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Hepatic Interleukin-7 Expression Regulates T Cell Responses

Yukihisa Sawa,[1] Yasunobu Arima,[1] Hideki Ogura,[1] Chika Kitabayashi,[1] Jing-Jing Jiang,[1] Toru Fukushima,[1] Daisuke Kamimura,[1] Toshio Hirano,[1,2] and Masaaki Murakami[1,*]
[1]Laboratory of Developmental Immunology and the CREST Program of the Japan Science and Technology Agency, Graduate School of Frontier Biosciences, Graduate School of Medicine, and WPI Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
[2]Laboratory of Cytokine Signaling, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan
*Correspondence: murakami@molonc.med.osaka-u.ac.jp
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SUMMARY

Systemic cytokine activity in response to Toll-like receptor (TLR) signaling induces the expression of various proteins in the liver after infections. Here we show that Interleukin-7 (IL-7), the production of which was thought to occur at a constant rate in vivo, was a hepatically expressed protein that directly controled T cell responses. Depletion of IL-7 expression in the liver abrogated several TLR-mediated T cell events, including enhanced $CD4^+$ T cell and $CD8^+$ T cell survival, augmented $CD8^+$ T cell cytotoxic activity, and the development of experimental autoimmune encephalitis, a Th17 cell-mediated autoimmune disease. Thus, T cell responses are regulated by hepatocyte-derived IL-7, which is expressed in response to TLR signaling in vivo. We suggested that TLR-induced IL-7 expression in the liver, which is an acute-phase response, may be a good diagnostic and therapeutic target for efficient vaccine developments and for conditions characterized by TLR-mediated T cell dysregulation, including autoimmune diseases.

INTRODUCTION

Conserved microbial structures associated with bacterial and viral infections are locally recognized by Toll-like receptors (TLRs), which then can induce both innate and adaptive immune responses (Janeway and Medzhitov, 2002). For example, after lipopolysaccharide (LPS) binds its receptor, TLR4, at least two signaling pathways are activated; one is dependent on the adaptor protein, myeloid differentiation primary response gene 88 (Myd88), whereas the other is mediated by other adaptor, Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF), Toll-like receptor adaptor molecule 1 (Ticam1) (Yamamoto et al., 2003; Hoebe et al., 2003). Myd88, a TLR-binding protein, contributes to the LPS-induced activation of the transcriptional factor, NF-κB and mitogen-activated protein kinases, thereby inducing inflammation. On the other hand, TRIF-dependent signaling via the TLR4 receptor leads to the activation of interferon regulatory factor 3 (IRF3) and subsequent type I interferon (IFN-I) expression (Yamamoto et al., 2003).

TLR-mediated signals in a variety of inflammatory cells, including dendritic cells, macrophages, neutrophils, and nonhematopoietic cells, result in the production of proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, TNF-α, IFN-α and -β, and IFN-γ (Takeda et al., 2003). These cytokines initiate various systemic responses and "acute-phase reactions," which are characterized by altered protein expression profiles in hepatocytes, including the production of such serum proteins as serum amyloid A (SAA), C-reactive protein (CRP), plasminogen, ferritin, and complement components (Fey et al., 1994), suggesting that the liver is a sensor of inflammation.

Interleukin-7 (IL-7), which is known to be important for T cell homeostasis, is mainly produced by stromal cells (Marrack et al., 2000). It is believed that the production of IL-7 in stromal cells occurs at a constant rate in vivo and is unaffected by most extrinsic stimuli (Ma et al., 2006; Mazzucchelli and Durum, 2007). Alternatively, several reports using cell lines have demonstrated that IL-7 expression is induced in vitro by IFN-γ and inhibited by TGF-β (Ariizumi et al., 1995; Tang et al., 1997). Nevertheless, IL-7 expression in vivo, which appears to limit the size of the lymphocyte pool, is thought to be regulated by IL-7 receptor α (IL-7Rα)-mediated consumption rather than the rate of IL-7 expression (Mazzucchelli and Durum, 2007). Indeed, the expression of IL-7Rα varies during the development of T and B cells (Mazzucchelli and Durum, 2007). Moreover, IL-7Rα expression in mature T cells can be regulated by extrinsic stimuli, including antigens and cytokines (Schluns et al., 2000). We, however, showed that excessive IL-6 signaling increased the amount of IL-7 expression in vivo, which was followed by the development of autoimmune arthritis (Sawa et al., 2006). Thus, whether IL-7 expression can be influenced by external stimuli, including TLR-mediated stimulation, requires further elucidation in vivo.

Here, we show that IL-7 expression induced in the liver in response to TLR signaling regulated T cell responses in vivo. This process likely controls a range of physiologic and pathologic responses by enhancing T cell survival.

RESULTS

IL-7 Expression in Hepatocytes Is Induced by LPS Injection In Vivo

We previously demonstrated that hyperactivation of the IL-6-gp130-STAT3 signaling axis in type I collagen$^+$ nonhematopoietic cells increased IL-7 expression (Sawa et al., 2006). This

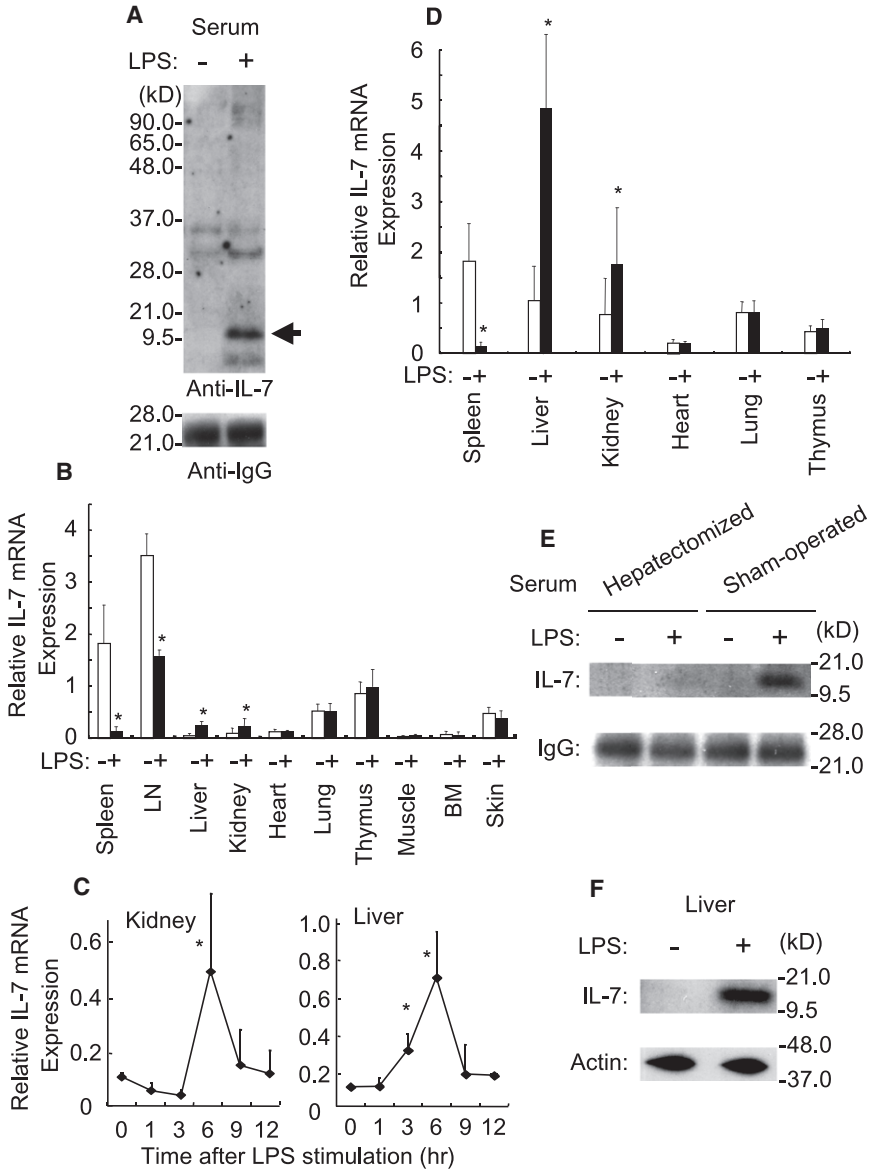

Figure 1. LPS Injection Induces IL-7 Production in the Liver

(A) IL-7 in serum was detected with immunoblotting 12 hr after LPS treatment. The arrow denotes the expected size of the IL-7 band.

