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Induction of hepatitis B virus surface antigen-specific cytotoxic T lymphocytes can be up-regulated by the inhibition of indoleamine 2, 3-dioxygenase activity

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Summary

Cytotoxic T lymphocytes (CTLs) are thought to be major effectors involved in viral clearance during acute infections, including hepatitis B virus (HBV) infection. A persistent HBV infection is characterized by a lack of or a weak CTL response to HBV, which may be reflective of tolerance to HBV. Efficient induction of HBV-specific CTLs leads to the clearance of HBV in patients with a chronic HBV infection. Previously, we reported that α-galactosylceramide (α-GalCer), a specific natural killer T (NKT) cell agonist, enhanced the induction of HBV surface antigen (HBsAg)-specific CTLs. In the present study, we found that inhibition of indoleamine 2,3-dioxygenase (IDO) activity enhanced the induction of HBsAg-specific CTLs after immunization with HBsAg and α-GalCer. The administration of HBsAg and α-GalCer increased the production of interleukin-2 and interleukin-12b, which are crucial for the induction of HBsAg-specific CTLs. The production of these cytokines was more strongly enhanced in IDO knockout mice compared with wild-type mice. In addition, α-GalCer induced the production of IDO in CD11b$^+$ cells, and these cells inhibited proliferation of HBsAg-specific CTLs. Our results lead to strategies for improving the induction of HBsAg-specific CTLs.

Keywords: 3-dioxygenase; cytotoxic T lymphocyte; hepatitis B virus. indoleamine 2; vaccination.

Introduction

Cytotoxic T lymphocytes (CTLs) have been shown to play a critical role in viral clearance in many types of viral infection.[1,2] Antigen-specific CTLs are generated during the T cytotoxic type 1 (Tc1) immune response, which is important in the immune response to hepatitis B virus (HBV). However, clearance of, or at least a continuous suppression of, HBV is not observed in all cases of chronic hepatitis, possibly, because of a weak CTL response to the HBV surface antigen (HBsAg). Therefore, devising an effective therapy for chronic hepatitis B requires elucidation of the mechanism underlying the weakness of the CTL response to HBsAg. Previous reports demonstrated that α-galactosylceramide (α-GalCer), a ligand for Vα14$^+$ natural killer T (NKT) cells, strongly induced the generation of antigen-specific CTLs.[3,4] Immunization with HBsAg plus α-GalCer enhanced the induction and proliferation of HBsAg-specific CTLs.[5] Moreover, immunization with HBsAg plus α-GalCer enabled the induction of HBsAg-specific CTLs even in HBsAg transgenic (HBsAg-Tg) mice, which generate an extremely tolerant cellular and humoral immune response to HBsAg. Hence, we decided to examine the HBsAg-specific CTL response generated in HBsAg-Tg mice following immunization with α-GalCer and HBsAg. This experiment required direct stimulation by HBsAg in vitro, because a sufficient number of HBsAg-specific CTLs may be difficult to induce in HBsAg-Tg mice in vivo.

Indoleamine 2, 3-dioxygenase (IDO) is an enzyme that participates in the catabolism of the essential amino acid L-tryptophan to L-kynurenine and is a potent immuno-modulator.[6,7] This enzyme is expressed in epithelial cells, macrophages and dendritic cells and is induced by pro-inflammatory cytokines, including type I and type II interferons (IFN).[8–10] The binding of cytotoxic T lymphocyte antigen-4 to CD80/CD86 on the surface of dendritic cells also stimulates transcription and activity of IDO.[11,12] Our previous study demonstrated that IDO expression was enhanced by treatment of mice with α-GalCer.[13]

Although the co-administration of HBsAg and α-Gal-Cer enhanced the induction of HBsAg-specific CTLs, the α-GalCer-induced IDO may inhibit the induction of the CTLs to some extent. Hence, in the absence of IDO, immunization with the HBsAg-α-GalCer combination may elicit a strong antigen-specific Tc1 response and numerous HBsAg-specific CTLs.

In this work, we examined the effect of IDO on the induction and proliferation of HBsAg-specific CTLs in mice immunized with α-GalCer and HBsAg. Our results indicate that elimination of IDO activity enhances the antigen-specific Tc1 response and increases the number of HBsAg-specific CTLs in mice immunized with HBsAg plus α-GalCer.

Materials and methods

Mice

Wild-type (WT) male B10.D2 (H-2^d) mice (age 8–10 weeks; weight 25–30 g) were obtained from Japan SLC Inc. (Shizuoka, Japan). IDO knockout (KO) mice on a C57BL/6J background were obtained from Jackson Laboratory (Bar Harbor, ME) and backcrossed with B10.D2 (H-2^d) mice. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the guidelines for the care and use of animals established by the Animal Care and Use Committee of Gifu University, Japan.

Cell lines and reagents

The H-2^d mastocytoma cell line P815 was obtained from the American Type Culture Collection (Rockville, MD). P815 cells expressing HBV-preS1, 2 and S regions (P815preS1) and the HBsAg-specific CD8$^+$ CTL clone 6C2 were generously provided by Francis V. Chisari.[14,15] The HBsAg peptide S$_{28-39}$ was synthesized at KURABO (Osaka, Japan). α-GalCer was obtained from Funakoshi Co. Ltd. (Tokyo, Japan) and stored as a 200 μg/ml stock solution in vehicle (0.5% weight/volume polysorbate-20). 1-methyl-D-L-tryptophan (1-MT) was purchased from Sigma-Aldrich (St Louis, MO).

ELISPOT assay

The WT and IDO-KO mice were intraperitoneally inoculated with either HBsAg (10 μg/mouse) or HBsAg (10 μg/mouse) plus α-GalCer (2 μg/mouse). Single-cell suspensions were prepared from the whole spleen on day 7 after the inoculation. A total of 2.0 × 10^5 splenocytes/well were stimulated for 14–16 hr with 0, 0.1 or 1 μg/ml of HBsAg peptide (S$_{28-39}$) in 96-well Multi-Screen filter plates (Millipore, Billerica, MA) pre-coated with a rat anti-IFN-γ monoclonal antibody (mAb; R4-6A2; BD Biosciences, Franklin Lakes, NJ). The plates were washed and then incubated with a biotinylated polyclonal goat anti-IFN-γ antibody (R&D Systems, Minneapolis, MN) and, after that, with streptavidin–alkaline phosphatase. Spots were visualized by the addition of a 5-bromo-4-chloro-3-indolyl phosphatase solution (Sigma-Aldrich) and counted manually under a microscope (× 40 magnification). The number of cytokine-secreting cells was determined by a single blinded observer, and all data were generated by analysing three separate wells per sample.

