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Humoral immunity-mediated chronic rejection in liver transplantation is associated with predominant IL-10 expression

Renhua Wan[1], Liping Tang[1], Renfeng Shan[1], Linxiang Zeng[2], Haimin Chen[1], Lianghui Gao[1]

[1]Department of Hepatobiliary and Pancreatic Surgery and Center of Organ Transplantation, The First Affiliated Hospital of Nanchang University, Jiangxi, China, [2]Department of Respiratory, The Second Affiliated Hospital of Nanchang University, Jiangxi, China

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1. ABSTRACT

Chronic rejection is a major cause of graft dysfunction and retransplantation after liver allotransplantation. Recent studies have implicated humoral response in this chronic rejection reaction. However, the manner in which humoral response is activated has not been fully investigated. In the present study, we address this question using our previously established chronic allograft liver rejection model induced by low-dose immunosuppressive cyclosporine (CsA) following Dark Agouti (DA) to Brown Norway (BN) liver transplantation. High-level donor-specific antibodies (IgG1 isotype), C4d deposition and histological graft damage indicated the involvement of humoral rejection in this chronic rejection reaction. *In vitro* assay showed that alloantibodies from pre-sensitized BN recipients induced apoptosis of bile ductal cells isolated from donor livers and the production of pro-fibrosis factors (TGF-beta, PDGF and FGF). Statistical analysis showed that the serum level of IL-10 was positively correlated with that of donor-specific antibodies (IgG1 isotype). Blockade of IL-10 *in vivo* down-regulated the level of donor-specific antibodies and ameliorated the outcome of chronic rejection. This suggests that humoral response in chronic allograft liver rejection is associated with Th2 type cytokine IL-10 and that Th2 response might promote chronic rejection by inducing a humoral response.

2. INTRODUCTION

Chronic rejection is an important cause of liver allograft dysfunction (1), which is a great threat to patients after liver transplantation. Among the many factors that could lead to chronic rejection, the most critical one is the alloimmune response generated by foreign histocompatibility antigens (2-3). Despite much effort to control its incidence, chronic allograft liver rejection is still an obstacle for the long-term survival of liver recipients (4).

T cells are known to play a pivotal role in modulating the immune response. According to the pattern of cytokine production and effector function, $CD4^+$ T helper cells can be divided into at least two distinct subsets, Th1 and Th2 cells (5). Th1 cells produce interleukin (IL)-2, IL-3, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), whereas Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13. The Th1 response has been implicated in acute allograft rejection, whereas the Th2 response has been associated with long-term allograft survival in several experimental settings (6). For example, expression of Th2 cytokines, such as IL-4 and IL-10, are enhanced in long-surviving allografts with diminished levels of Th1 cytokines, IL-2 and IFN-γ (7). However, recent studies reported the predominant expression of the Th2 cytokines (IL-4 and IL-10) and Th2-regulated alloantibodies in

chronic cardiac allograft rejection, which suggested involvement of the Th2 response in the development of chronic rejection (8). Though this is consistent with the hypothesis that a Th2 response may be important to the initiation/maintenance of chronic rejection (6), it did not provide direct support for the role of the Th2 response in chronic rejection. Whether Th2 response actively plays a role in the development of chronic allograft liver rejection and the mechanism by which it induces this rejection remain unclear.

T cell response to allografts is dictated by two distinct pathways of recognition of foreign histocompatibility antigens: direct and indirect (8). The direct pathway includes the stimulation of T cells by donor antigen presenting cells (APCs), and is traditionally accepted as representing the driving force behind early acute graft rejection (9). The indirect pathway involves the recognition of donor antigen which has been processed and presented in peptide form in the context of self-MHC on the host's own APCs (10). Accumulating studies now indicate that indirect recognition is crucial to the onset of chronic rejection (6, 9, 11-15). It was hypothesized that $CD4^+$ Th2 cells that are preferentially induced by the indirect recognition of allogeneic histocompatibility antigens late in transplantation may play the most critical role in the initiation and/or maintenance of chronic allograft rejection (6). Illigens *et al.* have provided direct evidence for the first time that induction of an indirect $CD4^+$ Th2 cell alloresponse can induce *de novo* chronic rejection of a cardiac allograft (9). Other studies have shown that the indirect recognition pathway might play an important role in the production of alloantibodies (16-18) which are known to mediate the chronic rejection process (19-23).

