Hepatitis

Periportal and sinusoidal liver dendritic cells suppressing T helper type 1-mediated hepatitis

Tomohiro Watanabe, Hiroaki Katsukura, Tsutomu Chiba, Toru Kita, Yoshio Wakatsuki

Background: Recently, we found that portal vein tolerance is associated with generation of Th2 cells and apoptosis of Th1 cells in the liver, which is regulated by antigen (Ag)-presenting dendritic cells (DCs) in the periportal area and sinusoids.

Aim: In this study, we tested whether the periportal and sinusoidal DCs, which were loaded with an Ag in vivo, can inhibit liver injury caused by Th1 cells activated by the Ag administered systemically.

Methods: Ag-specific hepatitis model was created by adoptively transferring ovalbumin (OVA)-specific CD4+ T cells to BALB/c mice and venous injection of OVA-containing liposomes. Liver CD11c+ cells obtained from mice fed OVA were then transferred into these mice.

Results: The transfer of liver CD11c+ cells from OVA-fed mice completely inhibited hepatic injury, which was associated with apoptosis of OVA-specific CD4+ T cells and emergence of Th2 cells in the liver. Transfer of CD11c+ cells and subcutaneous OVA challenge led to enhancement of OVA-specific IgE Ab as well as Th2 cytokine responses in the recipient mice.

Conclusions: Periportal and sinusoidal DCs loaded with an Ag in the portal vein can induce Th2 response in the liver and prevent hepatic injury caused by Th1 cells.

Methods

Animals and protocol for immunisation

DO11.10 mice with T cells bearing the transgenic T cell receptor (TCR) that recognises the 323–339 peptide fragment of ovalbumin (OVA) in the context of IAd were crossed to Rag2−/− mice. BALB/c and Rag2−/−DO11.10 mice were housed under specific pathogen free conditions in the Animal Facility of Kyoto University. Male BALB/c mice were fed 100mg of OVA (Sigma Chemical Co., St Louis, Missouri, USA) or PBS alone, every other day for a total of five times by intragastric intubation. All animal experiments were performed in accordance with institutional guidelines and ethical permission for this study was granted by the review board of Kyoto University.

Histological analysis

Liver sections were stained with biotinylated anti-CD11c (Pharmingen, San Diego, California, USA), anti-IAd (Pharmingen), and anti-F4/80 Ab (Serotec Ltd, Oxford, England) as described previously. For the detection of apoptotic hepatocytes, TdT-mediated dUTP nick-end labelling (TUNEL)-staining was performed using a commercial kit (apoTACS-DAB, Trevigen, Gaithersburg, Maryland, USA).

Preparation of mononuclear cells from the spleen, the lymph nodes, and the liver

Lymphocytes from the spleen and draining lymph nodes (dLNs) were prepared as previously described. Intrahepatic lymphocytes (IHLs) were prepared following the method

Abbreviations: Ag, antigen; APC, antigen presenting cell; LSEC, liver sinusoidal endothelial cell; DC, dendritic cell; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; TUNEL, TdT-mediated dUTP nick-end labelling; IHL, intrahepatic lymphocyte; PI, propidium iodide; AST, aspartate aminotransferase; ALT, alanine aminotransferase; dLN, draining lymph nodes
described previously.\textsuperscript{16} Hepatic low density non-parenchymal cells were also obtained as described previously.\textsuperscript{8}

**Adoptive transfer of CD11c\textsuperscript{+} cells to naive BALB/c mice**

Splenic and hepatic CD11c\textsuperscript{+} cells were purified by positive selection using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.\textsuperscript{8} The recovered cells were more than 90% CD11c\textsuperscript{+} (confirmed by flow cytometric analysis). Six-week old BALB/c mice were injected intravenously with $5 \times 10^5$ CD11c\textsuperscript{+} cells purified from the spleen or the liver of BALB/c mice fed OVA or PBS. The next day after the transfer, the recipient mice were immunised subcutaneously with complete Freund's adjuvant (CFA, GIBCO BRL, Grand Island, New York, USA) and OVA (1mg/ml). OVA-specific IgG, IgG1, IgG2a, IgE Ab were measured by ELISA as described elsewhere.\textsuperscript{18}