Detection of HBsAg-specific CTLs by flow cytometry

Immunodominant HBsAg-specific CTLs in mice are known to be restricted by H-2L^d of MHC class I, and the shortest peptide that can show the maximal activity is HBsAg S$_{28-39}$, sequence IPQSLDSWWTSL.[14] The number of HBsAg-specific CTLs was assessed by flow cytometry, as described previously.[16] Peptide-loaded recombinant soluble dimeric murine H-2L^d:Ig (mouse IgG1; BD PharMingen, San Diego, CA) was prepared by mixing soluble dimeric H-2L^d:Ig for 48 hr at 4° with a 160-fold molar excess of HBsAg S$_{28-39}$. The peptide-loaded dimeric immunoglobulins were then incubated with CD8$^+$ T cells isolated from either immunized or re-stimulated splenocytes.[16] After incubation for 1 hr at 4°, the cells were stained with an FITC-conjugated anti-mouse CD8a antibody and a phycoerythrin (PE) -conjugated anti-mouse IgG1 antibody (BD PharMingen). The proportion of HBsAg-specific cells was measured by flow cytometry on a FACSCanto II instrument (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Cytotoxicity assay

The cytolytic activity of HBsAg-specific CTLs was assessed using a fluorescence-based dye, 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) as described previously.[17] Target cells (P815 or P815preS1) were labelled with CFSE as follows. The cells were suspended in PBS and diluted to 1 × 10^6/ml. For sensitive targets, 0.5 μl of CFSE stock solution (5 mM) was added to 1 ml of cell suspension and the mixture was incubated for 4 min at room temperature. For control targets, 0.5 μl of diluted CFSE solution (100 μM) was used for labelling, in a similar fashion. Labelled targets and various numbers of effector cells were added in a final volume of 200 μl to each well of 96-well round-bottom plates and incubated for 6 hr at 37°. After incubation, sensitive target cells were mixed with control target cells in one tube with PBS containing 1% fetal calf serum and 0.1% sodium azide. Mixed cells were washed once, suspended in 4% paraformaldehyde, and then stored at 4° in the dark before flow cytometric analysis, which was performed on a FACSCan-

to II instrument (Becton Dickinson Immunocytometry Systems). All samples were assayed in duplicate and the mean percentage of specific lysis was calculated as follows: % specific lysis = [(number of sensitive target cells in the control sample – number of sensitive target cells in the test sample)/number of sensitive target cells in the control sample] × 100. The control sample consisted of target cells incubated without added effector cells, whereas the test sample consisted of target cells incubated with added effector cells.

Flow cytometric analysis of splenocytes

Splenocytes were isolated from the immunized mice as described previously.[18] Cell viability and cell number were assessed using a trypan blue exclusion assay. For flow cytometry, 2×10^5 splenocytes were stained with labelled antibodies using a standard protocol. The following antibodies were used: PE-Cy7-labelled anti-mouse CD8 mAb, clone 53-6.7 (eBioscience, San Diego, CA); PE-Cy7-labelled anti-mouse CD11b mAb (clone M1/70; eBioscience); VioBlue-labelled anti-mouse CD11c mAb (clone N418; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), FITC-labelled anti-mouse CD86 mAb (clone 24F; BD Biosciences); FITC-labelled anti-mouse Ly-6G mAb (clone RB6-8C5; BD Biosciences); PE-labelled anti-mouse CD80 mAb (clone BB1; BD Biosciences); PE-labelled anti-mouse Ly-6c mAb (clone AL-21; BD Biosciences); PE-labelled anti-mouse CD40 mAb (clone FKG45.5; Miltenyi Biotec); and PE-labelled anti-mouse Foxp3 mAb (clone FJK-16s; Biosciences). Samples were acquired on a flow cytometer and data analysis was performed using FACSDIVA software (BD Biosciences).

Real-time reverse transcription-PCR

Total RNA was isolated and transcribed into complementary DNA (cDNA) using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and a high-capacity cDNA transcription kit (Applied Biosystems, Foster City, CA). The resulting cDNA was used as a template for real-time PCR along with primer-probe sets for IDO, interleukin-2 (IL-2), IL-4, IL-6, IL-12b and 18S rRNA (TaqMan Gene Expression Assays; Applied Biosystems) and TaqMan universal PCR master mix (Applied Biosystems) according to the manufacturer's instructions (Applied Biosystems). The 18S rRNA was used as an internal control. Real-time PCR was carried out using a Light-Cycler 480 system (Roche Diagnostic Systems, Basel, Switzerland).

Isolation of CD8$^+$ and CD11b$^+$ cells

Splenocytes were sorted into CD8$^+$ or CD11b$^+$ cells using magnetic beads conjugated with an anti-CD8a or anti-CD11b antibody (Miltenyi Biotec GmbH) as described in previous reports.[19,20] The magnetically labelled cells were purified using a quadroMACS system (Miltenyi Biotec GmbH).

HBsAg-specific CTL proliferation assays

Isolated CD11b$^+$ and CD11b$^-$ cells were co-cultured with an HBsAg-specific CTL clone (6C2) in a CFSE-based proliferation assay.[21] The 6C2 cells were labelled with CFSE as previously described.[21] The CFSE-labelled cells were co-cultured with the purified CD11b$^+$ or CD11b$^-$ cells in 96-well plates at the cell-count ratios of 1 : 2 and 1 : 5 for the 6C2/CD11b$^+$ and the 6C2/CD11b$^-$ combinations, respectively, for 5 days at 37° in a 5% CO_2 atmosphere. To induce proliferation, the 6C2 cells were stimulated with irradiated P815PreS1 cells.