For decades, studies have suggested that T cells are the central regulatory and effector cells in graft rejection (24), because skin allografts are not rejected in mice that lack T cells (25) or in humans who are depleted of T cells (26). In 1970, it was demonstrated that there was a strong correlation between the existence of stenotic arterial lesions of renal allografts (known as chronic allograft arteriopathy) and the presence of circulating donor-specific anti-HLA antibodies (27). However, this finding, together with only a few other similar studies, was confronted by skepticism about the importance of antibody-mediated rejection, because a mechanistic connection and direct link to allografted tissue was lacking: antibody and complement (deposition of C3) were not reliably detectable in grafts (24). In the 1990s, with the application of immunopathologic techniques to demonstrate a complement fragment, C4d, and solid phase detection of circulating donor-specific antibodies (DSA), the involvement of humoral rejection (or antibody-mediated rejection, AMR) in allograft dysfunction received increasing recognition (24, 28). The evidence also demonstrates that circulating HLA-specific alloantibodies are common in patients with long-term organ allografts (24). For example, DSA and the complement system were found to be associated with chronic rejection in 33 studies of kidney, heart, lung and liver allografts (29). Studies also show that DSA are mediators of the chronic rejection process (19-23). Although the diagnostic criteria of AMR after kidney or heart transplantation have already been established by a national conference held in the USA, criteria for liver transplantation have not (28). Nevertheless, a number of studies have revealed the involvement of DSA and C4d in chronic allograft liver rejection (30-31), whereas other studies have not (32-33). The question of whether humoral rejection actively participates in the development of chronic allograft liver rejection and the manner in which it is activated remain unclear.

Given the strong correlation of Th2 alloresponse and chronic rejection as well as the relationship between humoral and chronic rejection indicated above, there is a possibility that the manner in which Th2 response induces chronic rejection is by promoting the humoral alloresponse. Using our previously established low-dose-CsA-induced rat model of chronic allograft liver rejection, we investigated the possible relationship between Th2 response and humoral rejection. We demonstrated that the existence of high-level DSA and complement C4d deposition is common in chronic allograft liver rejection in rats, and that the levels of DSA are associated with predominant expression of Th2 cytokines, especially IL-10. This suggests that Th2 type cytokine IL-10 might promote chronic allograft liver rejection by inducing a humoral response.

3. MATERIALS AND METHODS

3.1. Experimental animals

Inbred Brown Norway (BN) and Dark Agouti (DA) rats were purchased from Shanghai Laboratory Animal Center (SLAC). Animals were kept in a specific pathogen-free facility in the animal center of the Shanghai Jiao Tong University School of Medicine (Shanghai, China). Animal care and use were in compliance with institutional guidelines.

3.2. Liver transplantation and anti-IL-10 mAb treatments

Liver grafting was performed using BN rats as recipients of DA donor livers. Male rats with donors of 250-300 g and recipients of 250-350 g were used in this study. Transplantation was performed as before (4), using the techniques described by Kamada and Calne (34) with additional rearterialization. Rats were assigned to one of three treatment groups (A, B and C). In group A (control), liver transplantation was performed from rat BN to BN (BN→BN) with low-dose CsA (1 mg/kg/d) treatment from transplantation to day 30 by subcutaneous injection. In group B, liver transplantation was performed from DA to BN (DA→BN) with no immunosuppression treatment. In group C, the rat strain combination was DA→BN with low-dose cyclosporine (CsA; 1 mg/kg/d) treatment from transplantation to day 30 by subcutaneous injection (4). All animals received ampicillin (200 mg/kg intramuscularly) on post-operative days 0, 1 and 2.

For anti-IL-10 mAb treatments, as described in a previous study (35), some of the recipient rats in group C

received 400 μg goat anti-rat IL-10 IgG (R & D SYSTEMS) intraperitoneally daily beginning the evening following transplantation. Control animals (group A) received a similar amount of preimmune IgG on the same dosing schedule.

Recipient rats were monitored for clinical signs of acute (36)/chronic (1) liver allograft rejection and survival, as previously described. Rats were sacrificed on post-operative days 7, 14, 30, 60 and 90, and specimens of livers were taken for histopathologic assessment of acute/chronic allograft liver rejection. Slides were coded without reference to rat type and prior treatment status and examined systematically by a pathologist. Sera were also collected for DSA analysis by flow cytometry (described below), pro-fibrosis genetic factor analysis by ELISA, and *in vitro* assay.

3.3. In vitro assay

For *in vitro* assay, bile ductal cells were isolated from BN and DA livers using the methods described in previous studies (37-38). Sera were collected from BN liver recipients in group A, B and C at post-operative day 14 (for group B) and day 90 (for group A and C) as described above. After co-culturing the sera with bile ductal cells, we determined cell apoptosis using the TUNEL assay.

3.4. Cytokine and pro-fibrosis genetic factor ELISA

Serum levels of cytokines (IL-2, IL-4, IL-10 and IFN-γ) in rat recipients and pro-fibrosis genetic factors in the sera (PDGF, TGF-β and FGF) were measured using the Rat IL-2/IL-4/IL-10/IFN-γ Quantikine ELISA Kit (R&D SYSTEMS), Rat PDGF-BB Quantikine ELISA kit (R&D SYSTEMS), Rat TGF-β1 Quantikine ELISA Kit (R&D SYSTEMS) and FGF-2 antibody (Santa Cruz) according to the manufacturers' instructions (R&D SYSTEMS).

