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CpG ODN TRIGGERS LIVER INFLAMMATORY REACTION AND ABROGATES SPONTANEOUS TOLERANCE*

Lian-Li Ma¹, Xiudan Gao¹, Liping Liu², Zhidan Xiang¹, Timothy S. Blackwell², Philip Williams¹, Ravi S. Chari¹, and Deng-Ping Yin^{1,3}

Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation, Vanderbilt University Medical Center, Nashville, TN

Abstract

Liver allografts are spontaneously accepted in the liver transplantation mouse model; however, the basis for this tolerance and the conditions that abrogate spontaneous tolerance to liver allograft is incompletely understood. We examined the role of CpG ODN in triggering liver inflammatory reaction and allograft rejection. Bioluminescence imaging (BLI) quantified the activation of NF- κ B at the different timepoints post-transplantation. Intrahepatic lymphocyte subsets were analyzed by immunofluorescence assay and flow cytometry. The results show that liver allografts survived for more than 100 days without requirement for any immunosuppressive therapy. Donor matched cardiac allografts were permanently accepted while third-party cardiac grafts were rejected with delayed kinetics, confirming donor-specific tolerance. NF- κ B activation in the liver allografts was transiently increased at day 1 and diminished by day 4, compared to that was elevated up to 10 days post-transplantation in the cardiac allografts. When CpG ODN was administered as a high dose (50 μ g per mouse $\times$ 1) into the recipients at day 7 post-transplantation, it induced an acute liver inflammatory reaction with elevated NF- κ B activation in both allogeneic and syngeneic liver grafts. Multiple doses of CpG ODN (10 μ g per mouse $\times$ 3) elicited acute rejection of the liver allografts with significant T cell infiltration in the liver allografts, reduced regulatory T cells, and enhanced IFN- γ -producing cells in the intrahepatic infiltrating lymphocytes. These data demonstrate that CpG ODN initiates inflammatory reaction and abrogates spontaneous tolerance in the liver transplantation mouse model.

Keywords

CpG ODN; liver allograft; tolerance; mice

INTRODUCTION

Liver transplantation is a successful treatment for select forms of liver terminal disease and allograft rejection is less severe in liver allograft transplantation than other solid organs (1–3). Human liver allografts require less immunosuppressant than other organs, and the liver allograft is spontaneously accepted without any immunosuppressant in the mouse liver transplantation model (4,5). Although the characterization of liver spontaneous tolerance has

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3Address for Correspondence: Deng Ping Yin, MD, PhD, Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation, Vanderbilt University Medical Center, Nashville, TN, Telephone number: (615)343-6990, Fax number: (312)343-1355, dengping.yin@vanderbilt.edu.

¹Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation, Vanderbilt University Medical Center.

²Department of Medicine, Vanderbilt University Medical Center.

been described for many years, the mechanisms are still unclear and whether the spontaneous tolerance can be abrogated remains understudied.