Statistics

Values were calculated as the mean ± SEM. Differences between experimental and control groups were analysed using the Kruskal–Wallis test followed by Scheffe's F-test. Significance was assumed at $P < 0.05$.

Results

Induction of the Tc1 immune response and HBsAg-specific CD8$^+$ T cells in WT and IDO-KO mice

Our previous study demonstrated that α-GalCer-activated invariant NKT cells strongly increased the number of HBsAg-specific CTLs after immunization with HBsAg *in vivo*.[5] Therefore, in the present study, we used α-GalCer as an adjuvant to enhance the HBsAg-specific immune response. To determine whether IDO contributes to the development of the HBsAg-specific immune response, WT and IDO-KO mice were immunized with HBsAg and α-GalCer intraperitoneally. As shown in Fig. 1(a), IDO-KO mice mounted strong Tc1 cellular responses against HBsAg, as indicated by the significant expansion of IFN-γ-producing cells in response to *ex vivo* re-stimulation with HBsAg peptide 28–39. Next, we examined CTL frequencies in the spleen using recombinant soluble fusion protein H-2L^d:Ig, which can be used to stain CD8$^+$ T cells that recognize the H-2L^d-bound HBsAg peptide S$_{28–39}$ (Fig. 1b,c). α-GalCer enhanced the numbers of WT as well as IDO-KO HBsAg-specific CD8$^+$ T cells. However, the induction of HBsAg-specific CD8$^+$ T cells was enhanced more strongly for the IDO-KO subset. Moreover, we examined the HBsAg-specific lysis caused by CD8$^+$ T cells from immunized WT and IDO-KO mice *ex vivo* (Fig. 1d). After immunization of mice with the HBsAg-α-GalCer combination, the HBsAg-specific lysis in CD8$^+$ T cells isolated from the IDO-KO mice was significantly enhanced compared with that induced by the WT cells.
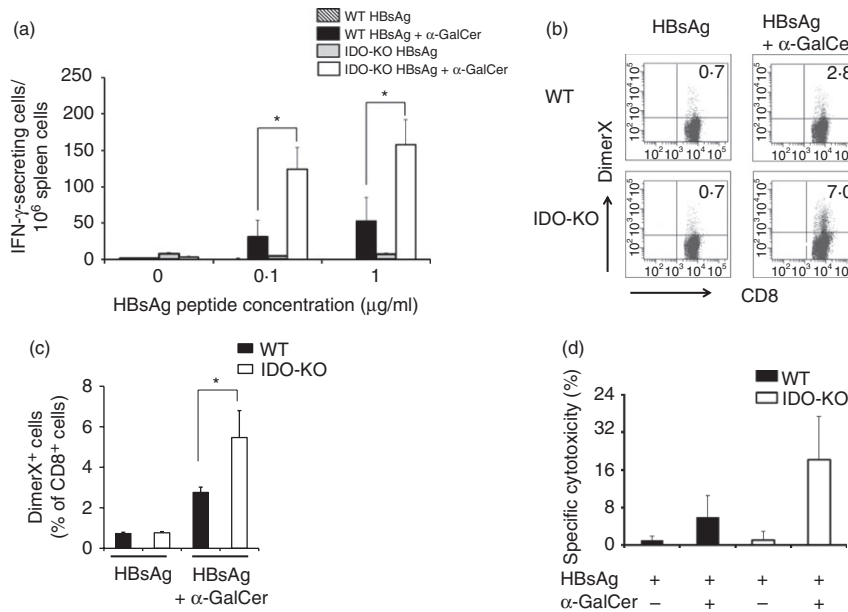
Figure 1. The induction of an hepatitis B virus surface antigen (HBsAg) S$_{28-39}$-specific T cytotoxic type 1 response and CD8$^+$ T cells in wild-type (WT) and indoleamine 2,3-dioxygenase knockout (IDO-KO) mice immunized with HBsAg alone or in combination with α-galactosylceramide (α-GalCer). Splenocytes were isolated from the animals 7 days after the immunization. (a) These cells were stimulated *ex vivo* with the HBsAg S$_{28-39}$ peptide and monitored for interferon-γ (IFN-γ)-secreting cells by means of an ELISPOT assay. Results are shown as mean ± SEM (four or five mice/group) for three independent experiments. (b) Induction of HBsAg-specific CD8$^+$ T cells was assessed by flow cytometric analysis using fluorescent dimeric H-2L^d-HBsAg S$_{28-39}$ complexes (DimerX). FACS profiles are shown for WT and IDO-KO mice immunized with either HBsAg or HBsAg plus α-GalCer. (c) Quantitative data on the frequency of dimeric H-2L^d-HBsAg-positive cell populations (mean ± SEM, $n = 4$). (d) Isolated effector cells (CD8$^+$ T cells) from WT and IDO-KO mice immunized with HBsAg and α-GalCer were incubated for 4 hr with CFSE-labelled target cells (preS1-transfected P815 cells) at an effector to target cell ratio of 20 : 1. The percentage of specific cytotoxicity was calculated by subtracting the percentage of P815 cells (HBsAg-negative) effector cell cytotoxicity from that for the preS1-transfected P815 cells (HBsAg-positive). Spontaneous release was always < 20% of the total. Each data point and error bar represents the mean and SEM, respectively, of results for triplicate samples. *Statistically significant differences.

Comparison of DC functions between WT and IDO-KO mice

Since α-GalCer can induce proliferation of the antigen-specific T cells via the activation of dendritic cells (CD11c$^+$ cells), we analysed dendritic cells from WT and IDO-KO mice immunized with HBsAg plus α-GalCer.[22] α-GalCer can up-regulate the expression of co-stimulatory molecules such as CD40, CD80 and CD86 in CD11c$^+$ cells (Fig. 2). Although the expression of these molecules on dendritic cells was enhanced bythe immunization with the HBsAg–α-GalCer combination, there was no difference between WT and IDO-KO mice.