3.5. Flow cytometry for donor-specific antibodies

To analyze the production of donor-specific antibodies, recipient sera were collected at post-operative days 7, 14, 30, 60 and 90 for determination of levels of IgG1 and IgM. Spleen cell suspensions from DA rats (1×10^7/ml) were prepared as described previously (39) and used as target antigens. 1×10^6 DA spleen cells were incubated with 10 μl of recipient sera for 30 min at 4 ^oC and then washed twice. To detect cell-bound donor-specific antibodies, cells were incubated with FITC mouse anti-rat IgGl and IgM (eBioscience) mAbs for 30 min at 4 ^oC. Dead cells were excluded by gating out low forward scatter/high propidium iodide-retaining cells. The data are expressed as a ratio of mean ± SD of the mean fluorescence channel of histogram per $l0^4$ cells analyzed.

3.6. C4d immunohistochemistry and scoring of C4d staining

Immunoperoxidase analyses were performed on snap frozen liver tissue obtained from sacrificed rats at post-operative days 14 (for group B) and 90 (for group A and C). Sections were fixed with acetone, dried and incubated for 30 min with rabbit anti-C4d polyclonal antibody (Abcam). After washing, samples were incubated with goat anti-rabbit IgG antibody (HRP) (Abcam). Peroxidase was detected with a 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories).

The slides were examined by one blinded observer. The blood vessels and the portal areas outside the blood vessels, the two sites previously showing positive C4d staining, were analyzed for C4d expression (30). The method for scoring of C4d staining was described in a previous study. Briefly, by semi-quantitating the intensity score (from 1 to 3) for C4d and counting the positive and negative portal fields and portal and central veins, total scores were intensity*proportion positive (30).

3.7. TUNEL assay

Apoptosis was assessed by detection of DNA fragmentation using a 3′ terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (APO-BrdU TUNEL Assay Kit; Invitrogen). Adherent cells were trypsinized into suspension and fixed with paraformaldehyde. Fixed cells were incubated with DNA-labeling solution containing TdT enzyme and BrdUTP for 60 min at 37^{o}C. Labeled cells were incubated with Alexa Fluor 488 dye-labeled anti-BrdU monoclonal antibody for 30 min at room temperature. A FACS device (BD) equipped with a 488 nm argon laser was used to quantify the number of apoptotic cells. Approximate fluorescence excitation and emission maxima were 495 nm and 519 nm respectively.

3.8. Statistical analysis

Results are shown as mean values ± standard deviations. Statistical differences were analyzed by Student t test and the log-rank test using SPSS 13.0 statistical software. The level of significance was set at $P < 0.05$.

4. RESULTS

4.1. Predominant expression of serum IL-10 in chronic allograft liver rejection

Cyclosporine (CsA) is an immunosuppressive drug used in clinical organ transplantation (40). IL-2, IFN-γ, IL-4 and other cytokine gene transcription was inhibited by CsA treatment (10 mg/kg/day), but IL-10 transcription was not affected (40). Using low-dose CsA (1 mg/kg/d) after liver transplantation as previously mentioned, we induced the chronic allograft liver rejection model 4).

Survival analysis showed significant differences between the three groups (Fig.1A). In the untreated DA→BN group, irreversible acute rejection developed and all BN rats died within 15 days (Fig.1A). In the low-dose CsA treated DA→BN group, all BN rats that survived more than 30 days developed moderate to severe manifestations of chronic liver rejection, with bile duct dystrophy or loss, obliterative arteriopathy, and liver fibrosis (4). The bile duct dystrophy or loss was progressive in chronic liver rejection (Fig.1B), and was consistent with the diagnostic criteria proposed for chronic liver rejection (1). No histological alterations were observed in the low-dose CsA treated BN→BN group, and all rats survived over 90 days (Fig.1A).

