Bilirubin Possesses Powerful Immunomodulatory Activity and Suppresses Experimental Autoimmune Encephalomyelitis

Yingru Liu, Ping Li, Jie Lu, Wei Xiong, Joel Oger, Wolfram Tetzlaff and Max Cynader

*J Immunol* 2008; 181:1887-1897; doi: 10.4049/jimmunol.181.3.1887

http://www.jimmunol.org/content/181/3/1887

**References**

This article cites 41 articles, 16 of which you can access for free at:

http://www.jimmunol.org/content/181/3/1887.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Bilirubin Possesses Powerful Immunomodulatory Activity and Suppresses Experimental Autoimmune Encephalomyelitis

Yingru Liu2,3✉, Ping Li,2 Jie Lu,* Wei Xiong,* Joel Oger,‡ Wolfram Tetzlaff,§ and Max Cynader*#

Bilirubin, an abundant bile pigment in mammalian serum, was once considered a toxic waste product and has more recently been recognized as a potent antioxidant of physiological importance. However, its potential biological functions in other fields are not well understood. Herein we show that bilirubin is also a powerful immunomodulatory agent. Bilirubin significantly inhibited Ag-specific and polyclonal T cell responses, while other similar antioxidants completely lacked this effect. Bilirubin suppressed CD4+ T cell responses at multiple steps. High levels of bilirubin could induce apoptosis in reactive CD4+ T cells. Bilirubin at nonapoptotic concentrations suppressed CD4+ T cell reactivity through a wide range of actions, including inhibition of costimulator activities, suppression of immune transcription factor activation, and down-regulation of inducible MHC class II expression. Further studies suggest that bilirubin actions were direct, rather than via induction of immune deviation or regulatory T cells. In vivo, treatment with bilirubin effectively suppressed experimental autoimmune encephalomyelitis in SJL/J mice. In contrast, depletion of endogenous bilirubin dramatically exacerbated this disease. In summary, our results identify bilirubin as an important immunomodulator that may protect mammals against autoimmune diseases, thereby indicating its potential in the treatment of multiple sclerosis and other immune disorders. The Journal of Immunology, 2008, 181: 1887–1897.

Materials and Methods

EAE induction and treatments

All animal care procedures were performed according to protocols approved by the Animal Care Committee of the University of British Columbia. Chronic EAE was induced in 6–8-wk-old female SJL/J mice (The
Jackson Laboratory) by immunization with 150 μg proteolipid protein peptide 139–151 (PLP139–151) emulsified in CFA (Difco Laboratories). To induce adoptive transfer EAE, lymph node cells were obtained from these mice on days 10–12 postimmunization and cultured in complete RPMI 1640 medium (Invitrogen) with 50 μg/ml PLP139–151. After 4 days of culture, cells were harvested and 2 × 10⁶ viable cells were injected i.p. into naive female SJL/J mice. Acute EAE was induced in male Lewis rats (Charles River Laboratories) with body weights between 175 and 200 g by immunization with 50 μg guinea pig myelin basic protein (Sigma-Aldrich) emulsified in CFA. Clinical EAE was scored daily as follows: 0, no signs; 1, limp tail; 2, partial paralysis of hind limbs; 3, complete paralysis of hind limbs; 4, paralysis of forelimbs and hind limbs; 5, moribund. In the chronic EAE paradigm, groups of mice were treated with bilirubin (Calbiochem) vs. glutathione (GSH) (Sigma-Aldrich) or α-tocopherol (Sigma-Aldrich) at increasing doses as indicated for 15 days starting from 6 days after immunization (DAI). Two additional groups were treated with high doses of bilirubin or α-tocopherol, respectively, for 15 days starting from the onset of symptoms. In the acute EAE paradigm, two groups of rats were treated with zinc protoporphyrin (ZnPP) (Frontier Scientific) or 1,4 bis[2-(3,5-dimethyl-2,4,6-triazin-1-yl)pyrimidin-5-yl]benzene (TCPBOP) (Calbiochem), respectively, for 10 days from 6 DAI. All the treatments were administered by i.p. injection. Control groups received injections with the same volume of vehicle on the same schedule.