Regulatory T cells and myeloid derived suppressor cells are stimulated by immunization with HBsAg and α-GalCer

Previous reports demonstrated that IDO was involved in the induction of regulatory T (Treg) cells.[23,24] We therefore examined the proliferation of Treg cells in WT and IDO-KO mice after inoculation with HBsAg and α-GalCer. Flow cytometry analysis revealed that the percentage of Treg cells in the spleen of WT and IDO-KO mice increased equally on day 7 after the immunization (Fig. 3a). The number of Treg cells did not decrease in IDO-KO mice after the HBsAg plus α-GalCer immunization in the present model. Myeloid derived suppressor cells (MDSCs) are identified by the expression of IDO, and were counted using flow cytometry. MDSCs are broadly defined as CD11b$^+$ Ly6G$^+$ mononuclear cells. The administration of HBsAg with α-GalCer expanded the MDSC population in WT and IDO-KO mice (Fig. 3b). This increase in MDSC frequency among spleen cells was observed 7 hr after the immunization, and was similar in both WT and IDO-KO mice.

IDO and cytokine production by WT and IDO-KO splenocytes in response to the intraperitoneal injection of HBsAg plus α-GalCer

Next, we examined whether the expression of IDO in the spleen is enhanced after the intraperitoneal adminis-

tration of the HBsAg–α-GalCer combination. This immunization promptly induced IDO expression in the spleen (Fig. 4a). Previous studies demonstrated that several cytokines play critical roles in the activation and proliferation of antigen-specific CTLs.[25,26] Accordingly, we measured mRNA levels for IL-2, IL-4, IL-6, IL-12b and IFN-γ in whole spleens from WT and IDO-KO mice after the co-administration of HBsAg and α-GalCer (Fig. 4b). The expression of IL-2, IL-4, IL-6 and IL-12b in the spleen of WT and IDO-KO mice was enhanced at 7 hr after the co-administration. In IDO-KO mice, however, compared with WT mice, the expression of IL-2 was significantly increased at 24 and 72 hr after the co-administration. Interleukin-12b expression in IDO-KO mice was also increased at 24 hr after the co-immunization with HBsAg and α-GalCer. These data indicate that after the co-administration, the enhancement of IL-2 and IL-12b expression persisted for longer in the spleen of the IDO-KO mice.

Induction and function of IDO in CD11b$^+$ cells after the immunization of mice with HBsAg and α-GalCer

As shown in Fig. 4(a), the expression of IDO mRNA was strongly enhanced at 7 hr after the administration of HBsAg and α-GalCer. Moreover, the number of CD11b$^+$ Ly6G$^+$ cells (MDSCs) in the spleen also increased at 7 hr after the immunization (Fig. 3b). For this reason, we examined the expression of IDO in CD11b$^+$ cells and CD11b$^-$ cells after the immunization of mice with HBsAg and α-GalCer. As a result of the immunization, the expression of IDO mRNA in CD11b$^+$ cells significantly increased compared with that in CD11b$^-$ cells (Fig. 5a). IDO was predominantly induced in CD11b$^+$ cells from the spleen. Next, we examined the function of CD11b$^+$ cells in HBsAg-specific T-cell proliferation in WT and IDO-KO mice. 6C2 cells are HBsAg-specific CTL clones. We labelled these CTLs with CFSE and stimulated them using irradiated P815PreS1 cells either in the presence or in the absence of CD11b$^+$ cells from WT or IDO-KO mice (Fig. 5c). 6C2 cell proliferation was evaluated by CFSE dilution. For WT mice, the presence of CD11b$^+$ cells inhibited the proliferation of the HBsAg-specific CTLs 6C2 in response to the target cells, after immunization of mice with HBsAg and α-GalCer. In contrast, enhanced cell proliferation was observed when IDO-KO cells were used under similar conditions. In addition, the expression of IL-12b in CD11b$^+$ cells from immunized WT and IDO-KO mice increased compared with that in the CD11b$^-$ subset (Fig. 5b). The enhanced IL-12b production by IDO-KO CD11b$^+$ cells may have contributed to the 6C2 cell proliferation in response to the P815PreS1 cells.

Effect of 1-MT on the induction of the HBsAg-specific immune response

1-Methyl-D-tryptophan is a potent inhibitor of IDO, and hence we used this agent to verify our finding. The WT mice were given 1-MT orally at either 0 or 5 mg/ml in drinking water 2 days before the immunization to 7 days after the immunization. ELISPOT analysis revealed that the HBsAg peptide S$_{28-39}$-specific Tc1 response in WT mice treated with 1-MT was significantly stronger compared with the untreated mice (Fig. 6a). The induction of HBsAg-specific CD8$^+$ T cells was enhanced by treatment with 1-MT (Fig. 6b,c). These data indicate that the inhibition of IDO activity leads to the enhancement of an HBsAg-specific Tc1 response after immunization with HBsAg and α-GalCer.

Discussion

In the present study, we demonstrated that inhibition of IDO activity enhances the HBsAg-specific Tc1 response and induction of CTLs after immunization with HBsAg and α-GalCer. The expression of IL-2 and IL-12b, which are critical cytokines for inducing the antigen-specific Tc1 response, increases in IDO knockout mice after immunization with HBsAg and α-GalCer. Moreover, CD11b$^+$ splenocytes up-regulate their expression of IDO and suppress the proliferation of HBsAg-specific CTLs after immunization of mice with HBsAg and α-GalCer treatment. Hence, the α-GalCer-induced increase of IDO expression reduced the antigen-specific Tc1 immunity. On the other hand, inhibition of IDO expression after the HBsAg plus α-GalCer immunization strongly enhanced the HBsAg-specific cellular immune response.