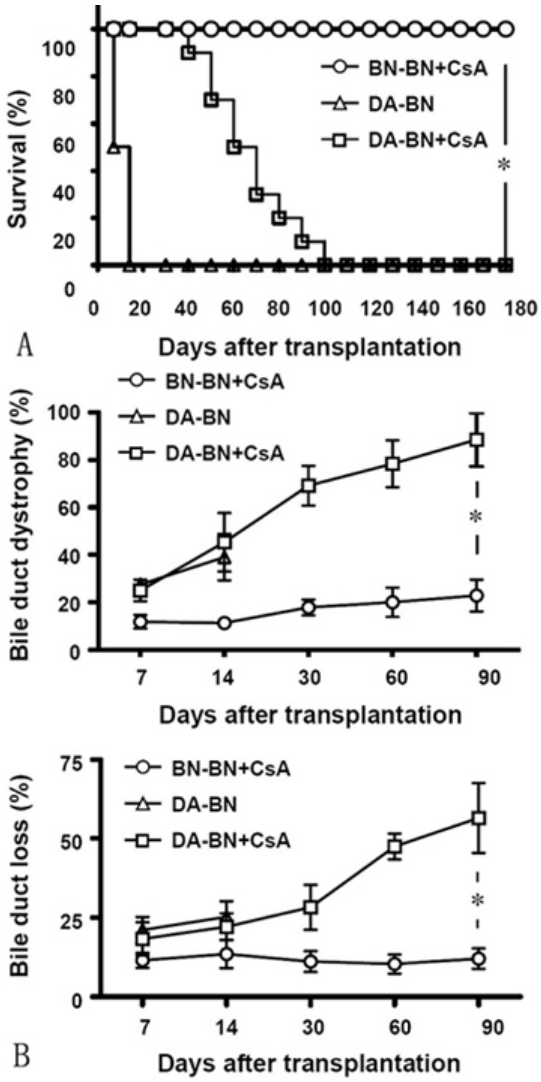

Figure 1. Establishment of the rat model of chronic allograft liver rejection. Liver transplantation was performed in 3 different groups: BN→BN with immunosuppressive CsA treatment (1 mg/kg/day), DA→BN with no additional treatment; DA→BN with immunosuppressive CsA treatment (1 mg/kg/day). A. The survival of liver recipients was monitored over time after transplantation. B. Bile duct dystrophy (BDD) and bile duct loss (BDL) were evaluated in liver slides. Each point was based upon a pool of 6 livers obtained from the recipients. The data represent mean value +/- SD of BDD and BDL of recipients from 3 independent experiments.

Whether Th2 response induces immune tolerance (41) or chronic rejection (8) in allograft transplantation remains controversial. Previous study has demonstrated the predominant expression of Th2 cytokines and the presence of Th2-regulated alloantibodies in chronic allograft heart rejection (8). To test whether chronic rejection in liver transplantation is associated with preferential activation of the Th2 response, we investigated allografts with acute or chronic rejection for systemic cytokine expression at the protein level. There were relatively high levels of IL-10 (117.9 pg/ml) (Fig.2A) and relatively moderate levels of IL-4 (19.5 pg/ml) (Fig.2B) in sera of allograft recipients with chronic rejection by day 30 following transplantation. IL-10 and IL-4 were first detected in sera of allograft recipients with chronic rejection by day 7 following transplantation, gradually increasing to peak levels on days 60-90 (Fig.2A, B). In contrast, sera from recipients with acute graft rejection had low levels of IL-10 and IL-4 (Fig.2A, B), but had relatively high levels of IL-2 (90.0 pg/ml) and IFN-γ (201.5 pg/ml) on day 7 following transplantation (Fig.2C, D). Systemic expression of IL-2, IFN-γ, IL-4 and IL-10 were low and stable in syngeneic recipients (BN→BN), regardless of the time of serum harvest (Fig.2).

4.2. Involvement of donor-specific antibodies and C4d deposition in chronic allograft liver rejection

Th2 cells are known to dictate the induction and regulation of humoral immunity. The Th2-type cytokine IL-10 regulates B cells for differentiation and production of antibody (42). The predominant expression of Th2 cytokines in allografts with chronic rejection, therefore, led us to investigate the humoral responses in this chronic allograft liver rejection model. Because circulating DSA is a major cause of humoral rejection, the detection of DSA in sera would be strong evidence for the presence of humoral rejection after transplantation. Also, studies have demonstrated the presence of Th2-regulated alloantibodies IgG1 and IgG2a in chronic cardiac allograft rejection in the rat (8). After collecting serum from allograft recipients at various time points, we examined the levels of donor-specific antibodies in serum samples. In rats with chronic rejection, high levels of IgG1 and relatively lower levels of IgM alloantibodies were detected, with IgG1 increasing progressively and reaching peak levels at post-operative days 60-90 (Fig.3A). Rats with acute rejection had high levels of IgM and lower levels of IgG1 at post-operative days 7 and 14. Rats with syngeneic grafts developed no obvious fluctuations in levels of IgG1 and IgM alloantibodies.

The complement split C4d is also a good indicator of antibody-mediated rejection and has been used as one of the diagnostic markers for humoral rejection after kidney or heart transplantation (28). Previous study has shown that, in liver allografts with chronic rejection, vascular deposition of complement C4d is increased (30). Therefore, in this study, we investigated the graft liver for C4d deposition. In the two structures studied (vessels and portal stroma), there was more C4d deposition in the livers from recipients with chronic rejection than in livers from recipients with acute rejection or from syngeneic recipients (Fig.3E). C4d positivity in the portal veins, central veins and stroma of the portal fields between the vascular structures were reproducible and clear.