(B) IL-7 mRNA expression in various organs after treatment with LPS. Organs were harvested from mice 6 hr after they were treated with or without LPS (n = 6 for spleen, n = 4 for lymph nodes [LN], n = 10 for liver, n = 14 for kidney, n = 6 for heart, n = 6 for lung, n = 6 for thymus, n = 4 for muscle, n = 4 for bone marrow [BM], and n = 4 for skin). IL-7 mRNA expression was measured by real-time PCRs. After LPS treatment, IL-7 expression in the spleen and lymph nodes significantly decreased, whereas it significantly increased in the liver and kidney ($p = 0.0026$, 0.042, 0.000011, and 0.0030, respectively).

(C) IL-7 mRNA expressions in liver and kidney tissues with or without LPS treatment were measured by real-time PCRs. The levels of IL-7 mRNA in the liver tissue 6 hr after treatment and in the kidney tissue 3 and 6 hr after treatment were significantly higher than before LPS treatment ($p = 0.021$, 0.000064, and 0.0023, respectively).

(D) The estimated amounts of IL-7 mRNA were calculated with the real-time PCR results from (C) and the average organ weights. After LPS treatment, IL-7 expression in the spleen significantly decreased, whereas it significantly increased in the liver and kidneys ($p = 0.0026$, 0.000011, and 0.003, respectively).

(E) IL-7 expression was analyzed in serum isolated from hepatectomized mice or sham-operated animals before and 12 hr after LPS treatment (n = 5 each). Representative data are shown.

(F) IL-7 in liver 12 hr after LPS treatment was detected with immunoblotting.

For (B)–(D), data represent the mean + SD; *$p < 0.05$ compared with control samples.

response was followed by enhanced $CD4^+$ T cell homeostatic proliferation, which was directly associated with the development of autoimmune arthritis in F759 mice (Sawa et al., 2006). Our results suggested that extrinsic stimuli, such as TLR signaling, can regulate IL-7 production under normal conditions in vivo. To examine this possibility, we investigated serum IL-7 amounts after LPS stimulation. We first enhanced the sensitivities of an IL-7-specific ELISA kit and immunoblotting system, in which IL-7 was detected with a commercially available antibody (Figure S1 available online; see Experimental Procedures). We found that mice injected with LPS showed increased serum amounts of IL-7 (Figure 1A; Figure S1A). The results showed that the serum concentration of IL-7 peaked 12 hr after LPS treatment (data not shown).

To identify the major source(s) of IL-7 after LPS injection, we examined the expression of IL-7 mRNA in various organs before and after LPS treatment by using real-time PCR. LPS induced an increase in the expression of IL-7 mRNA in kidney and liver tissues, whereas expression decreased in the spleen and lymph nodes in response to LPS (Figure 1B). On the other hand, LPS did not substantially affect IL-7 expression in other organs, including heart, thymus, lung, skin, and muscle (Figure 1B), suggesting that IL-7 mRNA expression is differentially regulated by TLR ligands in the various organs. Time-course experiments confirmed that the amount of IL-7 mRNA transiently increased in liver and kidney tissues, peaking 6 hr after the LPS injection (Figure 1C), whereas it decreased in lymph nodes (Figure S2).

In organs in which the major cell populations produce IL-7 in response to LPS, the total amount of IL-7 expressed in each organ should be related to the size of the organ. We estimated the total amount of expressed IL-7 by using the weight of the organ (average organ weights in 7- to 8-week-old C57BL/6 mice [n = 5]: spleen, 0.09 g; kidney, 0.32 g; heart, 0.13 g; liver, 0.95 g; thymus, 0.05 g; and lung, 0.14 g). As expected, a larger relative amount of IL-7 was expressed in the liver than in any of the other organs after LPS stimulation (Figure 1D). Thus, the liver appears to be a major source of LPS-induced IL-7 expression.

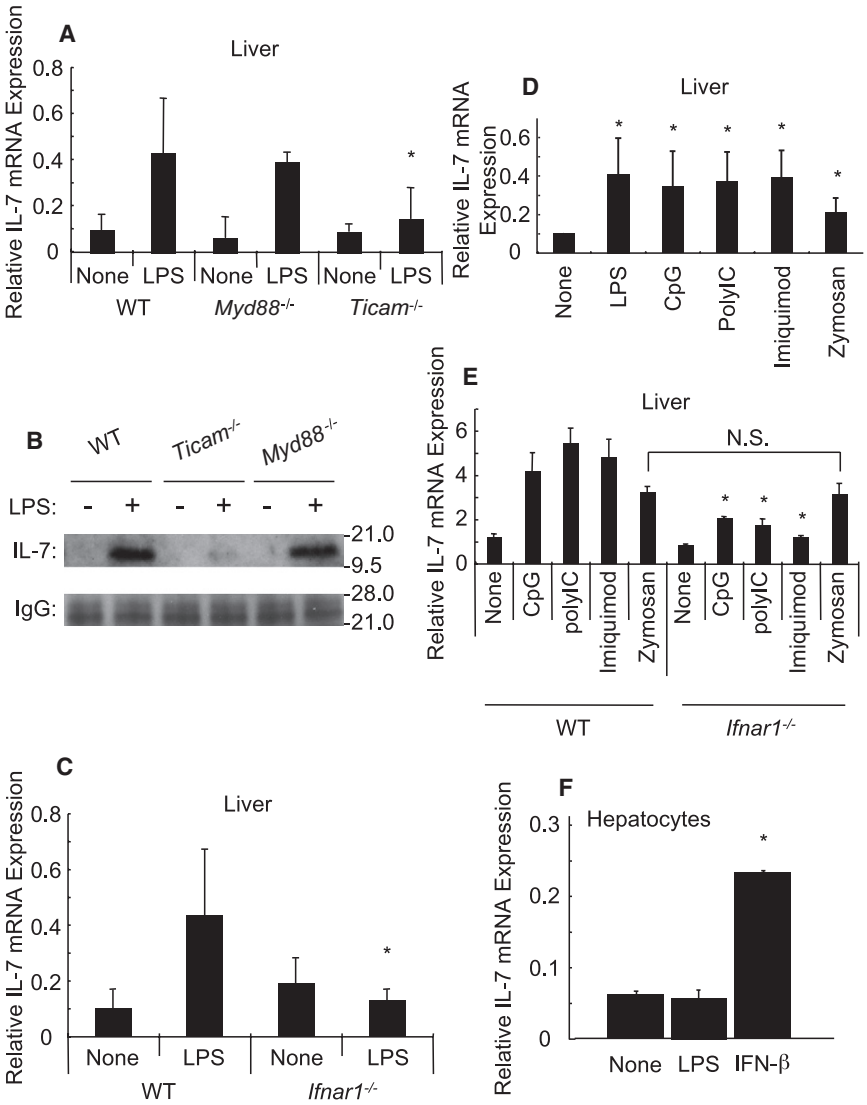

Figure 2. IL-7 Expression Is Induced in Hepatocytes after LPS Injection In Vivo

(A) *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$, and control wild-type mice (n = 5 each) were injected with LPS and the livers were harvested 6 hr later. IL-7 mRNA expression in the livers was analyzed with real-time PCRs. After treatment with LPS, the expression of IL-7 mRNA in the *Ticam1*$^{-/-}$ mice was lower than in the wild-type controls (p = 0.046).

(B) Serum expressions of IL-7 were examined in *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$, and control animals with or without LPS via immunoblotting 12 hr after injections.

(C) *Ifnar1*$^{-/-}$ and control mice (n = 5 each) were injected with LPS and the livers were harvested. IL-7 mRNA expression was analyzed with real-time PCR. 6 hr after LPS treatment, the expression of IL-7 mRNA in the IFN-I receptor-deficient mice was lower than in wild-type controls (p = 0.038).