Previously, we demonstrated that during immunization with HBsAg, α-GalCer works as an adjuvant and enhances the induction of an HBsAg-specific CTL response *in vivo*.[5] It appears that IL-2 and CD40–CD40 ligand interactions are involved in the enhancement of CTL induction caused by α-GalCer through NKT cell activation. On the other hand, α-GalCer enhances the activity of IDO in mice.[13] IDO catalyses the conversion of the essential amino acid L-tryptophan to L-kynurenine and has been identified as an enzyme that has powerful immunomodulatory effects. Furthermore, metabolites of the L-kynurenine pathway have been shown to act as immunosuppressive molecules in the tissue microenvironment.[27,28] IDO and the L-tryptophan pathway play critical roles in the generation of immune tolerance against foreign antigens in tissue microenvironments. In the present study, IDO expression in splenocytes also increased after HBsAg and α-GalCer administration (Fig. 5a). As shown in Fig. 1(a), the intraperitoneal injection of HBsAg and α-GalCer increased the HBsAg-specific Tc1 immune response, and this Tc1 response was enhanced more
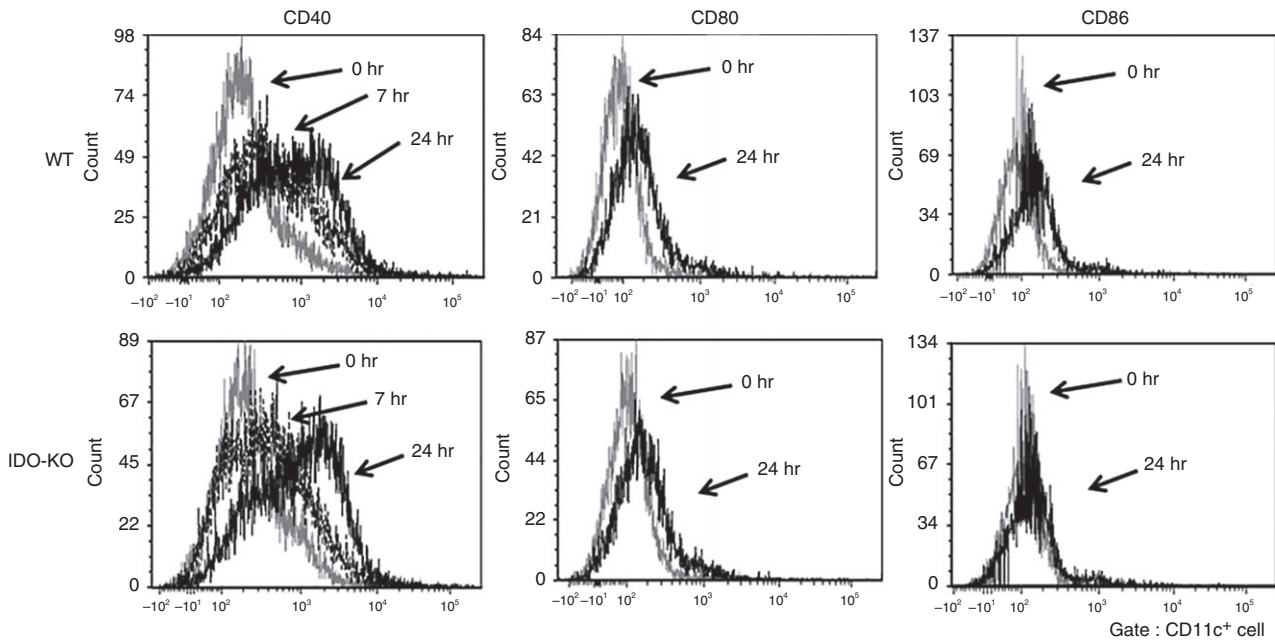
Figure 2. The expression of co-stimulatory molecules on dendritic cells from wild-type (WT) and indoleamine 2,3-dioxygenase knockout (IDO-KO) mice. At 0, 7 and 24 hr after the inoculation with hepatitis B virus surface antigen (HBsAg) and α-galactosylceramide (α-GalCer) (intraperitoneal injection), the expression of CD40, CD80 and CD86 on splenic CD11c+ cells was analysed using flow cytometry. The data represent three independent identical experiments.

strongly in IDO-KO mice compared with WT mice. Moreover, the number of CD8+ T cells that can specifically recognize HBsAg and cause HBsAg-mediated lysis also increased in IDO-KO mice ex vivo (Fig. 1b–d). These results suggest that the absence of IDO activity significantly enhances the HBsAg-specific Tc1 immune response after the immunization with HBsAg and α-GalCer. 1-MT is a competitive inhibitor of the IDO activity. As shown in Fig. 6, the administration of HBsAg, α-GalCer and 1-MT also enhanced both the HBsAg-specific Tc1 response and the induction of HBsAg-specific CD8+ T cells (Fig. 6a–c). However, the HBsAg-specific Tc1 response in the mice treated with HBsAg, α-GalCer and 1-MT was weak compared with that in the IDO-KO mice treated with HBsAg and α-GalCer. A previous report indicated that the L-kynurenine level in IDO-KO mice was lower than that in 1-MT-treated mice during Toxoplasma gondii infection in vivo.[29] Our previous study also indicated a difference in proliferation of splenocytes and viral replication between IDO-KO mice and 1-MT-treated mice during LP-BM5 murine leukaemia virus infection.[9] We assumed that complete local depletion of L-tryptophan is critical for the immune system, and the oral administration of 1-MT may fail to fully suppress local IDO activity. Several reports have demonstrated that the enhancement of the immune response by α-GalCer involves an up-regulation of co-stimulatory molecules such as CD80, CD86 and CD40.[22,30] In the present work, although α-GalCer enhanced the expression of CD40 and CD86 in CD11c+

cells, this effect was similar in WT and IDO-KO mice (Fig. 2). Real-time PCR analysis revealed that the expression of IL-2 and IL-12b was elevated at 24 and 72 hr after the HBsAg plus α-GalCer immunization in IDO-KO mice compared with that in WT mice (Fig. 4b). The expression of IL-2, IL-4, IL-6 and IL-12b increased at 7 hr after administration, and promptly decreased afterwards in WT mice. On the other hand, in IDO-KO mice, the enhancement of IL-2 and IL-12b expression was maintained between 24 and 72 hr after the immunization with HBsAg and α-GalCer. The persistence of the elevated expression of IL-2 and IL-12b in IDO-KO mice may play a role in the enhancement of the HBsAg-specific Tc1 immune response induced by HBsAg and α-GalCer in combination.