Despite donor-specific alloantibody, we also detected high expression of pro-fibrosis genetic factors, such as TGF-β (Fig.3D), PDGF (Fig.3F) and FGF-2 (Fig.3C), in the sera of recipients with chronic rejection. Those pro-fibrosis genetic factors have been implicated in

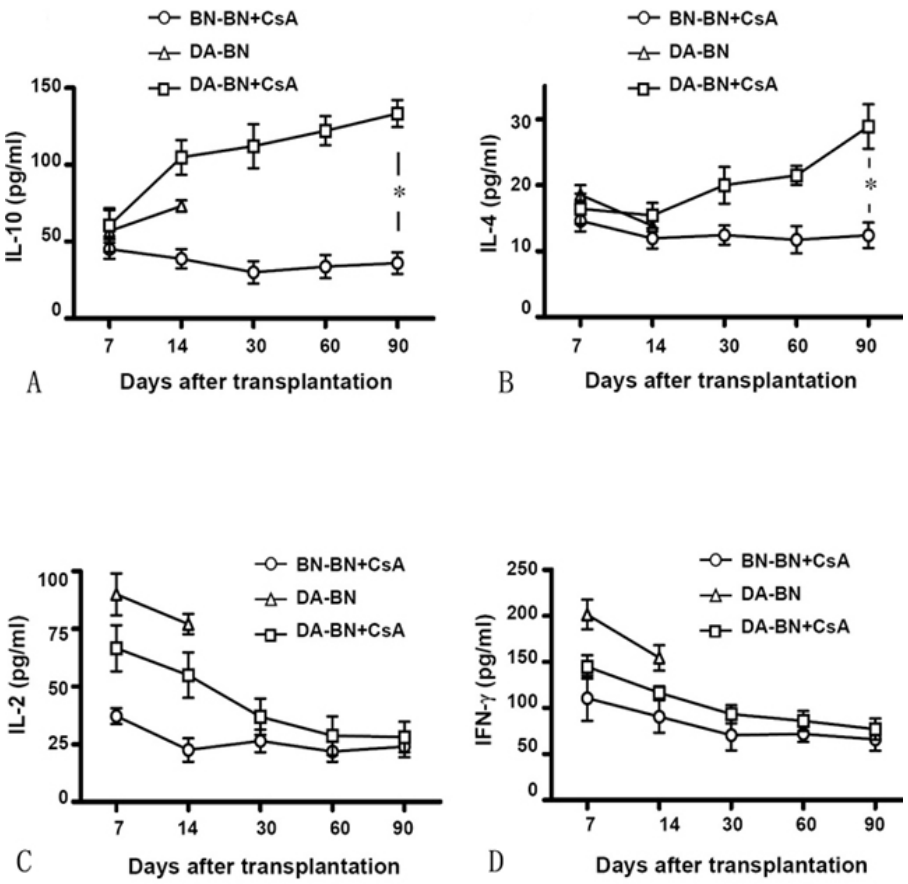

Figure 2. Predominant expression of IL-10 in chronic allograft liver rejection. Serum samples from liver recipients with acute rejection or chronic rejection, and syngeneic recipients were collected at post-operative days 7, 14, 30, 60 and 90. Levels of IL-10 (A), IL-4 (B), IL-2 (C) and IFN-γ (D) were analyzed using ELISA. The data are representative of three-independent experiments. Asterisk represents p value less than 0.05.

smooth muscle cell hyperplasia in chronic liver rejection induced by alloantibodies (6).

4.3. Donor-specific antibody induced apoptosis of donor liver cells and expression of pro-fibrosis factors *in vitro*

To investigate the effect of humoral alloresponse on donor cells, we used an *in vitro* assay to test whether sera from recipients would have an effect on cells from donor livers. After culture of bile ductal cells from donor DA livers with sera from recipient BN rats (with chronic or acute rejection), bile ductal cells underwent apoptosis (Figure 4). Significant apoptosis was observed in the co-culture with sera from recipient rats suffering chronic rejection, compared with that of acute rejection.

4.4. Donor-specific antibody level is positively correlated with IL-10 expression in chronic allograft liver rejection

Given the predominant Th2 response (especially Th2 cytokine IL-10) and humoral rejection detected in chronic allograft liver rejection, we then examined whether they correlated with each other. In the chronic allograft liver rejection model, the expression of Th2 cytokine IL-10 and alloantibody IgG1 increased progressively after transplantation (Figure 5). Moreover, the expression of IL-10 preceded that of DSA, suggesting that it was the increase in IL-10 that induced the promoted expression of DSA in sera (Figure 5).

4.5. Humoral rejection is inhibited upon IL-10 blockade

The predominant expression of IL-10 in chronic allograft liver rejection and the correlation between IL-10 and DSA prompted us to investigate the role of IL-10 further. Using anti-IL-10 mAb, endogenous IL-10 was blocked. In the low-dose CsA treated DA→BN group, recipient rats receiving control IgG survived for approximately 90 days; rats receiving anti-IL-10 showed a decelerated rejection process and some recipients survived for over 180 days (Figure 6A). Furthermore, in the low-dose CsA treated DA→BN group, recipient rats receiving anti-IL-10 showed significantly reduced levels of DSA (IgG1) as compared with rats receiving control IgG (Figure 6B).