Several hypotheses have been proposed to be responsible for tolerance induction of liver allografts. First, spontaneous tolerance to liver allografts may be due to the migration of a large population of donor liver leukocytes into the lymphoid tissues of the recipient (7). Second, a mechanism akin to activation-induced cell death with apoptosis of alloreactive recipient cells has been proposed as a mechanism responsible for the induction of spontaneous liver transplant tolerance (6). Third, donor cell chimerism, referred as to the persistence of donor-derived hematopoietic cells in the recipient, can lead to production of donor liver-derived immature dendritic cells (DCs), which cannot provide costimulatory signals and may mediate tolerance induction (7,8). Fourth, liver specific tolerance involves active suppression, as delayed-type hypersensitivity (DTH) analysis can detect donor antigen-linked suppression in liver transplant recipients (9,10). Recent studies suggest that donor- and/or recipient-derived T regulatory (Treg) cells mediate liver donor specific tolerance. Much of this tolerance can be attributed to the inherently tolerogenic manner and the presence of specialized regulatory lymphocyte populations. These Treg cells suppress the alloimmune response contributing to chimerism-associated tolerance after liver transplantation (2,11). Finally, the local lymphocyte population in the liver is enriched in natural killer (NK) cells and NK T cells that express NK1.1 with the specificity for major histocompatibility complex (MHC) class I-like restricted CD1d molecule in mice, which might have crucial roles in the recruitment of circulating T cells and is associated with tolerance induction (12,13). However, a recent report showed that tolerance to liver allografts required NK1.1(+) cells but was independent of CD1d-restricted NKT cells (14). The discovery of the Toll-like receptor (TLR) family has allowed the identification of a class of membrane-spanning receptors dedicated to microbial sensing. After activation of TLR, MyD88, an adaptor, is recruited into the TLR complex that induces IL-1 receptor associated kinase (IRAK) and TNF receptor associated factor-6 (TRAF-6). Both IRAK and TRAF-6 dissociate and activate the intracellular transcriptional factor, NF- κ B (15,16). NF- κ B activation amplifies the expression of multiple chemokine and cytokine genes, and NF- κ B is a potent proinflammatory signal transduction molecule in T cells involved in innate and adaptive immune reactions (17–19). TLR-4 activation and the TLR downstream signaling mediates the early inflammatory response and initiates exogenous antigen-independent innate immunity-mediated liver ischemia-reperfusion injury (IRI) (20).

Both unmethylated CpG motifs presenting in bacterial DNA and synthesized CpG ODNs mount strong innate immune responses. In the setting of liver transplantation, intestinal-derived bacteria and viruses perioperatively may stimulate intrahepatic macrophages to produce type I IFN to induce an inflammatory reaction. Previous reports and our finding demonstrated that TLR9 engagement with CpG ODNs prevents costimulation blockade-induced tolerance to mouse cardiac allograft (21–23); however, whether the TLR9 ligand, CpG ODNs, can trigger the liver inflammatory reaction and abrogate spontaneous tolerance to liver allograft in mouse remains unknown.

The role of the TLR/NF- κ B signaling pathway in innate and adaptive immune responses of liver transplantation remains unclear due to the difficulties in obtaining sufficient *in vivo* data. A quantitative method with transgenic mice for evaluating NF- κ B activation, *in vivo*, has been developed recently using bioluminescence imaging (BLI) (24–26). We have recently demonstrated that BLI using *NF- κ B-luc* mice is a non-invasive approach to visualize the activation of NF- κ B signaling in mouse cardiac allograft rejection and ischemia-reperfusion injury (23). In this study, we tested the role of CpG ODN in the initiation of liver inflammatory reaction and prevention of spontaneous tolerance to liver allografts using BLI to visualize the activation of NF- κ B. We show that stimulating TLR-9 with a high dose of CpG ODN elicits

liver inflammatory reaction, while multiple low doses of CpG ODN induce mouse liver allograft rejection associated with increased NF- κ B activation.

METHODS

Mice and liver transplantation

C57BL/6 (B6, H-2b), Balb/c (B/C, H-2d), and C3H/HeJ (C3H, H-2k) mice were purchased from the Jackson Laboratory or NCI at Frederic. *NF- κ B-luc* mice (C57BL/6, H-2b and Balb/c, H-2d) were utilized for the detection of NF- κ B activation and were kindly provided by Dr. Blackwell (24). All mice were housed in pathogen-free conditions in the Division of Animal Care at Vanderbilt University Medical Center, according to the National Institutes of Health guidelines.

Orthotopic liver transplantation was performed with revascularization of the portal vein and without hepatic artery reconstruction using microsurgical techniques as described previously (27). Briefly, the recipient's liver was removed, and anastomosis of the supra hepatic vena cava was performed using 10-0 sutures (Covidien, Norwalk). The portal vein and the infra-hepatic vena cava anastomosis were connected with cuff techniques. The bile duct was rejoined by end-to-end anastomosis over an indwelling stent. The rejection or liver failure was defined when the mouse died, excluding technical complications ($\leq 10\%$). Donor matched and third party cardiac allografts were performed as described in our previous reports (28). Briefly, cardiac allografts were transplanted into the neck area, and the donor aorta was anastomosed to the common arteria carotis and donor inferior vena cava to the internal venal carotis using 10-0 sutures. Rejection of the cardiac allograft was defined as complete cessation of heart beating.