IDO is strongly activated by IFN-γ or pro-inflammatory cytokines in macrophages/monocytes. As shown in Fig. 5(a), among splenocytes, the immunization of mice with HBsAg and α-GalCer induced IDO activity almost exclusively in CD11b+ cells. No enhancement of IDO expression was observed in CD11b− cells. In addition, CD11b+ cells from HBsAg-plus-α-GalCer-immunized WT mice reduced the proliferation of HBsAg-specific CTLs when the latter were stimulated with the specific antigen (Fig. 5c). In contrast, CD11b+ cells from HBsAg-plus-α-GalCer-immunized IDO-KO mice facilitated the proliferation of HBsAg-specific CTLs. These results indicate that IDO-deficient CD11b+ cells treated with α-GalCer have a strong ability to enhance proliferation of HBsAg-specific
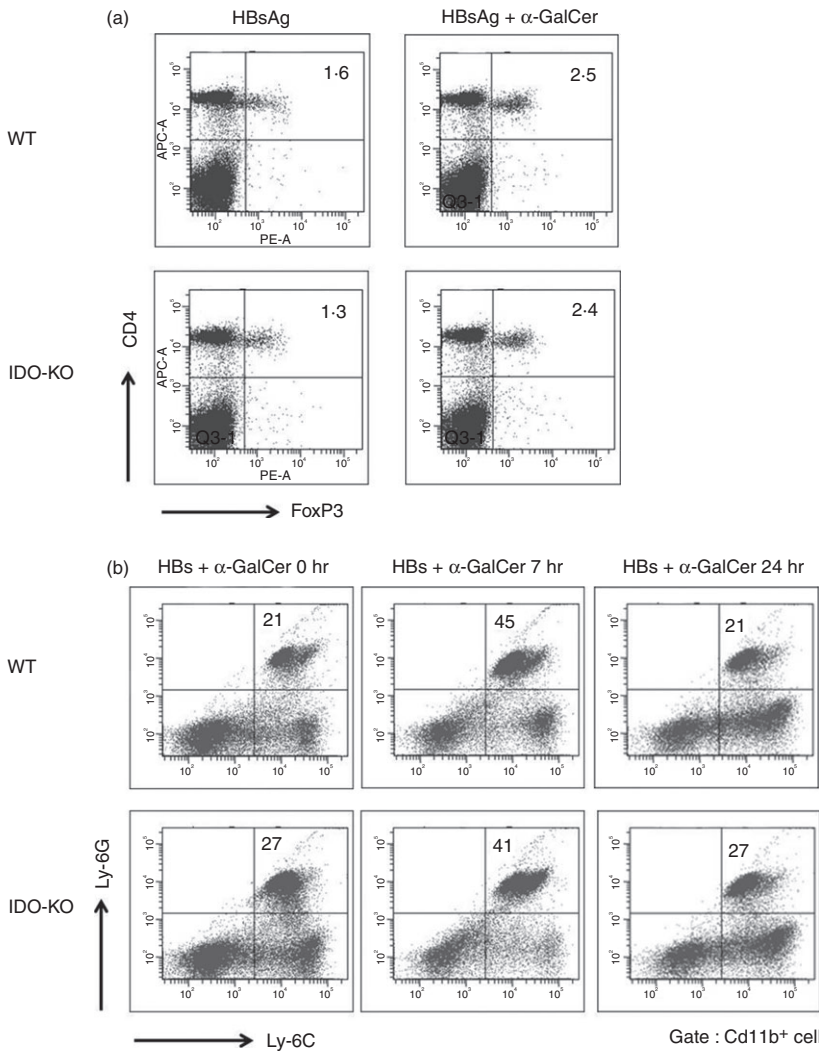
(a)

(b)

Gate : Cd11b⁺ cell

Figure 3. The induction of regulatory T cells and myeloid derived suppressor cells (MDSCs) in wild-type (WT) and indoleamine 2,3-dioxygenase knockout (IDO-KO) mice immunized with hepatitis B virus surface antigen (HBsAg) and α-galactosylceramide (α-GalCer). (a) Flow cytometric analysis of intracellular FoxP3 expression in CD4⁺ T cells obtained from WT and IDO-KO mice on day 7 after inoculation with HBsAg and α-GalCer. The data represent three independent identical experiments. (b) Flow cytometric analysis of surface Ly-6G and Ly-6C expression on CD11b⁺ cells from WT and IDO-KO mice at 7 and 24 hr after inoculation with HBsAg and α-GalCer. The data represent three independent identical experiments.

CTLs. The expression of IL-12b in CD11b⁺ cells was increased at 7 hr after the immunization with HBsAg plus α-GalCer (Fig. 5b). Moreover, the CD11b⁺ fraction included the CD11b⁺ CD11c⁺ cells, and the expression of co-stimulatory molecules on CD11c⁺ cells increased after the immunization (Fig. 2). Hence, the CD11b⁺ cells from the IDO-KO mice immunized with HBsAg and α-GalCer may enhance the proliferation of HBsAg-specific CTLs via an increase in IL-12b production and co-stimulatory molecule expression. The intraperitoneal administration of HBsAg and α-GalCer increased the proportion of CD11b⁺ Ly6G⁺ cells in the spleen (Fig. 3b). CD11b⁺ Ly6G⁺ cells are known as MDSCs and induce the expression of IDO, which inhibits the immune response.[31] Hence, the immunization with HBsAg and α-GalCer increases the proportion of CD11b⁺ Ly6G⁺ cells in the spleen as well as the number of IDO-producing cells and thereby inhibits the induction of an HBsAg-specific immune response. Although the immunization with HBsAg and α-GalCer also increased the proportion of CD11b⁺ Ly6G⁺ cells in IDO-KO mice,

these cells cannot up-regulate IDO and inhibit the HBsAg-specific immune response. Several reports demonstrated that IDO stimulates Treg cells and suppresses the immune response.[23,24] On the other hand, there was no difference in the percentage of Treg cells between the non-treated group and the 1-MT-treated group in a rheumatoid arthritis mouse model.[32] Our previous report also demonstrated that the expression of FoxP3 mRNA in WT and IDO-KO mice was up-regulated after LP-BM5 murine leukaemia virus infection and there was no difference in FoxP3 mRNA expression.[9] The inoculation with HBsAg and α-GalCer injection increased the proportion of CD4⁺ FoxP3⁺ T cells in the spleen (Fig. 3a), but there was no difference in Treg cell number between WT mice and IDO-KO mice. Therefore, in this study, Treg cells do not appear to be involved in the HBsAg-specific immune response following immunization with HBsAg plus α-GalCer.