5. DISCUSSION

In this study, based on a low-dose-CsA-induced rat model of chronic allograft liver rejection, we

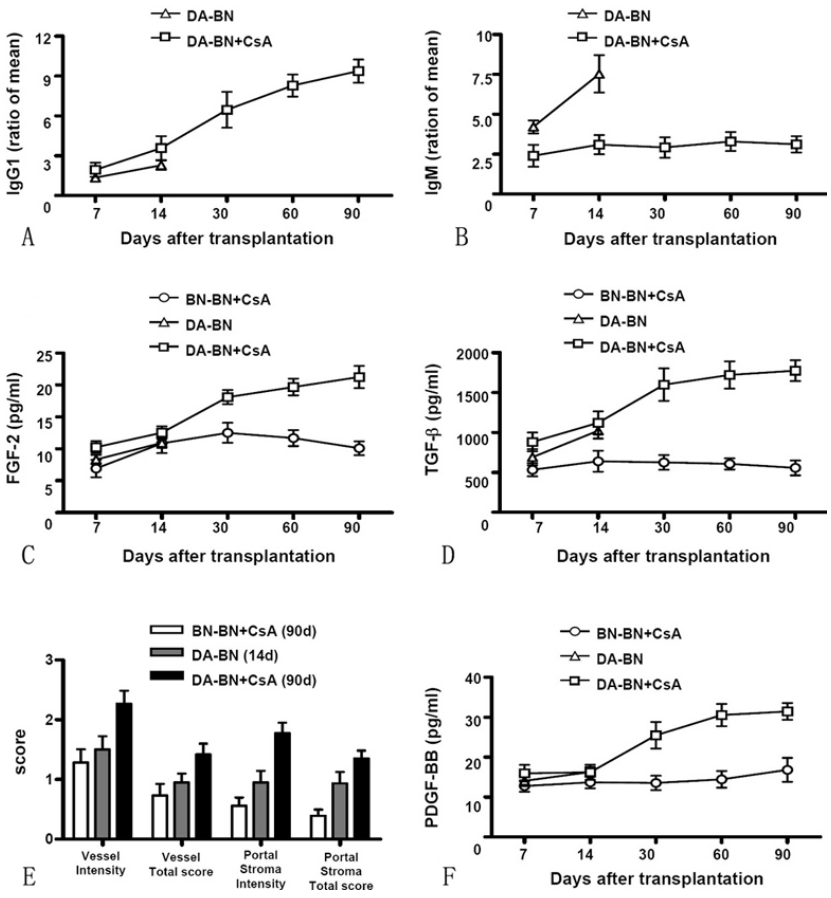

Figure 3. Detection of donor-specific antibodies and C4d deposition in chronic allograft liver rejection. A. Serum samples were collected at post-operative days 7, 14, 30, 60 and 90, and were analyzed using flow cytometry for donor-specific antibodies. Donor-type spleen cells were incubated with sera of recipient rats followed by FITC anti-rat IgG1 and IgM mAbs. Mean fluorescence intensity (MFI) of IgG1 and IgM in BN→BN rats with CsA treatment were used as control (=1). B. Frozen sections were stained with anti-C4d antibody. The intensity of C4d staining was scored in a blinded fashion from 0 to 3. Total scores for vessels and portal fields are given as products: staining intensity*proportion of positive vessels/all vessels, and staining intensity*proportion of positive portal fields/all portal fields (30). C. Serum levels of pro-fibrosis genetic factors (TGF-β, PDGF-BB and FGF-2) were analyzed by ELISA.

demonstrated the prevalence of DSA in the sera and C4d deposition in the graft liver during chronic rejection. In addition, we demonstrated high levels of Th2 cytokines, especially IL-10, and relatively lower levels of Th1 cytokines, IL-2 and IFN-γ in our model of chronic rejection. Levels of DSA (IgG1) in chronic allograft liver rejection were associated with that of Th2 cytokines, especially IL-10. Blockade of IL-10 reduced the levels of DSA (IgG1), and decelerated the chronic rejection process. Thus, this study suggests that Th2 response might play an important role in the development of chronic allograft liver rejection through regulation of the humoral response.

The liver has been traditionally considered resistant to antibody-mediated rejection (43). Postulated reasons for resistance include a large vascular bed, with a dual supply able to absorb antibody and complement, and secretion of soluble HLA molecules (43). However, previous studies reported the detection of antibody-mediated rejection in ABO incompatible and ABO compatible liver transplantation (44-46). DSA and the complement system were found to be associated with chronic rejection in 33 studies of kidney, heart, lung and liver allografts (29). Studies also show that DSA are mediators of the chronic rejection process (19-23). In our rat model of chronic allograft liver rejection, we demonstrated serological and histological signs of humoral rejection. We observed graft liver injury, high-level circulating DSA (IgG1), and C4d deposition in the allograft liver. Our detection of humoral rejection in chronic allograft liver rejection reminded us of Terasaki's humoral theory of transplantation, which proposes that chronic rejection is caused by alloantibodies (47). The primary effect of alloantibodies is endothelium injury, which includes a complement-dependent pathway (membrane attack complex formation, recruitment of inflammatory cells, and complement-complement receptor-mediated phagocytosis) and complement independent pathway antibody-dependent cell cytotoxicity (48). Secondary to endothelium injury, the following pathological reactions