For TLR engagement, different doses of CpG ODN (10 μ g, 50 μ g, and 100 μ g per mouse, Coley Pharmaceutical Group Ltd, Ottawa, Ontario, Canada) were administered intraperitoneally (i. p.) at day 7 post-transplantation.

In vivo bioluminescence imaging (BLI)

BLI was performed as previously described (24,26). After anesthesia with isoflurane, luciferin (Roche, Mannheim, Germany) was injected intravenously at a dose of 50mg/kg. Mice were housed inside a light-tight box and imaged with an ICCD camera (Hamamatsu C2400-32, Xenogen Corp). Bioluminescence in control mice without transplantation was also imaged. Light emission through the ventral body wall was detected as photon counts over a standardized area of the organs of interest using the ARGUS-50 software for image processing (24,29,30).

Pathological examination

The recently rejected and long-term surviving mice, engrafted with liver allografts, were sacrificed for histological examinations (28). The grafts were stored in 10% formalin solution. Four to six μ m sections were stained with H&E for the assessment of cell infiltration.

Immunofluorescence for T/B cell analyses

T and B cell infiltration in the liver grafts were analyzed by immunofluorescence. The liver grafts were harvested and imbedded in OCT media. The embedded tissues were sectioned and incubated with FITC-labeled B220 (RA3-6B2, BD PharMingen, San Diego, CA) and PE-labeled CD3 (145-2C11, BD PharMingen) antibodies. The samples were mounted on slides with DAKO fluorescent mounting media (Dako Cytomation, Inc., Carpinteria, CA) and analyzed on a fluorescence microscope.

Liver infiltrating lymphocyte analyses

Intrahepatic lymphocytes were purified from the liver by perfusing the liver with 1x Hank's solution containing collagenase 0.5mg/ml, pressing the liver through a stainless still mesh, and centrifuging at 1500 rpm for 5 min. The pellets were suspended in a 35% Percoll-PBS-heparin (100 U/ml) solution and centrifuged at 800 $\times g$ for 20 min at room temperature. The pellets of mononuclear cells are lysed by ACK solution and washed in 1x PBS twice. Lymphocyte subsets including CD4 (GK1.5), CD25 (61.5), CD3 (2c11), CD19 (1D3), F4/80, IL17 (TC11-18H10), and IFN- γ (XMG1.2) (BD PharMingen and eBioscience) in the liver infiltrating lymphocytes were analyzed by flow cytometry.

Statistics

The graft survival days were presented as mean survival time $\pm$ standard deviation (MST $\pm$ SD) and evaluated for statistical significance by ANOVA analysis (Abacus Concepts, Berkeley, CA). *P*-value of < 0.05 or less was taken as significant.

RESULTS

Donor specific tolerance was induced in mouse liver allograft transplantation

The liver allografts were accepted without any requirement for immunosuppressive therapy, which are consistent with previous reports (4,27). To determine donor-specific tolerance, donor-matched heart allografts were transplanted into long-term surviving mice engrafted with liver allografts after 60 days of transplantation. The results show that donor-matched second cardiac allografts were accepted (mean survival time $\pm$ standard deviation, MST $\pm$ SD > 30 days), and third-party cardiac grafts were rejected (MST $\pm$ SD = 16.0 $\pm$ 5.83 days, Fig. 1), suggesting that the liver allograft confers protection on other organ from allograft rejection.