A recent study evaluated the effect of IDO on the humoral immune response, and they demonstrated that inhibition of IDO by the administration with 1-MT at

Figure 4. Indoleamine 2,3-dioxygenase knock-out (IDO) and cytokine production by splenocytes from wild-type (WT) and IDO knockout (IDO-KO) mice in response to an intraperitoneal injection with hepatitis B virus surface antigen (HBsAg) and α-galactosylceramide (α-GalCer). In all graphs, the y-axis shows arbitrary units. (a) The relative expression levels of IDO mRNA in the spleen of WT and IDO-KO mice were measured using quantitative real-time RT-PCR. The results were normalized for the expression of 18S rRNA. Each value is shown as mean (SEM) for three mice. (b) The relative expression levels of interleukin-2 (IL-2), IL-4, IL-6 and IL-12b mRNA in the spleen of WT and IDO-KO mice were measured using quantitative real-time RT-PCR. The results were normalized for the expression of 18S rRNA. Each data point and error bar represents the mean and SEM, respectively, of data from triplicate samples. *Statistically significant differences.
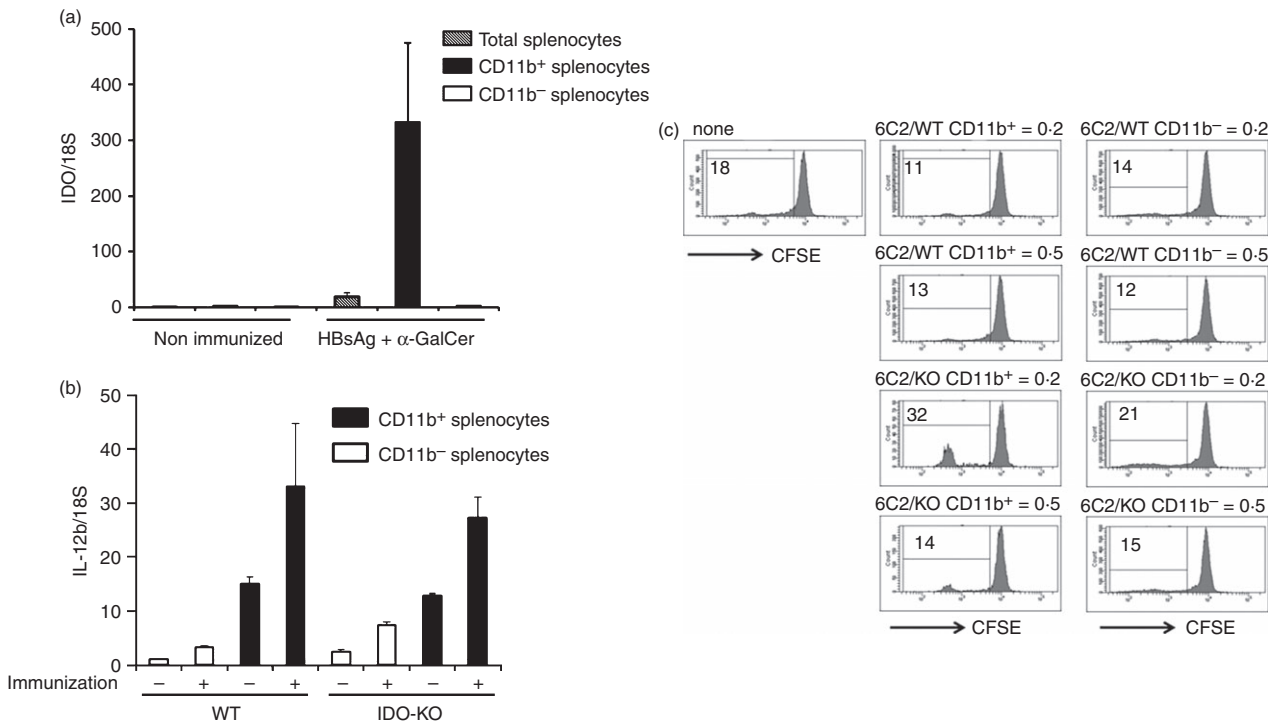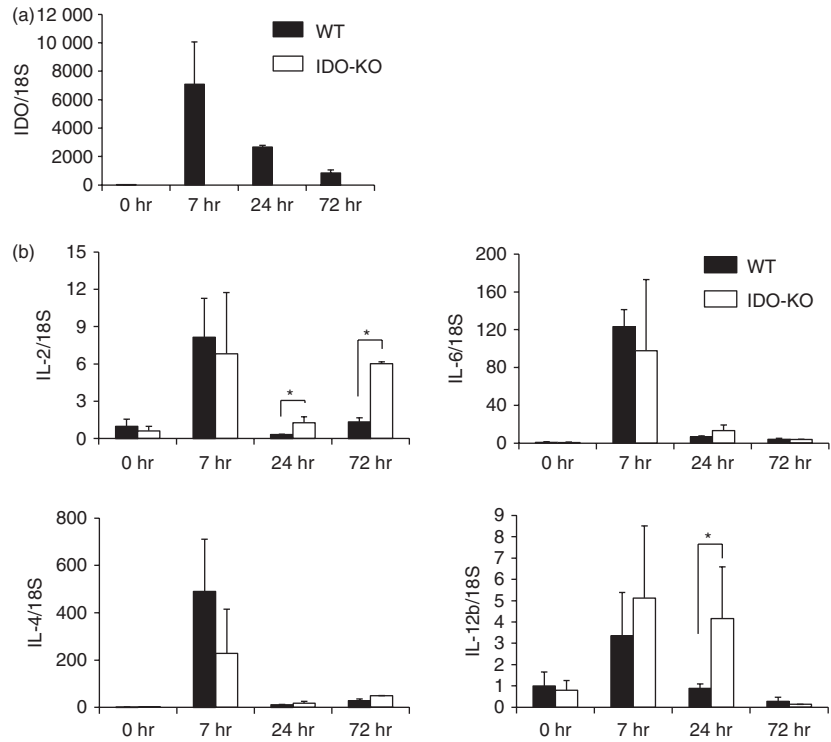