(D) IL-7 mRNA in liver with or without TLR ligand treatment were measured with real-time PCRs. The IL-7 mRNA in liver increased 6 hr after treatment with LPS (2.5 μg), CpG (25 μg), poly(I-C) (100 μg), imiquimod (25 μg), or zymosan (500 μg) (p = 0.002, 0.0036, 0.00035, 0.0017, and 0.012, respectively).

(E) IL-7 mRNA in the liver with or without TLR ligand treatment were measured in IFN-I receptor-deficient mice or control animals by real-time PCRs. The IL-7 mRNA in the liver were lower in IFN-I receptor-deficient mice than in control mice 6 hr after treatment with CpG (25 μg), poly(I-C) (100 μg), or imiquimod (25 μg) (p = 3.2 × 10^{-8}, 3.7 × 10^{-7}, and 6.3 × 10^{-6}, respectively).

(F) IL-7 expression in primary hepatocytes was significantly elevated 6 hr after treatment with IFN-β (p = 0.00022). Data represent the mean + SD; *p < 0.05 compared with control samples; N.S., not significant.

We confirmed the important role of liver tissues in LPS-mediated upregulation of serum IL-7 by using partial hepatectomies (Mitchell and Willenbring, 2008). We showed that the LPS-mediated increases in serum IL-7 were smaller in mice that underwent hepatectomy than in sham-operated animals (Figure 1E). Importantly, IL-7 protein expression increased in liver cells after LPS stimulation (Figure 1F). Thus, we concluded that liver is the major source of LPS-induced IL-7 expression.

Hepatic IL-7 Expression Is Dependent on TRIF-IFN-I Pathway after LPS Injection In Vivo

LPS signaling can occur through either of two adaptor molecules, TRIF or Myd88, both of which intracellularly bind to the LPS receptor TLR4 (Yamamoto et al., 2003). To investigate which of these pathways is important for LPS-mediated IL-7 expression in liver tissues, we employed two mouse strains: *Ticam1*$^{-/-}$ (TRIF-deficient) and *Myd88*$^{-/-}$ mice (Adachi et al., 1998; Yamamoto et al., 2003). IL-7 expression markedly increased in *Myd88*$^{-/-}$ and wild-type control liver tissues and sera but not in *Ticam1*$^{-/-}$ livers after LPS treatment (Figures 2A and 2B). To investigate whether IFN-I, a target of the TRIF-dependent pathway, is critical for the induction of IL-7 expression in the liver after LPS injection, we prepared *Ifnar1*$^{-/-}$ mice (Müller et al., 1994). Although LPS injection increased IL-7 mRNA expression in the livers of wild-type mice, this effect was not observed in *Ifnar1*$^{-/-}$ mice (Figure 2C). Collectively, these results demonstrated that LPS-mediated upregulation of IL-7 expression in the liver is dependent on the TRIF-IFN-I signaling pathway.

To determine whether IL-7 expression in the liver is generally induced by TLR signaling, which is known to increase the expression of IFN-I, we treated mice with various TLR ligands, including CpG, poly(I-C), imiquimod, and zymosan, to mimic physiologic responses to infection. All of the tested TLR ligands increased IL-7 expression in liver tissues (Figure 2D). Furthermore, we investigated whether CpG-, imiquimod-, zymosan-, and poly(I-C)-mediated upregulation of IL-7 expression in the liver was dependent on IFN-I by using mice deficient for the IFN-I receptor. The results showed that the increased IL-7 expression induced in the liver by each of the TLR ligands except zymosan was dependent on IFN-I (Figure 2E).

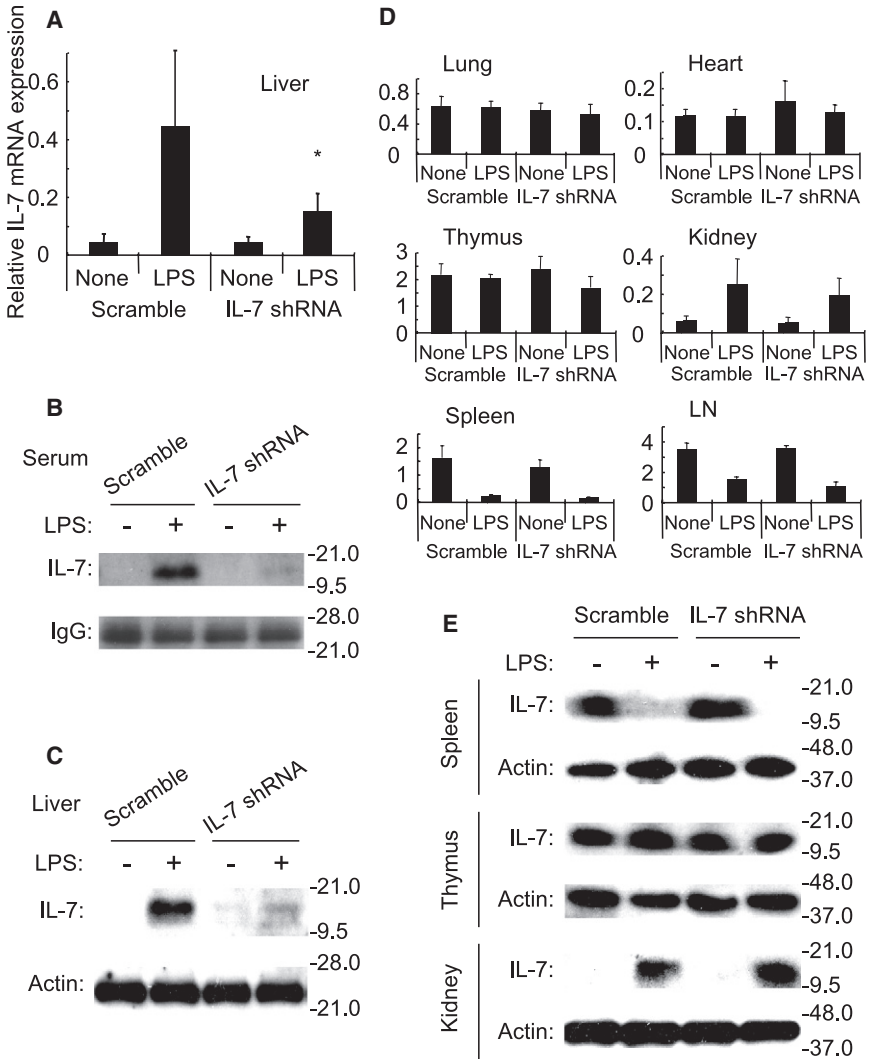

Figure 3. Liver-Specific IL-7 Depletion in Mice

C57BL/6 mice were treated with plasmid vectors coding for IL-7 shRNA or scrambled control shRNA. The mice were then injected with LPS and the IL-7 mRNA expression in the liver (A) and other organs (D) were analyzed 6 hr after the injection with real-time PCRs. The amount of LPS-mediated IL-7 expression in liver was lower in the mice treated with IL-7 shRNA (n = 20 for scrambled shRNA plus LPS and n = 20 for other conditions; p = 0.005). (B) IL-7 in serum 12 hr after LPS treatment, (C) IL-7 in the liver 12 hr after LPS treatment, and (E) IL-7 in other organs 12 hr after LPS treatment were detected with immunoblotting. Data represent the mean + SD; *p < 0.05 compared with control samples.

Because the liver contains a number of cell types that potentially could serve as sources of IL-7, including stromal cells, dendritic cells, Kupffer cells, natural killer (NK) cells, NK T cells, conventional T cells, and hepatocytes, we next sought to determine which cells in the liver produce IL-7 in response to LPS. We obtained a primary culture of mouse hepatocytes and stimulated the cells with LPS or IFN-I (IFN-β). We found that IFN-β but not LPS markedly induced the expression of IL-7 mRNA in the primary hepatocytes (Figure 2F). These results strongly suggested that IFN-β, which is upregulated via LPS-TRIF signaling in nonhepatocytes, induces IL-7 expression in hepatocytes. Thus, IL-7 is likely an acute-phase reactant expressed in response to TLR-IFN-I signaling in hepatocytes.