Figure 1 Characterisation of CD11c\textsuperscript{+} cells in the liver of BALB/c mice fed OVA. BALB/c mice were fed 100mg of OVA or PBS every other day for a total of five times and killed 3 days after the fifth feeding. (A) Localisation of CD11c\textsuperscript{+}, IAd\textsuperscript{+}, F4/80\textsuperscript{+} cells in the liver. Frozen liver sections were stained with anti-CD11c mAb, anti-IAd mAb, or anti-F4/80 mAb. (B) Flow-cytometric analysis of non-parenchymal low density cells in the liver. Cells were stained with PE-conjugated anti-CD11c mAb and biotin-conjugated anti-F4/80 mAb followed by incubation with streptavidin-FITC. Dead cells were excluded by propidium iodide (PI) staining. The number shows the percentage of cells in each region. (C) CD11c\textsuperscript{+} cells are isolated from the liver of mice fed PBS or OVA. These cells were stained with PE-conjugated control Ab, anti-IAd mAb, anti-CD80 mAb, and anti-CD86 mAb. The results shown are representative one of two experiments ($n = 4$ in each group).
Induction of Ag-specific hepatic injury
Six-week old BALB/c mice were injected intravenously with 5×10⁶ CD11c⁺ cells purified from the liver of BALB/c mice fed OVA or PBS as described above. OVA-specific liver injury using OVA-liposome was induced in these recipient mice as reported previously with some modifications. Briefly, splenocytes from Rag²−/−DO11.10 mice were stimulated for 3 days with OVA123–339 peptide (1µg/ml) and then activated OVA-specific CD4⁺ T cells were purified by positive selection using autoMACS (>90% KJ1-26, confirmed by flowcytometric analysis). BALB/c mice that received CD11c⁺ cells were treated with intravenous injection of OVA-containing liposome (15mg, NOF corporation, Tokyo, Japan) followed by the transfer of activated splenic CD4⁺KJ1-26⁺ T cells 2 hours later (2.5×10⁶/mouse). Mice were killed 16 hours later and then serum, IHLs, and splenocytes were prepared. The liver injury was assessed by serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities by a commercial kit (Wako, Osaka, Japan).

Stimulation of draining lymph nodes (dLN)-cells, spleen cells, and IHLs
CD4⁺ T cells were purified from the spleen, dLN, and the liver by positive selection using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). These CD4⁺ T cells (1×10⁷/well) were stimulated in vitro with OVA (0.1mg/ml) or OVA-peptide (1µg/ml) presented by irradiated splenocytes (5×10⁷/well) from BALB/c mice. The cultures were incubated for 72 hours and 1µCi of [³H] thymidine was added for the final 16 hours. Proliferation and cytokine production were evaluated as described previously. In some experiments, splenic OVA-specific CD4⁺ T cells (1×10⁷/well) were stimulated with OVA-peptide (1µg/ml) presented by hepatic CD11c⁺ cells (1×10⁷/well) in the presence of mouse IL12 (10ng/ml, Peprotec), rat IgG (10µg/ml, Pharmingen), or neutralising anti-IL12p40 mAb (10µg/ml, Pharmingen).

mAbs and flow cytometry
FITC-conjugated KJ1-26 recognising transgenic TCR specific to OVA and biotin-conjugated F4/80 mAb were purchased from Caltag (San Francisco, California, USA). FITC or PE-conjugated anti-mouse CD11c mAb, PE-conjugated anti-mouse CD4, CD14, CD80, CD86 mAb and biotin-conjugated Annexin V were purchased from BD Biosciences. FITC-conjugated KJ1-26 recognising transgenic TCR specific to OVA were used as control, and the Ab titre equivalent to 2¹⁰ dilution of immune sera was defined as one arbitrary unit (AU). Proliferative responses, IFN-γ and IL4 secretion by dLN-CD4⁺ T cells were measured 1 day after the subcutaneous immunisation. CD4⁺ T cells (1×10⁵/well) were stimulated by OVA (0.1mg/ml) presented by irradiated splenocytes (5×10⁶/well) from BALB/c mice. For proliferative responses, the cultures were incubated for 72 hours and 1µCi of [³H] thymidine was added for the final 16 hours. For cytokine analysis, culture supernatants were collected at 48 hours for IFN-γ production and at 72 hours for IL4 production. The results are expressed as mean±SD. *p<0.05, **p<0.01 versus PBS control. The results shown are representative one of two independent experiments (n=4 in each group).

RESULTS
Activation of liver DCs upon oral administration of an Ag
As our previous study indicated that a part of OVA administered orally is carried to the liver and co-localise with class II (IAd)⁺ cells in the periportal area, we analysed tissue localisation of DCs in the liver by staining class II and CD11c antigens (fig 1A). Class II⁺ and CD11c⁺ cells were mainly localised in the periportal area of the liver of PBS-fed mice. In mice fed OVA, numbers of class II⁺ and CD11c⁺ cells increased not only in the periportal area but also in the sinusoidal area. No increase was seen in F4/80⁺ Kupffer cells that localised mainly sinusoidal area. To analyse quantitatively and to discriminate between DCs and Kupffer cells, we did dual staining flow-cytometric analysis by using hepatic low density...
non-parenchymal cells (fig 1B). Most CD11c+ cells were negative for F4/80 staining, suggesting that these CD11c+ cells are DCs rather than Kupffer cells. Consistent with the results of tissue staining, the percentage of CD11c+ cells was markedly increased in the liver of OVA-fed mice. As shown in fig 1C, the percentages of CD11c+ DCs expressing class II, CD80, and CD86 were all increased in the liver by oral administration of OVA. Thus, these flow-cytometric and immunohistochemical analyses indicated that OVA feeding associates with activation of DCs in the liver.