Figure 5. Induction and function of indoleamine 2,3-dioxygenase (IDO) in CD11b$^+$ cells from wild-type (WT) and IDO knockout (IDO-KO) mice after the inoculation with hepatitis B virus surface antigen (HBsAg) and α-galactosylceramide (α-GalCer). (a, b) Splenic CD11b$^+$ and CD11b$^-$ cells were purified using immunomagnetic separation 7 hr after the inoculation and used to prepare total mRNA. The expression of IDO and interleukin-12b (IL-12b) mRNA was analysed using real-time RT-PCR. Results were normalized for the expression of 18S rRNA. (c) CFSE-labelled 6C2 cells were co-cultured with the purified CD11b$^+$ or CD11b$^-$ cells in 96-well plates at a cell-count ratio of 1 : 2 or 1 : 5 for 6C2/ myeloid derived suppressor cell or 6C2/control combination, respectively, for 5 days. To induce proliferation, the 6C2 cells were stimulated with irradiated P815PreS1 cells.
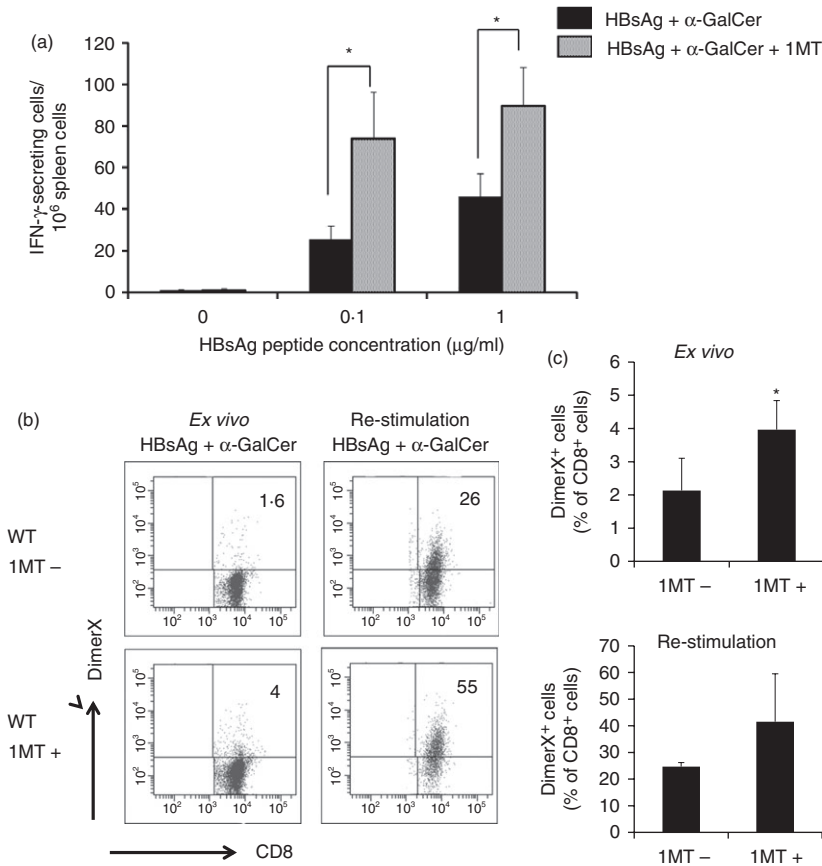
Figure 6. The effects of 1-methyl-D-L-trypto-phan (1-MT) on the induction of an hepatitis B virus surface antigen (HBsAg) -specific immune response. (a) Wild-type (WT) mice received 1-MT orally at a dose of either 0 or 5 mg/ml in drinking water 2 days before the immunization to 7 days after the immunization. Splenocytes from these mice were isolated 7 days after the immunization. These cells were stimulated *ex vivo* with the HBsAg S$_{28-39}$ peptide and monitored for interferon-γ-secreting cells by means of an ELISPOT assay. The results are shown as mean $\pm$ SEM (four or five mice/group) for three independent experiments. (b) Induction of HBsAg-specific CD8$^+$ T cells was assessed by means of flow cytometric analysis using fluorescent dimeric H-2L^d-HBsAg S$_{28-39}$ complexes (DimerX). FACS profiles are shown for WT mice and for 1-MT-treated WT mice after the inoculation with HBsAg and α-galactosylceramide (α-GalCer). (c) Quantitative data on the frequency of dimeric H-2L^d-HBsAg-positive cell populations (mean $\pm$ SEM, $n = 4$). *Statistically significant differences.

the time of vaccination decreased the serum anti-HBs antibody level after HBsAg vaccination.[33] Another report showed that 1-MT inhibit the B cells' ability to differentiate into autoantibody-secreting cells and improve autoimmune arthritis.[34] These studies indicated that 1-MT inhibits the humoral immune response. The enhancement of HBs-antibody induction is critical to prevent HBV infection. On the other hand, the induction of a cellular immune response to HBV antigen is pivotal to the complete elimination of HBV during chronic infection.[35] Enhancement of the induction of HBsAg-specific CTLs may be helpful in developing strategies to clear HBV in patients with chronic hepatitis.

We can conclude that the inhibition of IDO activity significantly enhanced the induction of HBsAg-specific CTLs following immunization with HBsAg and α-GalCer. The addition of α-GalCer enhances IDO activity, which inhibits the activation and proliferation of antigen-specific CTLs. Hence, abrogation of the α-GalCer-mediated enhancement of IDO activity makes the HBsAg-specific Tc1 response much more powerful. These results lead to strategies for improving the induction of HBsAg-specific CTLs.

Disclosures

The authors have no financial or commercial conflict of interest.

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