Depletion of IL-7 Expression in the Liver Reduces Memory and Naive CD4$^+$ and CD8$^+$ T Cells

To uncover a direct link between the IL-7 expression induced via TLR-IFN-I signaling and T cell responses in vivo, we attempted to deplete IL-7 expression in hepatocytes by using hydrodynamics-based transfection of plasmid DNA (Zhang et al., 1999). It has been reported that the transduction of genes by this method results in increased gene transfection efficacy and higher target molecule expression in hepatocytes than in other cell types. In fact, this method produces a degree of expression that is more than three orders of magnitude higher in liver cells than in other organs, including kidney, spleen, thymus, lymph nodes, muscle, lung, skin, and heart (Figure S3 and data not shown; Zhang et al., 1999). Plasmids coding for either IL-7-specific short hairpin RNA (shRNA) or a scrambled control sequence were introduced into liver cells by this transfection method. After LPS injection, IL-7 mRNA and protein expression in the liver as well as the serum amount of IL-7 protein was lower in mice transfected with the plasmid coding for IL-7 shRNA compared with mice treated with the plasmid coding for scrambled control shRNA (Figures 3A–3C). We also confirmed that the expression of IL-7 in other organs, including lymph nodes, spleen, lung, heart, thymus, and kidney, was not affected markedly by the injection of the IL-7 shRNA plasmid (Figures 3D and 3E). These results demonstrated that the hydrodynamics-based transfection of a plasmid coding for IL-7 shRNA specifically depleted the expression of IL-7 protein in hepatocytes in vivo.

By using the hydrodynamics-based transfection method, we evaluated the physiological roles of TLR-IFN-I-mediated expression of IL-7 in immune cell responses. We first investigated the sizes of various cell populations before and after LPS-TLR stimulation in the presence or absence of hepatocyte-specific depletion of IL-7 expression. LPS-TLR stimulation increased the numbers of CD4$^+$ T cells, CD8$^+$ T cells, NK cells, B cells, and granulocytes, whereas the dendritic cell population was not affected (Figures S4A and S4B). Importantly, TLR-mediated hepatic IL-7 expression only increased the CD4$^+$ and CD8$^+$ T cell numbers but not those of NK cells, B cells, and granulocytes (Figures S4A and S4B). Moreover, the populations of both naive and memory CD4$^+$ and CD8$^+$ T cells expanded in

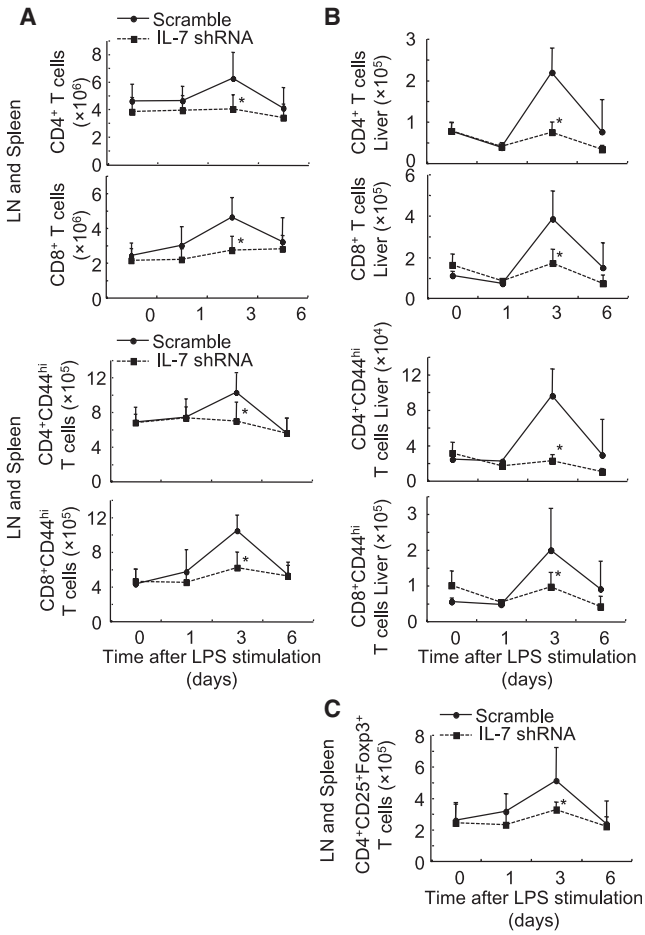

Figure 4. CD4$^+$ and CD8$^+$ T Cell Numbers Increase in Response to LPS-Induced IL-7 Production in the Liver

C57BL/6 mice were treated with plasmid vectors coding for IL-7 shRNA or scrambled control shRNA 1 day before LPS injection. The mice were then intraperitoneally injected with LPS (2.5 μg). (A) The numbers of CD4$^+$ and CD8$^+$ T cells in lymph nodes and spleen or liver, (B) the numbers of CD44^{hi} cells among the CD8$^+$ and CD4$^+$ T cells in lymph nodes and spleen or liver, and (C) the numbers of CD4$^+$CD25$^+$ cells in lymph nodes and spleen were analyzed on days 0, 1, 3, and 6 after LPS injection with a flow cytometer. LPS treatment increased the numbers of CD4$^+$ and CD8$^+$ T cells in lymph nodes (A), spleen (A), and liver (B); the numbers of memory CD44^{hi} cells among the CD8$^+$ and CD4$^+$ T cells in the lymph nodes (A), spleen (A), and liver (B); and the numbers of memory CD4$^+$CD25$^+$Foxp3$^+$ cells in the lymph nodes and spleen (C). The numbers of CD4$^+$, CD8$^+$, CD4$^+$CD44^{hi}, and CD8$^+$CD44^{hi} T cells on day 3 after the LPS stimulation were significantly lower in mice treated with IL-7 shRNA compared with mice injected with scrambled control shRNA ($p = 0.0164$, 0.00249, 0.0124, and 0.00049, respectively [A]; $p = 0.0001$, 0.0062, 0.00017, and 0.048, respectively [B]). The numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells on day 3 after the LPS stimulation were significantly lower in mice treated with IL-7 shRNA than in mice treated with scrambled control shRNA ($p = 0.049$) (C). Data represent the mean + SD; *$p < 0.05$ compared with control samples.

response to TLR-induced hepatic IL-7 expression (Figure 4A; Figure S4A). Time-course experiments showed that the T cell number peaked on day 3 after LPS injection (Figure 4A). After LPS treatment, a larger increase in the T cell number was observed in liver tissues than in lymphoid tissues (Figure 4B). Further, expansion of the T cell population was dependent on

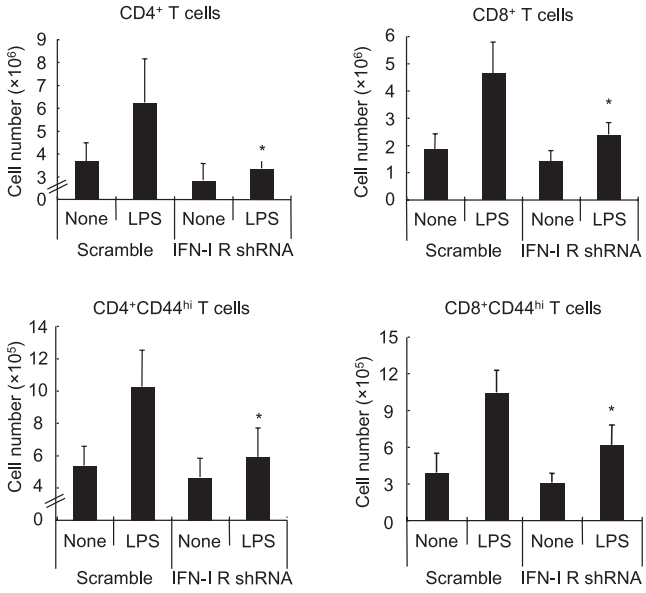

Figure 5. CD4$^+$ and CD8$^+$ T Cell Numbers Increase in Response to LPS-Mediated IFN-I Signaling in the Liver

C57BL/6 mice were treated with plasmid vectors coding for IFN-I receptor shRNA or scrambled control shRNA 1 day before LPS injection. The mice were then intraperitoneally injected with LPS (2.5 μg) and the CD4$^+$ and CD8$^+$ T cells, and the CD44^{hi} cells among the CD8$^+$ and CD4$^+$ T cells were counted. On day 3 after LPS stimulation, the numbers of CD4$^+$, CD8$^+$, CD4$^+$CD44^{hi}, and CD8$^+$CD44^{hi} T cells were significantly lower in mice treated with IFN-I receptor shRNA compared with mice injected with scrambled control shRNA ($p = 0.0054$, 0.00093, 0.0016, and 0.0052, respectively). Data represent the mean + SD; *$p < 0.05$ compared with control samples.