Systemic Th2 response elicited by liver DCs

Next, we assessed the functional properties of in vivo Ag-loaded CD11c+ cells by adoptively transferring them into naïve recipient mice. For this purpose, hepatic or splenic CD11c+ cells from donor BALB/c mice fed OVA or PBS were transferred into the recipient BALB/c mice, which were immunised subcutaneously with OVA/CD4+ T cells isolated from mice fed PBS, BSA, or OVA. A) Cells were cultured for 48 hours and the percentage of Annexin V+ cells in OVA-specific CD4+KJ1-26+ T cells was determined. Cells were stained with FITC-conjugated Annexin V and PE-conjugated KJ1-26 mAb. Analysis gate was set on KJ1-26+ cells. The number in each panel shows the percentage of Annexin V+ cells. (B) Production of IFN-γ and IL4 by OVA-specific CD4+ T cells. Culture supernatants were collected at 48 hours for IFN-γ production and at 72 hours for IL4 production. (C, D) Effects of IL12 signalling on induction of apoptosis and generation of Th2 responses by hepatic CD11c+ cells. Anti-IL-12p40 mAb (10μg/ml), control Ab (10μg/ml), or mouse IL12 (10ng/ml) were added to the culture. The percentage of Annexin V+ cells in OVA-specific CD4+KJ1-26+ T cells (C) and production of IFN-γ and IL4 (D) were determined as described above. *p<0.05, **p<0.01 versus PBS control (B) or Control Ab (D). The results shown are representative one of two independent experiments (n = 3 in each group).

A mechanism of Th2 differentiation by liver DCs

As we knew that liver CD11c+ cells loaded an Ag in vivo can induce Th2 differentiation of naïve CD4+ T cells by Fas-mediated apoptosis of Th1 cells, we assessed whether Ag-specific killing was occurring in our model. For this purpose, hepatic CD11c+ cells isolated from mice fed either OVA or PBS did not alter cytokine or Ab responses. Taken together, CD11c+ DCs in the liver, not in the spleen, of mice administered OVA orally were sufficient to transfer Th2 response to OVA in the recipient mice.
Liver dendritic cells (DCs) involve Ag-presentation and apoptosis of Ag-specific CD4+ T cells.

To assess how the above phenomenon relates to the property of DCs, which are deficient in IL12 secretion and cause apoptosis,8 we neutralised and restored IL12 signalling by anti-IL12p40 mAb and recombinant IL12, respectively. The percentage of Annexin V+ OVA-specific CD4+ T cells and IL4 production were markedly increased when anti-IL12 mAb was added to the culture containing liver DCs of PBS-fed mice (fig 3C, D). In contrast, the percentage of apoptotic cells and IL4 production were decreased when IL12 was added to the culture containing liver DCs of OVA-fed mice. Thus, blockade of IL12 signalling in liver DCs of PBS-fed mice had Th2-inducing function similar to that of DCs of OVA-fed mice. Conversely, restoration of IL12 signalling in liver DCs of OVA-fed mice abrogated Th2 induction. Taken together, these data suggest that deficiency in IL12 secretion by liver DCs of OVA-fed mice is responsible for Th2 differentiation and apoptosis of naïve OVA-specific CD4+ T cells.