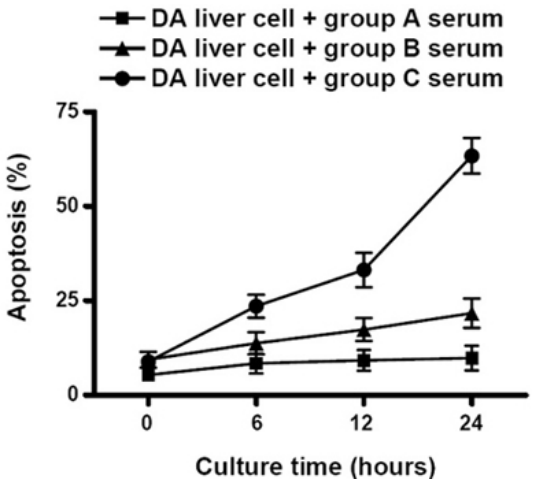

Figure 4. Donor-specific antibody induced apoptosis of donor liver cells *in vitro*. Bile ductal cells isolated from BN and DA livers were co-cultured with sera collected from BN liver recipients in group A, B and C. Apoptosis was analyzed by TUNEL assay on a flow cytometer.

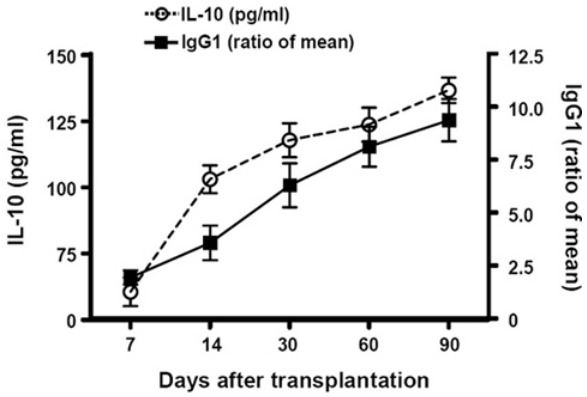

Figure 5. Correlation between donor-specific antibody and IL-10 in chronic allograft liver rejection.

are found to be responsible for progressive tissue injury and final graft function loss: platelet activation and thrombosis, pathological smooth muscle and endothelial cell proliferation, and humoral and/or cellular infiltrate-mediated parenchyma damage after endothelium injury (48). In our *in vivo* assay, the expression of pro-fibrosis genetic factors (including TGF-β, PDGF and FGF) in the sera of the recipients was increased in chronic rejection. In our *in vitro* assay, we demonstrated that sera from DA recipients induced apoptosis of cultured bile ductal cells from donor livers. These data showed that the effects of sera from DA recipients with chronic rejection on donor cells were similar to the proposed effects of donor-specific alloantibodies on donor cells (48).

In our liver transplantation model, the predominant expression of Th2 cytokine IL-10 correlated with high-level DSA and IgG1 isotype. Upon IL-10 blockade, DSA level decreased. Our data are consistent with a study demonstrating the predominant expression of IL-10 as well as the presence of IgG1 and IgG2a antibody isotypes in chronic cardiac allograft rejection (8). It is well known that, during B2 lymphocyte maturation, T cells help B cells to produce antibody against T cell-dependent antigens and to switch the isotype of the antibody produced from IgM to other isotypes, including IgG. $CD4^+$ T cells can be divided into Th1 and Th2 subsets according to their pattern of cytokine production and effector function (5). Th2 cells are known to dictate the induction and regulation of humoral immunity. The Th2-type cytokine IL-10 regulates B cells in differentiation and production of antibody (42). Some *in vitro* studies have shown the inhibitory effects of IL-10 on Th1 cytokine synthesis, APC function or cell-mediated immunity (39). A study has also demonstrated the early emergence of alloantibody and accumulation of IL-10 mRNA in chronic allograft heart rejection, but the role of IL-10 in the model studied was not clear (39). Here, we propose that, in our model of chronic allograft liver rejection, IL-10 contributes to humoral rejection by regulating B cell maturation and production of antibody. In this current study, we did not detect the predominant expression of another Th2 type cytokine, IL-4, in chronic allograft liver rejection sera samples. This observation is consistent with the study which detected high levels of IL-10 but not IL-4 in chronic cardiac rejection (8). To explain this phenomenon, further work is needed.