IL-7 expression (Figures 4A and 4B). Upregulation of IL-7 expression in the liver also expanded the Treg cell (CD4$^+$CD25$^+$Foxp3$^+$ T cells) population in vivo (Figure 4C).

We then examined whether activation markers of T cells, such as CD69 and CD44, were regulated by TLR-IFN-I-mediated hepatic IL-7 expression. The percentage of CD69$^+$ T cells among the CD4$^+$ and CD8$^+$ T cells increased after LPS treatment, whereas that of CD44$^+$ T cells did not (Figure S5). Furthermore, TLR-induced hepatic IL-7 expression did not contribute to the increased percentage of CD69$^+$ T cells (Figure S5).

Because it is known that IFN-I is pleiotropic, serving both suppressive and stimulatory functions at various points during T cell immune responses in vivo (Theofilopoulos et al., 2005), we examined the specific contributions of IFN-I to IL-7-mediated increases in T cell numbers in the liver. We prepared mice with depleted IFN-I receptor expression in their livers and injected them with LPS; the LPS-mediated increases in serum IL-7 and the T cell number were dependent on IFN-I receptors in the liver (data not shown and Figure 5). These data suggest that IFN-I functions to enhance T cell-mediated adaptive immunity in the liver via IL-7.

We next investigated whether TLR-IFN-I-induced hepatic IL-7 expression increased the T cell number by enhancing the proliferation or survival of T cells. BrdU was placed in the drinking water of the mice to allow us to examine the percentage of proliferating T cells before and after LPS treatment in the presence or absence of IL-7 depletion in the liver. LPS treatment increased

the percentage of proliferating BrdU$^+$ memory-phenotype CD8$^+$ T cells but not that of BrdU$^+$ naive-phenotype CD8$^+$ T cells (Figure S6); the populations of both BrdU$^+$ memory- and naive-phenotype CD4$^+$ T cells also did not expand, as demonstrated previously (Sun et al., 1998; Tough et al., 1996). Moreover, LPS-mediated hepatic IL-7 expression did not markedly contribute to the increase in the number of proliferating BrdU$^+$ memory-phenotype CD8$^+$ T cells (Figure S6). These results strongly suggested that TLR-IFN-I-induced hepatic IL-7 expression increased the numbers of naive and memory CD4$^+$ and CD8$^+$ T cells by enhancing their survival.

Hepatic IL-7 Expression Induced via LPS Signaling Plays a Role in CD8$^+$ and CD4$^+$ T Cell Responses

To examine the relationship between increased hepatic IL-7 expression and T cell responses, we first performed infection experiments with *Listeria* and LCMV to investigate antigen-specific CD8$^+$ T cell responses with or without depleted IL-7 expression in the liver. By using *Listeria* and LCMV, we showed that upregulated IL-7 expression in the liver does not markedly contribute to pathogen-specific CD8$^+$ T cell proliferation (Figure S7). We also confirmed that the liver showed high amounts of IL-7 mRNA expression in response to both infections, and that IL-7 shRNA but not scrambled control shRNA effectively attenuated observed hepatic IL-7 mRNA expression (Figure S8). Consistent with these results, it was reported that IL-7 signaling is dispensable for antigen-induced expansion of the CD8$^+$ T cell population, whereas it is essential for CD8$^+$ memory T cell production (Schluns et al., 2000). Additionally, we showed that the *Listeria*-specific and the LCMV-specific CD4$^+$ T cells were not affected by the hepatically expressed IL-7 (Figure S9 and data not shown).

IFN-I has been shown to induce an increase in the number of CD44^{hi} memory-activated T cells, a process referred to as bystander activation (Marrack et al., 1999). This IFN-I-dependent increase in the memory T cell population occurs independently of signaling through the T cell receptor (TCR) and is believed to play a role in long-lasting memory T cell maintenance in the absence of specific antigen (Kolumam et al., 2005). Therefore, we examined the involvement of TLR-IFN-I-mediated IL-7 expression in the prolongation of antigen-specific responses. LPS treatment increased the number of antigen-specific memory-activated CD8$^+$ T cells, whereas depletion of IL-7 expression in hepatocytes suppressed these effects (Figure 6A; Figure S10). Moreover, cytotoxic T-lymphocyte (CTL) activity and IFN-γ expression in the CD8$^+$ T cells were higher in mice treated with LPS than in untreated mice (Figures 6B and 6C). Importantly, IL-7 depletion in the liver cells inhibited the LPS-mediated enhancement of CTL activity and IFN-γ expression (Figures 6B and 6C).

We next investigated whether hepatic IL-7 expression in response to LPS plays a role in the homeostatic proliferation of CD8$^+$ T cells under lymphopenic conditions, which are known to increase the number of memory-activated CD8$^+$ T cells. As expected, CD8$^+$ T cell homeostatic proliferation was enhanced by upregulated IL-7 expression in the liver (Figures 6D and 6E). Taken together, these results demonstrated that the upregulation of IL-7 expression in the liver increases the number of memory-activated CD8$^+$ T cells in an antigen-nonspecific manner.

To investigate the role of TLR-induced hepatic IL-7 expression in the development of CD4$^+$ T cell responses, we employed an experimental autoimmune encephalomyelitis (EAE) system, in which mice develop a Th17 cell- and Th1 cell-mediated autoimmune disease; in this model, the percentages of IL-17- and IFN-γ-expressing CD4$^+$ T cells are determinants of disease severity (Stromnes et al., 2008). EAE was induced with MOG peptides in addition to the TLR ligands pertussis toxin (PTx) to stimulate TLR4 and/or complete Freund's adjuvant (CFA) to stimulate TLR2 and TLR4 (Jo et al., 2007; Racke et al., 2005). We hypothesized that TLR-induced hepatic IL-7 expression enhances pathogenic CD4$^+$ T cell responses, because IL-7 expression increased after treatment with PTx and/or CFA (data not shown), and the serum IFN-I concentration increased after immunization with the peptide (Figure 7A). Th17 cell numbers and serum expression of IL-17 in the EAE mice were then examined in the presence or absence of the IL-7 shRNA plasmid. Depletion of IL-7 expression in the liver decreased the numbers of Th17 and Th1 cells in both the spinal cord and draining lymph nodes (Figures 7B and 7C), as well as the serum concentrations of IL-17 and IFN-γ (Figure 7D). Thus, these results strongly suggest that hepatically derived IL-7 contributes to the induction of pathogenic CD4$^+$ T cell responses associated with EAE. Importantly, the IL-7 deficiency attenuated the development of EAE (Figure 7E).

DISCUSSION

We have demonstrated that both CD4$^+$ and CD8$^+$ T cell responses are enhanced by TLR-mediated increases in hepatic IL-7 expression in vivo. It was previously thought that the degree of intracellular IL-7-mediated signaling depends primarily on the amount of IL-7Rα expressed on the cell surface (Mazzucchelli and Durum, 2007; Schluns et al., 2000). Moreover, IL-7 is thought to be expressed at a constant amount in vivo, unaffected by extrinsic stimulation (Fry and Mackall, 2005). Therefore, many studies have focused on the expression of the receptor rather than the ligand. Indeed, memory-activated T cells have been shown to express high amount of IL-7Rα (Kaech et al., 2003). However, we recently showed that excessive IL-7 production in nonhematopoietic cells from F759 mutant mice, which show augmented IL-6-gp130-STAT3 signaling, contributes to the development of autoimmune arthritis (Atsumi et al., 2002; Sawa et al., 2006). Moreover, our results here demonstrate that IL-7 production induced via TLR-IFN-I signaling in wild-type mice contributes to T cell survival and enhanced T cell responses. Thus, intensity of IL-7 signaling in T cells is dependent on both the amount of IL-7, which is regulated by such signaling pathways as IL-6-gp130-STAT3 and TLR-IFN-I, and the expression of IL-7Rα, which is controlled by various signaling molecules, including TCRs and cytokines.