**Inhibition of hepatitis by adoptive transfer of liver DCs**

In our final series of experiments, we assessed the in vivo function of liver DCs in the OVA-specific hepatitis model which utilises intravenous injection of OVA-liposome and adoptive transfer of pre-activated OVA-specific CD4+ T cells.14 In the mice that received hepatic CD11c+ cells of control mice, elevations of serum AST and ALT levels were seen (fig 4A). Compatible with this, H&E and TUNEL staining revealed mononuclear cell infiltration in the periportal region and focal necrosis of hepatocytes in the liver of these mice (fig 4B, C). In contrast, elevation of serum levels of transaminases or necrosis of hepatocytes was not seen in the mice which received cells obtained from OVA-fed mice (fig 4A, C). Thus, adoptive transfer of liver DCs from OVA-fed mice abolished the development of hepatitis in the recipient mice. The prevention of hepatitis was associated with OVA-specific Th2 responses in the liver. As shown in fig 5A, OVA-specific IL4 production by hepatic CD4+ T cells was markedly increased in these mice. In contrast, OVA-specific proliferative response and IFN-γ production were significantly decreased in these mice. Finally, we studied the population of hepatic CD4+KJ1-26+ T cells that were specific to OVA and causing hepatitis. As shown in fig 5B, compared to control, the percentage of CD4+KJ1-26+ T cells in the liver was remarkably reduced in the mice that received hepatic CD11c+ cells of OVA-fed mice. The total number of IHLs was significantly reduced in
and IL10. Another report shows that liver-derived cells secreting anti-inflammatory cytokines such as IL4. The DCs, upon injection to allogenic recipients, activate T immune responses elicited are different in their spectra of Kupffer cells, DCs, and LSECs. Depending on the type of APCs, caused by apoptosis as the percentage of Annexin V+ OVA-specific CD4+ and sinusoidal CD11c+ cells. Depending on the type of APCs, counter-regulation was achieved by the function of periportal response to OVA migrating via the portal blood flow. This indicated by the fact that an inflammatory response elicited by a systemic OVA challenge is counter-regulated by another indicated by the fact that an inflammatory response elicited by a systemic OVA challenge is counter-regulated by another.

**DISCUSSION** Utilising an experimental hepatitis model, we show here that the dual blood flow system, systemic and portal, serves to maintain immunological homeostasis in the liver. This was indicated by the fact that an inflammatory response elicited by a systemic OVA challenge is counter-regulated by another response to OVA migrating via the portal blood flow. This counter-regulation was achieved by the function of periportal and sinusoidal CD11c+ cells.

Three populations of APCs are known to reside in the liver: Kupffer cells, DCs, and LSECs. Depending on the type of APCs, immune responses elicited are different in their spectra of inflammation in the liver. One such example is the case with DCs derived from GM-CSF stimulated progenitors in the liver. The DCs, upon injection to allogenic recipients, activate T cells secreting anti-inflammatory cytokines such as IL4 and IL10. Another report shows that liver-derived DEC205+ B220+ CD19+ DCs induce apoptosis of activated T cells. Thus, induction of Th2 responses, rather than Th1, and apoptosis of activated T cells seem to be shared properties of some hepatic DCs. This notion fits well to our previous finding in that CD11c+ class II+ cells in the portal and sinusoidal areas take a soluble Ag (OVA) administered orally and present the Ag to Ag-specific CD4+ T cells, which finally generates regulatory Th2 cells expressing Fas ligand. Thus, some liver DCs, capturing dietary Ags and being activated by materials contained in portal blood flow, generates tolerogenic Th2 cells. The similar property was shared by LSECs which can induce apoptosis of activated T cells. Furthermore, this inhibition was caused by apoptosis as the percentage of Annexin V+ cells in CD4+ KJ1-26+ T cells was significantly increased in the liver of mice received hepatic CD11c+ cells of OVA-fed mice (fig 5C).

**Figure 5** Cytokine production and apoptosis of OVA-specific CD4+ T cells in the liver. OVA-specific hepatic injury was induced in BALB/c recipient mice transferred with hepatic CD11c+ cells as described in fig 4. (A) Proliferative responses (left), IFN-γ secretion (midde), and IL4 secretion (right) by hepatic CD4+ T cells were measured 16 hours after the induction of hepatitis. Hepatic or splenic CD4+ T cells (1×10⁶/well) were stimulated with OVA peptide (1 μg/ml) presented by irradiated splenocytes (5×10⁵/well) from BALB/c mice. (B, C) Flow cytometric analysis of IHLs or splenocytes in the mice transferred with hepatic CD11c+ cells from mice fed PBS or OVA followed by the induction of OVA-specific hepatitis. IHLs or splenocytes were stained with KJ1-26 FITC mAb, anti-CD4 PE mAb, and biotinylated Annexin V followed by streptavidin-RED670. An analysis gate was set on CD4+ KJ1-26+ cells for Annexin V binding assay. The number in each panel shows the percentage of CD4+ KJ1-26+ T cells (B) or Annexin V+ cells in CD4+ KJ1-26+ T cells (C). **p<0.05, ***p<0.01 versus PBS control. The results are expressed as mean±SD. The results shown are representative one of two independent experiments (n=3 in each group).

those mice (PBS versus OVA, 3.81 (SD 8)×10⁶/mouse versus 1.90 (SD 6)×10⁶/mouse)**.
Liver dendritic cells suppressing hepatitis

Tomohiro Watanabe, Tsutomu Chiba, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Toru Kita, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

Funding: This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan, the Japan Society for the Promotion of Science (JSPS) and the Ministry of Health, Labour and Welfare.

Competing interests: None.

REFERENCES