con A mice was similar to that in GVHR mice [11]. Although IL-10 might be produced in liver by a reactive mechanism, its role in the GVHR hepatic lesions is still unknown.

IL-10 is a Th2-derived cytokine that suppresses the ability of macrophages to stimulate the production of the IFN-γ and other cytokines by Th1 cells [38] and inhibits the macrophage-dependent development of Th1 cells [39,40]. Moreover, Groux *et al.* have reported that T regulatory cells 1 (Tr1) with immunoregulatory properties produce high level of IL-10 [41]. The ability of IL-10 to decrease hepatotoxicity in several animal models [16-22] and to ameliorate the inflammation of human HCV hepatitis [23] was shown. Therefore, we hypothesized that the production of IL-10 might inhibit the progression of the hepatic lesions induced by GVHR.

In the present study we administered anti-IL-10 mAbs into recipient mice before the induction of GVHR to determine the participation of IL-10 in the progression of GVHR hepatic lesions. It turned out that the spleen index was elevated and portal cellular infiltration was worsened in these mice, indicating that GVHR hepatic lesions deteriorated by neutralizing IL-10. Namely, these

results suggest that IL-10 might play an important role in our GVHR system by suppressing the inflammation of hepatic lesions. Neutralizing IL-10 at an early stage of GVHR deteriorated hepatic lesions, suggesting that IL-10 produced from the activated alloreactive donor $CD4^+$ T cells may be blocked by anti-IL-10 mAbs, and IL-10 from donor cells may contribute to regulating the hepatic lesions in this GVHR. Blazar BR *et al.* have reported that the deficiency of donor IL-10 accelerated the recipient lethality in another combination of acute GVHR [29]. Together, it is suggested that IL-10 produced from donor $CD4^+$ T cells may be important in influencing the cytokine profile and autoimmunity in the recipient. Although it was not determined exactly which IL-10, that from either the donor or recipient cellular source, was blocked by anti-IL-10 mAbs, according to the matter mentioned above, we speculated that IL-10 from recipient-origin liver-infiltrating lymphocytes might be suppressed as a secondary event following the neutralization of IL-10 produced by donor cells and be neutralized by anti-IL-10 mAbs directly.

Next, we performed quantitative RT-PCR of IFN-γ (Th1) and IL-4 (Th2) mRNA from liver-infiltrating lymphocytes using real-time RT-PCR. In our GVHR mice model up-regulated IFN-γ and unaltered IL-4 levels suggest that this system may be mediated by Th1 response and IL-4 may not contribute to the formation of GVHR hepatic lesions. In GVHR plus Anti-IL-10 mice, both expression levels of IFN-γ and IL-4 mRNA were significantly higher than those in GVHR mice with control mAbs. The elevation of IFN-γ is conceivable from other animal models using anti-IL10 mAb [42,43] and IL-10 knockout mice [44,45]. However, the expression level of IL-4 mRNA was also elevated by neutralizing IL-10. IL-10 has been reported not to impair the synthesis of cytokines by Th2 cells *in vitro* [38,46]. Moreover, The level of IL-4 is increased in some animal study using IL-10 knockout mice [44,45,47,48], indicating that IL-10 is not necessary for the development of Th2 cells. Therefore, the elevated expression of IL-4 mRNA cannot be explained by the natural consequence of the deficiency of IL-10. Hoffmann KF *et al.* reported that the increased production of both IFN-γ and IL-4 was observed in IL-10–deficient mice infected with *Schistosoma mansoni* [45]. Similarly, in other studies using IL-10 knockout mice, the production of IL-4 was increased by infection or allergens, whereas spontaneous production was the same as that in the wild-type mice [44,47]. Taken collectively, it is suggested that IL-4 may be

elevated by a compensatory mechanism with certain stimulation under the deficiency in IL-10. Although the functional significance and the precise mechanism of IL-4 up-regulation await evaluation, GVHR hepatic lesions was deteriorated under Th1 up-regulated conditions despite the presence of a significant Th2 response. Hoffmann *et al.* have shown that the maintenance of IL-10, rather than a classical Th2-type response, is the more critical factor contributing to host survival in *S. mansoni* infected mice [45]. Together, these findings suggest that IL-10, rather than IL-4, may be the key mediator in down-regulating GVHR hepatic lesions. Namely, it is possible that IL-10 may play a role as an imunosuppressor in this GVHR system.

As the results of the present study, it is shown that the hepatic lesions in GVHR plus anti-IL-10 mAbs mice indicated the increase of the degree of inflammation. However, these lesions do not progress into interface hepatitis and fibrosis. Although the focal intraepithelial lymphocyte infiltration and loss of continuity of the bile duct wall was shown, destructive changes of bile ducts were not observed. This suggests that other mediators contribute to these findings. The role of the effector cells and the participation of other cytokines in these GVHR hepatic lesions have to be examined in future experiments.

5. Summary

Our studies indicate an important role of IL-10 in murine GVHR due to MHC class II disparity. The portal cellular infiltration in GVHR hepatic lesions is deteriorated by the administration of antibodies against IL-10. Neutralizing IL-10 caused the elevation of not only IFN-γ, but also IL-4 mRNA. These results suggest that IL-10 may play a crucial role in down-regulating autoimmune–related hepatic lesions.