We have also shown that the liver is a primary source of IL-7 in response to TLR stimulation, although IL-7 expression is limited in the liver under steady-state conditions. TLR-mediated systemic cytokine expression alters protein expression profiles in hepatocytes, which produce a number of serum acute-phase reactant proteins, markers of systemic inflammation that include SAA, CRP, plasminogen, ferritin, and complement components (Fey et al., 1994). Thus, IL-7, an important survival factor for T cells, is expressed in the liver in response to inflammation to

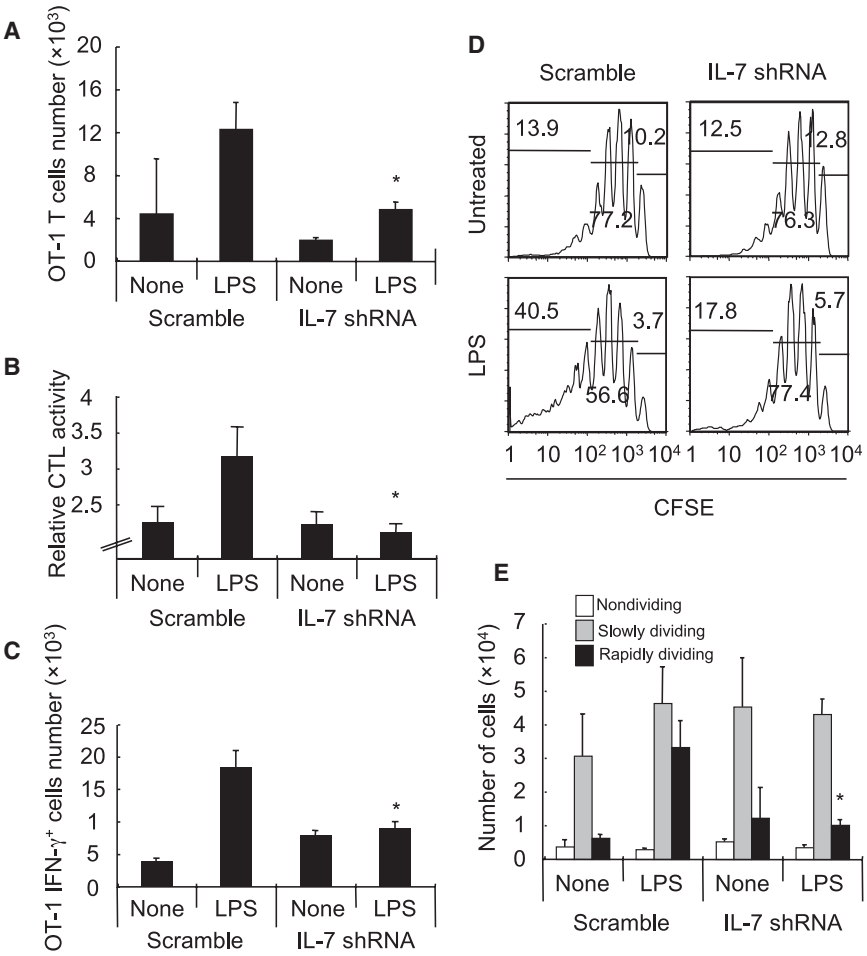

Figure 6. CD8⁺ T Cell Responses Are Regulated by LPS-Mediated IL-7 Production in the Liver

(A–C) OT-1 $CD8^+$ T cells were transferred to congenic mice and activated with OVA and CFA. 4 days after activation, $CD8^+CD44^{hi}CD90.2^+$ T cells were sorted and transferred to B6.PL mice (day 0). The mice were then treated with LPS (days 0, 2, 4, and 6) and IL-7 shRNA or scrambled control shRNA (days −1, 1, 3, and 5). OT-1 $CD8^+$ T cell numbers and CTL activity were measured on day 8. In the presence of LPS, significant decreases in the number of OT-1 T cells (A), in the in vivo CTL activity (B), and in the number of $CD90.2^+IFN\gamma^+$ OT-1 T cells (C) were observed after treatment with IL-7 shRNA compared with results observed after treatment with scrambled control shRNA ($p = 0.015$, 0.017, and 0.01, respectively).

(D) Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD8^+CD44^{lo}$ T cells from C57BL6-SJL (CD45.1) were transferred to sublethally irradiated (5 Gy) C57BL/6 mice and the mice were treated with IL-7 shRNA or scrambled control shRNA in the presence or absence of LPS. Proliferation of the transferred $CD8^+CD45.1^+$ T cells was assessed by FACS-measured dilution of CFSE. We have divided the CFSE-labeled cells into three populations: nondividing (high CFSE concentration), slowly dividing (1 to 4 divisions; midlevel CFSE concentration), and rapidly dividing (more than 5 divisions; low CFSE concentration). The numbers represent the percentage of cells in these populations.

(E) The actual cell numbers in each population from (D): nondividing, white columns; slowly dividing, gray columns; rapidly dividing, black columns. In the presence of LPS, a larger increase in the rapidly dividing population was observed for $CD8^+$ T cells from mice treated with scrambled control shRNA than for those from mice treated with IL-7 shRNA ($p = 0.021$). Data represent the mean + SD; *$p < 0.05$ compared with control samples.

allow the immune system to respond against pathogens as an acute-phase reactant. It should be noted that IL-7 expression increased only in liver and kidney tissues after TLR stimulation, whereas it decreased in the spleen and lymph nodes. These results suggested that systemic IL-7 expression driven by hepatocytes, but not by lymphoid organs, play an important role in IL-7-mediated T cell homeostasis in response to infections. How IL-7 expression is differentially regulated in the different organs after TLR activation, however, has not yet been elucidated. Interestingly, IL-7 transgenic mice develop autoimmune diseases, such as colitis (Watanabe et al., 1998) and dermatitis (Uehira et al., 1998). Because T cells in lymphoid organs are activated by the systemic expression of IL-7 in IL-7 transgenic mice even under steady-state conditions (Watanabe et al., 1998), heightened IL-7 expression in lymphoid organs likely leads to an increased number of autoreactive T cells and autoimmune diseases. Thus, the decreased IL-7 expression in lymphoid organs after TLR stimulation may counteract these processes.

IL-7 expression in hepatocytes is critical for enhanced antigen-specific $CD8^+$ T cell responses, a process that occurs via antigen-nonspecific bystander proliferation. Moreover, the fact that hepatic IL-7 expression was induced by all of the tested TLR ligands may be advantageous for vaccine development, because the use of TLR ligand combinations synergistically increases the intensity of signaling followed by enhanced cytokine expression (Napolitani et al., 2005). TLR ligand combinations also may synergistically increase the amount of IL-7 expression from hepatocytes, which would enhance antigen-specific memory and activated T cell responses. Treatment with TLR ligands after a period of antigen-specific T cell activation, a contraction phase and/or a memory phase, would efficiently increase memory-activated T cell responses, because increased IL-7 expression in the liver did not play a major role in the proliferation of antigen-specific $CD8^+$ T cells in response to *Listeria* and LCMV at a period of antigen-specific T cell activation. Future studies about these issues may increase the efficacies of vaccines in vivo through a hepatic IL-7-dependent enhancement of memory-activated T cell maintenance. Regulation of IL-7 production in the liver must be tightly controlled, however, because IL-7 expression in hepatocytes contributes to the induction of pathogenic T cell activity and may enhance autoimmune diseases, such as EAE.