Not only is the exact role of Th2 response in humoral rejection undetermined, but also the role of Th2 response in chronic rejection. Whether Th2 response induces immune tolerance (41) or chronic rejection (8) in allograft transplantation remains controversial. Our data demonstrate the predominant expression of Th2 response in chronic rejection, and that chronic rejection could be ameliorated upon blockade of IL-10. In one study, the administration of IL-10 to heart allograft recipients resulted in increased levels of Th2 cytokines and accelerated chronic rejection (49). In another study, chronic allograft aortic rejection was prevented by the treatment of graft recipients with anti-IL-4 antibodies (50). As discussed above, Th2 response may promote humoral response in chronic allograft liver rejection. The Th2 response may initiate and/or perpetuate the process of chronic rejection by regulating the production of specific alloantibodies (8). Therefore, Th2 response might contribute to chronic allograft liver rejection by promoting humoral response.

A recent study on heart transplantation has demonstrated for the first time that induction of an indirect $CD4^+$ Th2 response can induce *de novo* chronic rejection(9). It is in agreement with previous data which reported the accelerated course of chronic allograft vasculopathy in heart-transplanted miniature swine upon injection of donor MHC class I peptides (51). Therefore, it is reasonable to propose that, in our chronic allograft liver transplantation model, $CD4^+$ Th2 responses in chronic rejection might also be activated through an indirect recognition pathway. As discussed above, Th2 response might play a critical role in chronic rejection by regulating humoral alloresponse. Thus, we hypothesize that $CD4^+$ Th2 cells that are activated by an indirect recognition pathway play a critical role in the development of humoral rejection

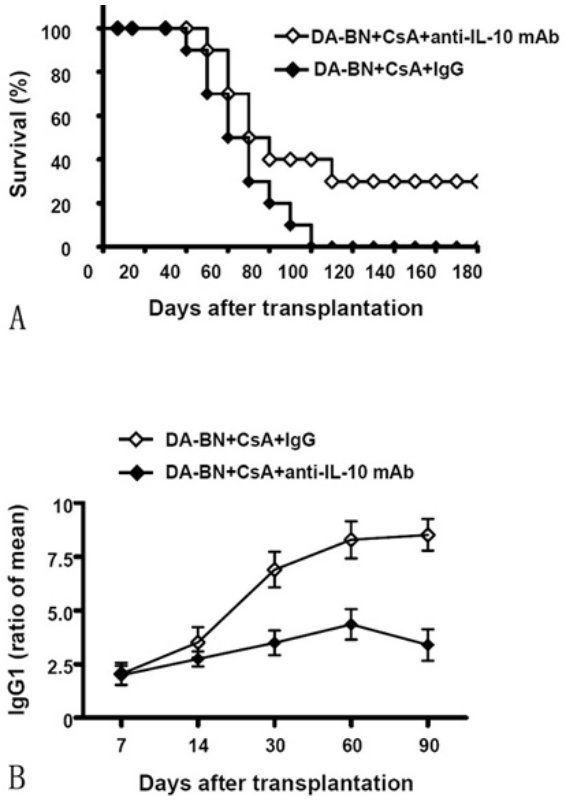

Figure 6. IL-10 blockade reduced levels of donor-specific antibodies in sera and decelerated the chronic rejection process. Part of the recipient rats in group C received 400 μg anti-IL-10 IgG intraperitoneally daily beginning the evening following transplantation. Control animals in group C received a similar amount of preimmune IgG on the same dosing schedule. A. The survival of liver recipients with antibody treatment was monitored over time after transplantation. B. Serum samples were collected at post-operative days 7, 14, 30, 60 and 90, and were analyzed through flow cytometry for donor-specific antibodies. Donor-type spleen cells were incubated with sera of recipient rats followed by FITC-conjugated anti-rat IgG1 mAbs. IgG1 of BN-BN rats with IgG treatment was used as control (=1).

in chronic allograft liver rejection. Further investigation into the indirect T cell response in chronic allograft liver rejection will be important for understanding the mechanism of the development of humoral alloresponse after liver transplantation and is greatly needed. Furthermore, a deeper understanding of chronic allograft liver rejection will shed light on the development of better treatments targeting chronic rejection.

In summary, our data provide evidence for a link between humoral rejection and Th2 response in a chronic allograft liver rejection model. We demonstrate that, after liver transplantation, a predominant Th2-type cytokine IL-10 expression correlates with humoral response which plays a role in the development of chronic rejection. Given the importance of Th2 cytokines in regulating humoral immunity, our findings shed light on the pathophysiology of chronic rejection in liver recipients.

6. ACKNOWLEDGEMENTS

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Abbreviations: AMR: antibody-mediated rejection, APC: donor antigen presenting cell, BN: Brown Norway, CsA: cyclosporine, DA: Dark Agouti, DSA: donor-specific antibody, PDGF: platelet derivative growth factor, FGF: fibroblast growth factor, IFN-γ: interferon-γ, IL: interleukin, TGF-β: transforming growth factor – beta, TNF-α: tumor necrosis factor-α

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Send correspondence to: Lianghui Gao, Department of Hepatobiliary and Pancreatic Surgery, Center of Organ Transplantation, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Nanchang City, Jiangxi 330006, China, Tel: 86-791-8694129, Fax: 86-791-8695112, E-mail: lh_gao@163.com

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