CpG ODN induces acute inflammatory reaction in the liver allograft

To define the role of TLR activation in the induction of acute inflammatory reaction of liver allografts, CpG ODN was administered (i. p.) into recipients at day 7 post-transplantation. Our results indicate the following: (i) administration of 100 μ g of CpG ODN per mouse resulted in 100% mice death within 24 hr (data not shown), (ii) mice died within 1 to 3 days with 50 μ g of CpG ODN per mouse, and (iii) the liver allografts were rejected within 20 days with 10 μ g per mouse (mean survival time, B6 to B/C, MST $\pm$ SD = 9.6 $\pm$ 2.51 days; B/C to B6 MST $\pm$ SD = 11.8 $\pm$ 4.92 days, Fig. 2a). In syngeneic liver transplantation model (from C57BL/6 to C57BL/6), a high dose of CpG ODN (50 μ g per mouse $\times$ 3) resulted in 50% of recipients death, and all mice survived or more than 30 days treated with low doses of CpG ODN (10 μ g per mouse $\times$ 3, Fig. 2b). These results suggest that a higher dose of CpG ODN is required to induce liver inflammatory reaction in the syngeneic liver graft than that in allogeneic liver graft.

The liver grafts were removed at 4 hr after CpG ODN injection for histological and immunofluorescence examinations. Massive thromboses in the central vein and leukocyte infiltration were observed in CpG ODN-treated liver allografts (Fig. 3a, panel B), compared to the liver allograft without treatment (Fig. 3a, panel A). Immunofluorescence examinations show a slight increase of T and B cells infiltration (Fig. 3b panel D), compared to naive liver (Fig. 3b, panel A) and liver allograft without any treatment (Fig. 3b, panel B). To test whether CpG-induced liver inflammatory reaction was identical to liver IRI, allogeneic (Balb/c, H-2d) and syngeneic (C57BL/6, H-2b) liver grafts were preserved in cold University of Wisconsin (UW) solution (4°C) for 16 hr and transplanted into C57BL/6 recipients. H&E staining showed more severe damage of liver allografts (Fig. 3a, panel D) than syngeneic liver grafts (Fig. 3a, panel C), which are identical to CpG-induced liver inflammatory reaction histologically (Fig. 3a, panel B).

In flow cytometry analyses, macrophages (F4/80) and IFN- γ -producing cells in intrahepatic infiltrating lymphocytes were increased at 4hr after injection of a single high dose of CpG ODN in both allogeneic and syngeneic liver grafts. CD4⁺ T cells and macrophages have the ability to produce IFN- γ that triggers innate and adaptive immune responses (Fig. 4 shows data of liver allografts). These observations collectively suggest that CpG ODN induces liver inflammatory reaction.

CpG ODN elicits acute rejection of the liver allografts

TLR engagement prevents induction of transplantation tolerance in a cardiac allograft transplantation mouse model (22). It is unclear, however, whether spontaneous tolerance to liver allografts can be abrogated by TLR-9 engagement. CpG ODN (10 μ g per mouse, i. p.) was administered into the recipient starting on day 7 post-transplantation (referred to as day 0) followed by injection of the same dose of CpG ODN at day 3 and day 6. In contrary to a high dose of CpG ODN (50 μ g per mouse $\times$ 1) that induced recipient death within 1 to 3 days, all liver allografts survived for more than 7 days and were rejected within 20 days after multiple low doses of CpG ODN (10 μ g per mouse $\times$ 3, Fig. 2a).

Previous results suggest an important role of regulatory T cells (Tregs) in the induction of spontaneous tolerance, and increased CD4⁺/CD25⁺/FoxP3⁺ subsets were observed in the liver allograft and the spleen (2). To define whether CpG-induced liver allograft rejection is associated with reduced Tregs, liver infiltrating lymphocytes were obtained for lymphocyte subset analyses. Figure 4 shows relative increases of CD4⁺/CD25⁺, and CD4⁺/FoxP3⁺ subsets in the liver allografts without any treatment (LTx). In CpG ODN-treated mice, CD4⁺/CD25⁺ and CD4⁺/FoxP3⁺ cells were reduced, and IFN- γ -producing cells were increased in the intrahepatic lymphocytes, however, no increased IL-17-producing cells were observed. (Fig. 4, LTx+CpGx3). In combination with Figure 3 that a large amount of T and B cells were infiltrating in the liver allografts (Fig. 3b, panel C), the results show that multiple doses of CpG ODN induce lymphocyte infiltration in the liver allografts and elicit acute liver allograft rejection with a reduced Treg subset.