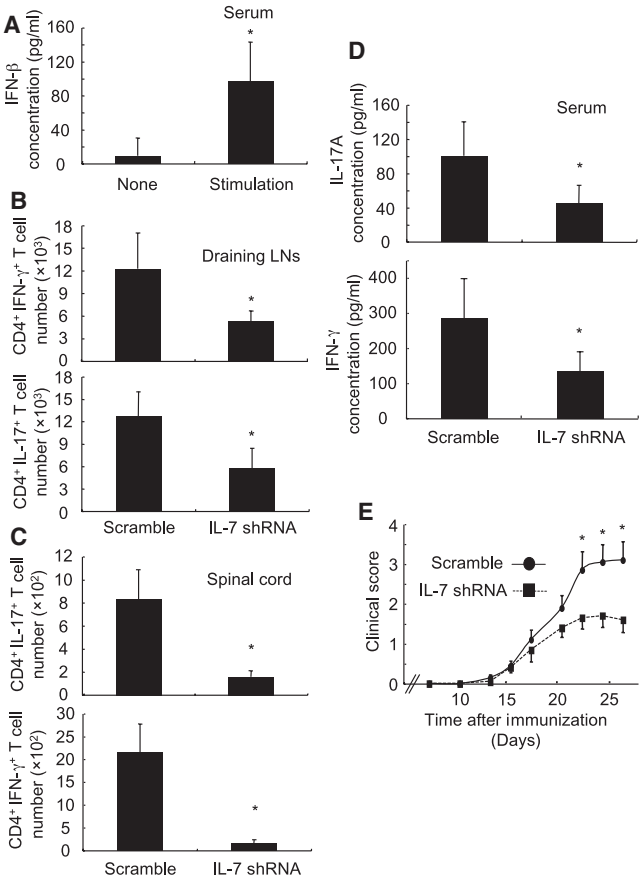

Figure 7. CD4$^+$ T Cell Responses Are Regulated by LPS-Mediated IL-7 Production in the Liver

(A) C57BL/6 mice were injected with MOG peptide and CFA (day 0) and intravenously with PTx (days 0, 2, and 7). IFN-β concentrations in serum were measured 12 hr after the last PTx injection in immunized C57BL/6 mice and compared with results observed in control nonimmunized mice ($p = 0.0054$).

(B and C) C57BL/6 mice treated with IL-7 shRNA or scrambled control shRNA (days −1, 1, and 6) were injected subcutaneously with MOG peptide and CFA (day 0) followed by intravenous injection of PTx (days 0, 2, and 7). (B) CD4$^+$ T cells from the inguinal lymph nodes of immunized C57BL/6 mice were sorted with magnetic beads on day 10 and (C) mononuclear cells from the spinal cords of immunized C57BL/6 mice were isolated on day 21. The resulting cell populations were stimulated in vitro with MOG peptide and bone marrow-derived dendritic cells. 6 hr after in vitro stimulation, intracellular IL-17 and IFN-γ were examined. The CD4$^+$IL-17$^+$ and CD4$^+$IFN-γ$^+$ T cells from immunized C57BL/6 mice treated with IL-7 shRNA or scrambled control shRNA were counted (days −1, 1, and 6). The numbers of CD4$^+$IL-17$^+$ and CD4$^+$IFN-γ$^+$ T cells in the inguinal lymph nodes ($p = 0.00299$ and 0.00362, respectively) and the spinal cords ($p = 0.00491$ and 0.0147, respectively) were significantly lower in the immunized mice treated with IL-7 shRNA than in those treated with scrambled control shRNA.

(D) C57BL/6 mice treated with IL-7 shRNA or scrambled control shRNA (days −1, 1, and 6) were injected subcutaneously with MOG peptide and CFA (day 0) followed by intravenous injection of PTx (days 0, 2, and 7). On day 10, the serum IL-17A and IFN-γ concentrations were significantly lower in the immunized mice treated with IL-7 shRNA than in those treated with scrambled control shRNA ($p = 0.0026$ and 0.000479, respectively).

(E) C57BL/6 mice treated with IL-7 shRNA or scrambled control shRNA (days −1, 1, and 6) were injected subcutaneously with MOG peptide and CFA (day 0) followed by intravenous injection of PTx (days 0, 2, and 7) and analysis for EAE development. EAE development was attenuated in the C57BL6 mice injected with IL-7 shRNA compared with mice injected with scrambled control shRNA ($p = 0.003$, 0.002, and 0.003 on days 22, 24, and 26 after the first immunization, respectively). All data are shown as the mean + SD, except for the T cell numbers in the spinal cord (C) and EAE development (E) (mean + SEM). *$p < 0.05$ compared with control samples.

TLR-mediated hepatic IL-7 expression was dependent on IFN-I. The role of IFN-I in T cell responses is complicated. IFN-I may play contradictory roles in the regulation of immune responses, especially for the development of autoimmune diseases; this cytokine enhances several autoimmune diseases (Stewart et al., 1993), whereas it suppresses others (Treschow et al., 2005). In humans, one of the most commonly used therapies for multiple sclerosis is systemic administration of IFN-I, in particular IFN-β. In patients with multiple sclerosis, IFN-β has been shown to reduce the frequency of clinical exacerbations, the progression of disability, and cumulative disease activity. Despite these clinical benefits, however, IFN-β is only partially effective and is not a cure for multiple sclerosis. Furthermore, many patients do not respond to this treatment modality. Because IFN-I treatment may produce an unwanted augmentation of IFN-I-IL-7 signaling, as demonstrated here, targeting the TLR-IFN-I-IL-7 signaling axis may provide an effective therapeutic approach for autoimmune diseases, including multiple sclerosis.

In summary, we identified hepatically derived IL-7 as an effecter molecule of TLR-IFN-I-mediated signaling in vivo. The TLR-IFN-I-IL-7 signaling axis in the liver, which was found to contribute to increased numbers of naive and memory CD4$^+$ and CD8$^+$ T cells, enhances LPS-mediated increases in the antigen-specific memory-activated CD8$^+$ T cell number, antigen-specific CTL activity, and EAE, a CD4$^+$ T cell-induced autoimmune disease. Thus, we concluded that the induction of IL-7 expression in liver cells via TLR-IFN-I signaling is important for T cell responses and subsequent enhancement of adaptive immunity in vivo.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Japan SLC. B6.PL (Thy-1.1) congenic mice were purchased from the Jackson Laboratory. B6.SJL (CD45.1) congenic mice were obtained from Taconic. C57BL/6-Myd88-deficient (Adachi et al., 1998) and C57BL/6-TRIF-deficient (Yamamoto et al., 2003) mice were obtained from S. Akira (Osaka University, Japan). IFN-I receptor-deficient mice were obtained from T. Kaisho (RIKEN-RCAI, Japan). C57BL/6-OT-1 TCR transgenic mice were kindly provided by W. Heath (WEHI, Australia). Mice were maintained in the medical science animal facilities at Osaka University under specific pathogen-free conditions (SPF). Animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees of the Graduate School of Frontier Biosciences and the Graduate School of Medicine (Osaka University). We used 7- to 12-week-old animals for all of the experiments.

Antibodies and Reagents

FITC-conjugated anti-mouse CD19, NK1.1, I-A^b, Gr-1, CD44, Foxp3, and IgG$_{2a}$; PE-conjugated anti-mouse CD4, Dx5, CD69, CD44, IL-17A, IgG$_{2a}$, and CD25; PE-Cy5-conjugated anti-mouse CD4 and CD8; PE-Cy7-conjugated anti-mouse CD4 and CD8; APC-conjugated anti-mouse CD8, CD90.1, and CD45.1; PB-conjugated anti-mouse CD44; biotin-conjugated anti-mouse CD8; and APC-conjugated streptavidin were purchased from eBioscience. FITC-conjugated anti-mouse CD4 and CD8; APC-conjugated anti-mouse CD4, IFN-γ, and IgG$_1$; biotin-conjugated anti-mouse CD19, Dx5, TCRβ, and

I-A^b; and PE-Cy5-conjugated streptavidin were purchased from BioLegend. FITC-conjugated anti-mouse BrdU, PE-conjugated anti-mouse IFN-γ, IgG_1, and APC-conjugated anti-mouse CD90.2 were purchased from BD Biosciences. PE-conjugated D^bGP33 tetramer and H-$2K^b$ OVA tetramer were used. Anti-mouse actin was purchased from Santa Cruz Biotechnology. HRP-conjugated anti-mouse IgG was purchased from Zymed Laboratories. LPS, poly(I-C), and partially purified zymosan were purchased from Sigma. CpG was obtained from Operon and imiquimod was purchased from InvivoGen.