CpG ODN stimulates NF- κ B activation

TLR activates the TLR downstream transcriptional factor, NF- κ B, that is a rapid response transcription factor for genes whose products are critical for the inflammatory reaction and the alloimmune response (31,32); however, direct evidence for NF- κ B activation in liver transplantation is lacking because of the absence of an effective method to test NF- κ B activity. The availability of genetically modified transgenic mice expressing luciferase under the control of NF- κ B promoter (*NF- κ B-luc*) provides a useful reporter-based assay system to analyze NF- κ B enhancer activity in response to a variety of inflammatory signals (23,25). The current results show that luciferase activity in both allogeneic and syngeneic liver grafts was elevated at day 1 post-transplantation and diminished at day 4 in NF- κ B promoter-driven luciferase transgenic C57BL/6 and Balb/c liver allografts (Fig. 5a). Luciferase activity in the liver syngeneic grafts with 16 hr preservation in cold (4°C) (UW) solution (Fig. 5b, B/C to B/C, LTx, IRI) was elevated up to 10 days, which is consistent with luciferase activity in the cardiac allografts (Fig. 5b, B6 to B/C, HTx).

To detect the role of CpG ODN on the activation NF- κ B in both syngeneic and allogeneic liver grafts, CpG ODN was injected at day 7 post-transplantation (referred to as day 0), and BLI was performed at 4 hr after each time injection of CpG ODN. Luciferase activity was elevated at day 0 after injection of CpG ODN and gradually diminished after 3 days (Fig. 5c, B/C to B6, LTx plus CpG $\times$ 1). To test NF- κ B activation in multiple doses TLR ligand-treated syngeneic and allogeneic liver grafts, CpG ODN (10 μ g per mouse) was injected at day 7 post-transplantation (referred to as day 0) for 3 times at days 0, 3, and 6, and BLI was performed at

4 hr after CpG ODN injection. Luciferase activity was elevated at each timepoint (Fig. 5c, B/C to B6, LTx plus CpG x 3). NF- κ B activation was also elevated in a high dose of CpG (50 μ g per mouse $\times$ 1)-treated syngeneic liver grafts; however, multiple low doses of CpG ODN could not significantly enhance NF- κ B activation (Fig. 5d, B6 to B6, LTx, CpGx3). These observations reveal the following: (i) NF- κ B activation in untreated liver allografts is lower than that in untreated cardiac allografts; (ii) liver IRI stimulates increased NF- κ B activation; and (iii) CpG ODN activates NF- κ B activation that triggers inflammatory reaction in both syngeneic and allogeneic liver grafts and induces liver allograft rejection.

DISCUSSION

We demonstrate that a single high dose of CpG ODN induces leukocyte infiltration, emboli, NF- κ B activation, and production of proinflammatory cytokines, including TNF α and IL-1 β in the liver grafts, which is identical to liver IRI. Multiple low doses of CpG ODN increase T cell infiltration in the liver allografts and IFN- γ producing cells in the intrahepatic infiltrating lymphocytes, which abrogates spontaneous tolerance to liver allografts.

The role of TLR-4 in the mediation of liver IRI has been defined (33–35); however, the well-defined molecular links between TLR-9 and liver IRI have not been established. It is now clear that bacterial DNA and synthetic CpG ODN are potent immunostimulatory agents. Both can bind TLR-9 to activate B cells, natural killer cells, and antigen presenting cells (APCs), including DCs, macrophages, and monocytes (36). After activation of TLR, MyD88, an adaptor, is recruited into the TLR complex that induces IL-1 receptor associated kinase (IRAK) and TNF receptor associated factor-6 (TRAF-6). IRAK and TRAF-6 dissociate and activate the intracellular transcriptional factor, NF- κ B (21,32), which has pleiotropic functions involving the up-regulation of multiple inflammatory and immunogenes to elicit alloimmune responses. The liver is located downstream of the intestine and exposed to bacteria and viruses containing TLR ligands, such as CpG DNA, which have the ability to trigger TLR activation in the liver and stimulate the cells of the innate and adaptive immune system. Our results show that a single high dose of CpG ODN stimulates a series of events involving leukocyte infiltration and thrombosis in the liver allografts. CpG ODN results in an increased macrophage population in liver infiltrating lymphocytes, compared to those of untreated mice in the early period post-transplantation. Stimulation of macrophages triggers activation of NF- κ B, which in turn up-regulates gene expression patterns involved in proinflammatory responses (37). Proinflammatory cytokines, including TNF α and IL-1 β , are increased after CpG ODN treatment in both allogeneic and syngeneic liver grafts (Western blot, data not shown). CpG-induced activation of macrophages can also promote the maturation of dendritic cells (DCs) and MHC class II expression (38,39). These cellular and molecular events may bridge liver ischemia reperfusion injury in the early period post-transplantation and subsequent alloimmune responses.