IL-7 ELISA

Precoated plates from an IL-7 ELISA kit (R&D Systems) were blocked with 1 × ELISA Ultrablock (AbD Serotec) for 3 hr and 150 × human serum for 3 hr at 4°C. The plates were then washed three times with PBS containing 0.05% Tween-20 (PBST). Then, samples were applied to the resulting plates (overnight at 4°C), washed five times with PBST, and incubated with biotin-labeled polyclonal IL-7 antibodies (R&D Systems) overnight at 4°C followed by treatment with 10 μg/ml polyHRP80 (Stereospecific Detection Technologies). The sensitivity of the ELISA system was determined with a titration of rIL-7 (Peprotech; Figure S1B). IL-7 was not detected in the sera of normal C57BL/6 mice and IL-7-deficient mice kept under SPF conditions (less than 8 pg/ml). The specificity of the system was confirmed by two methods. First, both intravenous injection of rIL-7 and IL-7 expression in hepatocytes after hydrodynamics-based transfection resulted in an increased serum IL-7 concentration (data not shown). Second, supernatants from L929 cells transfected with cDNA encoding murine IL-7 were found to contain approximately 20 pg/ml mIL-7, whereas those from mock-transfected L929 cells did not contain a detectable level (less than 8 pg/ml; data not shown).

Immunoblotting for IL-7

Mice sera were collected and concentrated as described previously (Sawa et al., 2006). The concentrated sera were mixed with SDS buffer. Western blotting for IL-7 was performed as described previously (Sawa et al., 2006). After SDS-PAGE on a 10%–20% gel, the protein fraction was used for western blotting analysis with biotinylated anti-mouse IL-7 antibodies (R&D Systems; the same antibody used as the detector antibody for the IL-7 ELISA system) and streptavidin-conjugated horseradish peroxidase secondary antibodies (Sigma). The samples were visualized with an enhanced chemiluminescence kit (Perkin Elmer). The sensitivity of the immunoblot system was determined with a titration of rIL-7 (Peprotech) (Figure S1C). IL-7 was not detected in the sera of normal C57BL/6 mice (less than 5–10 pg/ml). The sensitivity of the IL-7 western blotting system was similar to that of the IL-7 ELISA system. The specificity of the system was confirmed with the same two methods described for the ELISA system.

IL-7 immunoblotting with organ samples yielded similar results to those observed with serum samples. In brief, liver, spleen, thymus, and kidney cells were minced, homogenized in hypotonic buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 0.25 M sucrose, and 1% protease inhibitor at pH 7.4 [Nacalai Tesque]), and centrifuged. The resulting supernatants from spleen and thymus were subjected to SDS-PAGE followed by immunoblotting. The resulting supernatants from liver and kidney were subjected to immunoprecipitation with IL-7-specific monoclonal antibodies (M25) and protein G Sepharose. Then, the immunoprecipitants were separated with SDS-PAGE followed by immunoblotting.

Cell Preparation and Cell Sorting

A T cell-enriched sample was prepared with a MoFlo high-performance cell sorter (Beckman Coulter) as described previously (Ogura et al., 2008). The purities of the T cell samples were routinely >98%.

Flow Cytometry

Flow cytometry analysis was performed as described previously (Ogura et al., 2008).

Intracellular Cytokine Staining

The in vivo numbers of MOG-specific IL-17$^+$ and IFN-γ$^+$ T cells and OT-1 IFN-γ$^+$ T cells were determined with intracellular cytokine staining as previously described (Nishihara et al., 2007).

ELISA

IFN-β, IL-17A, and IFN-γ levels in serum were determined with ELISA kits (BD Biosciences, eBiosciences, or R&D).

BrdU Labeling

Mice were given 0.8 mg/ml BrdU (Sigma) in their drinking water. BrdU staining for flow cytometry was performed with a BrdU Flow kit (BD Biosciences).

Generation of Antigen-Specific Activated CD8$^+$ T Cells and an In Vivo Cytotoxicity Assay

The lymph nodes and spleen were harvested from OT-1 TCR transgenic mice, passed through a 100 μm cell strainer with RPMI-1640, and washed. Erythrocytes were eliminated with 0.165 M NH_4Cl. A T cell-enriched sample was then prepared with a nylon wool column. The purified cell samples containing 8 × 10^6 CD8$^+$CD44^{lo} T cells were intravenously injected into B6.PL (CD90.1) mice on day −1. These mice were then injected with ovalbumin (OVA; Calbiochem, Germany) in CFA (Sigma) at the base of the tail on day 0. Activated OT-1 cells (CD8$^+$CD44^{hi}CD90.2$^+$) were sorted on day 4 and injected into B6.PL mice (2 × 10^6 cells per mouse). The resulting mice then received an intravenous injection of shRNA plasmid (10 μg/mouse) with or without LPS (2.5 μg/mouse) on days 0, 2, 4, and 6 after the transfer of the activated T cells. Splenocytes from B6 mice were incubated for 30 min at 37°C with or without 10 μg/ml $OVA_{257-264}$ peptide (Operon Biotechnologies). In vivo cytotoxic activity was evaluated as described previously (Kamimura et al., 2006).

Real-Time PCRs

Real-time PCRs were performed as described previously (Ogura et al., 2008). The PCR primer pairs used for real-time PCRs were as follows: mouse HPRT primers, 5′-GATTAGCGATGATGAACCAGGTT-3′ and 5′-CCTCCCATCTCC TTCATGACA-3′; mouse IL-7 primers, 5′-CTGCAGTCCCAGTCATCAGTA-3′ and 5′-GTGGCACTCAGATGATGTGACA-3′. The conditions for the real-time PCRs were 40 cycles of 94°C for 15 s followed by 60°C for 60 s. The relative mRNA expression levels were normalized to the levels of HPRT mRNA.

Primary Hepatocyte Culture and Stimulation

Mouse primary hepatocytes (the purities of the cultures were more than 99%) were purchased from Cosmo Bio Co. and stimulated with LPS (500 ng/ml; Sigma) or mouse IFN-β (5 ng/ml; Peprotech) for 6 hr after 12 hr of starvation in DMEM plus 0.1% FCS.

Construction and Injection of the shRNA Plasmid

RNAi-Ready pSIREN-RetroQ-ZsGreen vector was purchased from Clontech Laboratories. shRNA plasmid construction was performed according to the manufacturer's instructions (IL-7 shRNA sequence: 5′-GCCCGAATAATGAAC CAAACT-3′; control shRNA sequence: 5′-CGAACACACGAACGACGATA-3′; IFNαR shRNA sequence: 5′-GCACATGTGATGGACTCAATT-3′; control shRNA sequence: 5′-CAACAAGATGAAGAGCACCAA-3′). The resulting shRNA plasmids (10 μg) were intravenously injected into mice in 2 ml of TransIT-QR Hydrodynamic Delivery solution (Mirus). We investigated the degree of IL-7 mRNA knockdown in the liver after a single dose of shRNA to determine the appropriate interval between the shRNA injections. We injected mice with IL-7-specific shRNA on day 0 and stimulated the mice with LPS on days 1, 3, and 7 after the shRNA injection. The inhibition of IL-7 expression peaked on day 1 (approximately 70%–80% suppression) and gradually subsided from day 3 to day 6 (approximately 40%–50% and 10%–20% suppression on day 3 and day 6, respectively). Therefore, we injected the mice with siRNA every 2 days during the EAE experiments. Importantly, during the EAE experiments, the degree of IL-7 mRNA knockdown in the liver remained at approximately 70%–80% on day 25 after immunization, and no marked suppressive effects were observed in other organs, including the spleen, lung, heart, and kidney on day 25 after immunization.

Partial Hepatectomy

Two-thirds partial hepatectomies were performed as described previously (Mitchell and Willenbring, 2008).

Induction of EAE

EAE was induced and scored as described previously (Ogura et al., 2008).

Mononuclear Cell Isolation from Spinal Cords and Livers

Mononuclear cells from the spinal cords were isolated after cardiac perfusion with PBS as described previously (Matsushita et al., 2008). Mononuclear cells from the livers were isolated after portal vein perfusion with PBS. Liver tissues were digested with type IV collagenase (0.5 mg/ml; Sigma) at 37°C for 30 min. Mononuclear cells were isolated by passing the tissue through 100 μm cell strainers (BD Biosciences), followed by Percoll gradient (33%) centrifugation. After elimination of erythrocytes, lymphocytes were collected from the pellet and washed.

Statistical Analysis

All comparisons of the data were performed with Student's t tests (two tails).

SUPPLEMENTAL DATA

Supplemental Data include ten figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00105-8.

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