Liver allografts survive for more than 100 days without requirement for any immunosuppressive therapy. Donor matched 2nd heart allografts are accepted, and third party grafts are rejected, suggesting that donor specific tolerance is induced. The mechanisms of liver spontaneous tolerance are incompletely understood. Previous results have shown that a single TLR engagement is sufficient to prevent transplantation tolerance to cardiac allograft by anti-CD154 monoclonal antibody (mAb) and to elevate NF- κ B activation (21–23,40). The current data show that spontaneous tolerance to liver allograft is abrogated by a challenge of multiple doses of CpG ODN, which is consistent with previous reports that CpG ODN must be co-localized with the alloantigen, but not CpG ODN alone, in order to induce antigen-specific immune response (41,42).

Liver spontaneous tolerance is mediated by increased Tregs, and elimination of Tregs can prevent liver allograft tolerance in a liver transplantation mouse model (43). A recent report shows an important role of CpG ODN in regulation of Tregs that anti-CD154 and rapamycin increase the proportion of CD4⁺FoxP3⁺ cells among the alloreactive responder cells although this regimen could not enhance absolute Treg number (44). We indeed observe an increased CD4⁺FoxP3⁺ population in liver infiltrating lymphocytes in untreated liver allografts, compared to naive livers, and CpG ODN reduces the percentage of CD4⁺FoxP3⁺. CpG ODN has the capacity of enhancing release of IFN- γ resulting in functional polarization of innate and adaptive immune responses (45). The current results show that one dose and multiple doses of CpG ODN have induced increased IFN- γ producing cells that derive from CD4⁺ T cells as well as from non-T cells, such as macrophages. There is no significant increase of IL-17, and additional IL-6 and TGF- β s may be required for Th17 generation (44).

We have recently shown that NF- κ B activation is significantly increased in the cardiac allografts when luciferase transgenic mice are used as recipients as well as donors. Costimulation blockade inhibits luciferase activity, and CpG ODN enhances luciferase activity (23). NF- κ B activation is less severe in untreated liver allografts than that in cardiac allografts. A single high dose of CpG ODN induces increased NF- κ B activation in both allogeneic and syngeneic liver grafts, which is associated with the liver inflammatory reaction. Multiple doses of CpG ODN maintain luciferase activity at higher levels. To our knowledge, this is the first time for visualizing NF- κ B activation in liver allografts using BLI. These observations suggest that CpG ODN triggers the TLR downstream signaling pathway and induces liver inflammatory reaction and subsequent acute allograft rejection.

Mouse liver allograft is spontaneously accepted; however, the liver can display destructive immunologic processes, such as allograft rejection although the allograft rejection is less severe in liver allograft transplantation than other solid organs in the clinic. Determining exactly how the state of spontaneous tolerance arises and how it can be induced may make it possible to induce “operational” tolerance with a reduced immunosuppressant. The current study using a liver transplantation mouse model shows that liver allografts are spontaneously accepted, and donor-specific tolerance is induced. A high dose of CpG ODN induces early liver inflammatory reaction, and engagement of a single TLR with multiple low doses of CpG ODN elicits acute liver allograft rejection. Understanding these cellular and molecular mechanisms of liver inflammatory reaction and liver allograft rejection induced by CpG ODNs may result in the development of strategies to interfere with early liver graft failure and subsequent allograft rejection.

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ABBREVIATIONS

BLI	Bioluminescence imaging
MST	Mean survival time
NF- κ B	Nuclear transcriptional factor κ B
<i>NF-κB-luc</i>	Luciferase transgenic mice under the control of a NF- κ B promoter
and Tregs	Regulatory T cells
TLR	Toll-like receptor

and WT Wild-type

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Figure 1. Liver allografts were spontaneous accepted

Liver allografts (C57BL/6, B6, H-2b) survived for more 100 days without any immunosuppressant in C3H/HeJ (C3H, H-2k) mice. Donor matched 2nd cardiac allografts (C57BL/6, B6, H-2b) were accepted, and third party cardiac allografts (Balb/c, B/C, H-2d) were rejected.

Figure 2a

Figure 2b

Figure 2. Survival of allogeneic and syngeneic liver grafts in CpG ODN-treated mice

a) Survival of CpG ODN-treated mice engrafted with liver allografts. b) Survival of CpG ODN-treated mice engrafted with syngeneic liver grafts and CpG-treated naive mice. One dose of CpG ODN (50 μ g per mouse $\times$ 1, i. p.) and multiple doses of CpG ODN (10 μ g per mouse $\times$ 3, i. p.) were administered into recipients starting at d 7 post-transplantation (referred to as d 0) for 3 times (at d 3 and d 6). Naive mice were treated with one dose of CpG ODN (50 μ g or 100 μ g per mouse).

Figure 3a

Figure 3b

Figure 3. Histological examinations of the liver allografts

a) H&E staining of allogeneic and syngeneic liver grafts. Panel A shows an untreated liver allograft, panel B shows a liver allograft treated with 10µg CpG ODN, panel C shows a syngeneic liver graft (C57BL/6, H-2b) preserved in cold UW solution for 16hr and transplanted into a C57BL/6 recipient (H-2b), and panel D shows a liver allograft (H-2d) preserved in cold UW solution for 16hr and transplanted into a C57BL/6 recipient. Leukocyte infiltration and thrombosis were observed in panel B, C, and D. b) Immunofluorescence examinations. Panel A, naive liver; panel B, the liver allograft at 7 d without any treatment; panel C, CpG ODN-treated liver, 10µg per mouse x 3; and panel D, CpG ODN, 10µg per mouse x 1. The experiments were repeated three times.

Figure 4. Flow cytometry analyses of liver infiltrating lymphocytes

Balb/c liver allografts were transplanted into C57BL/6 mice. One dose (10 μ g per mouse $\times$ 1, LTx+CpGx1) or multiple doses of CpG ODN (10 μ g per mouse $\times$ 3, LTx+CpGx3) were administered into recipients at day 7 post-transplantation. Liver infiltrating lymphocytes were obtained at 4 hr after CpG ODN injection for lymphocyte subset analyses. The experiments were repeated three times.

Figure 5a

Figure 5b

Figure 5c

Figure 5d

Figure 5. Luciferase activities in NF-κB-luc liver and cardiac grafts

a) Luciferase activities in both syngeneic (B6 to B6 LTx) and allogeneic liver grafts (B/C to B6 LTx; and B6 to B/C LTx) at d1, d4, and d7 post-transplantation. b) Luciferase activities in both syngeneic liver grafts (B/C to B/C, LTx, IRI) preserved in UW solution for 16 hr and allogeneic cardiac grafts (B6 to B/C, HTx) at d1, d4, d7, and 10d post-transplantation. c) Luciferase activities in liver allografts treated with one dose of CpG ODN (10μg per mouse, B/C to B6, LTx, CpGx1) and multiple doses of CpG ODN (10μg per mouse, B/C to B6, LTx, CpGx3) starting at d7 post-transplantation. BLI was examined at 4hr after injection of CpG ODN. d) Luciferase activities in syngeneic liver grafts treated with one dose of CpG ODN (50μg per mouse, B6 to B6, LTx, CpGx1) and multiple doses of CpG ODN (10μg per mouse x 3, B6 to B6, LTx, CpGx3) starting at d7 post-transplantation. BLI was examined at 4hr after injection of CpG ODN. The experiments were repeated three to four times.