Proliferation assays
To analyze polyclonal T cell proliferation, spleens were harvested from naive SJL/J mice. RBCs were deleted with ammonium chloride lysing buffer. Cell suspension was prepared and CD4⁺ T cells were purified through negative selection using CD4⁺ T cell isolation kit (Miltenyi Biotech). Purified CD4⁺ T cells (5 × 10⁶/well) were then cultured in 96-well tissue culture plates in complete RPMI 1640 medium. Cells were stimulated with Con A (Sigma-Aldrich) or coated anti-CD3 mAb (BD Biosciences) with or without soluble anti-CD28 mAb (BD Biosciences) in the presence of different concentrations of bilirubin or other antioxidants. After 72 h of stimulation, cells were pulsed with [³H]thymidine (0.5 μCi/well), and 16 h later, thymidine incorporation was measured using a liquid scintillation counter. To analyze Ag-specific T cell proliferation, purified CD4⁺ T cells were isolated from the spleens of SJL/J mice immunized 10–12 days previously with PLP139–151. The cells were then stimulated with PLP139–151 in the presence of APCs (irradiated splenocytes) with different concentrations of bilirubin or other antioxidants. Cell proliferation was also determined by [³H]thymidine incorporation after 72 h stimulation. In some cases, whole PLP-immune spleen cells were used for proliferation assays. Human PBMC polyclonal proliferation assays were performed according to a similar protocol. Blood was collected from healthy donors in accordance with local ethics committee standards. PBMCs isolated by Ficoll-Hypaque density gradient centrifugation were cultured in 96-well tissue culture plates (2 × 10⁶/well).

Cell viability and apoptosis assays
Cell viability was measured using MTT colorimetric assay after the indicated treatments. For detection of apoptosis, after washing in PBS, the cell pellets were resuspended in binding buffer containing annexin V-FITC (BD Biosciences) and propidium iodide (PI) (BD Biosciences) for 20 min at room temperature. The samples were analyzed on a FACSCalibur cytometer within 1 h.

Cytokine ELISA
PLP-specific CD4⁺ T cells were stimulated as described above. For TGF-β1 analysis, T cells were cultured in X-VIVO serum-free medium (Lonza). Culture supernatants were collected 48 h later, and IL-2, IFN-γ, IL-4, IL-10, and TGF-β1 levels were measured in triplicate using commercially available ELISA kits (BD Biosciences).

Flow cytometry
mAbs against mouse CD4, CD11b, CD11c, CD28, CTLA-4, B7-1, B7-2, Foxp3, 1A-1, IL-2, IL-4, IL-10, and IFN-γ conjugated with FITC or PE were purchased from BD Biosciences or eBioscience. Cultured cells were washed with staining buffer and then incubated with the indicated mAbs in staining buffer for 30 min on ice, washed twice, and analyzed on a FACSCalibur cytometer. For intracellular staining, cells were first fixed with Cytofix/Cytoperm (BD Biosciences).

Preparation of purified macrophages and dendritic cells (DCs) and real-time RT-PCR
For purification of macrophages and DCs, cells were incubated with FITC-conjugated anti-CD11b or anti-CD11c Ab for 30 min. Labeled CD11b⁺ or CD11c⁺ cells were isolated by a fluorescence cell sorter. Total cellular RNA of purified macrophages or DCs was isolated with TRIzol reagent (Invitrogen). RNA was transcribed to cDNA using random hexamer primers (Invitrogen). Real-time RT-PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems). The primers used were as follows: CIITA, 5'-CAAGTCTCCTGAAGGTGTTGA-3', 5'–AGGTCATCAGCCGGAGGAC-3'; β-actin, 5'–GTGGCCGCTCTAGGCAACAA-3', 5'–CTCTTTGAATGTCGACACATTT-3'. Relative quantification of target genes was analyzed based on a comparative Ct method as suggested by Applied Biosystems.

Nuclear protein extraction and EMSA
Nuclear extracts were prepared using a nuclear extraction kit (Panomics) according to the manufacturer's protocol. For EMSA, the NF-κB consensus oligonucleotide probe double strand (5'-AGTGTGAGGGACTTTCCAG-3') (Promega) was labeled with [³²P]ATP. Nuclear extracts (4 μg) were used for each binding reaction in gel shift binding buffer with [³²P]-labeled NF-κB consensus oligonucleotide. The samples were analyzed by electrophoresis on 4% acrylamide gels. The gels were dried and exposed to x-ray film. Double-stranded mutated oligonucleotide (5'-AGTTGAGGCCATTTCCCCAGG-3') was used to verify the specificity of NF-κB binding to DNA.

Western blot assays
Nuclear and cytoplasmic protein extracts were prepared using the nuclear extraction kit mentioned above. Total cell protein extracts were prepared with lysis buffer. Equal amounts of protein were separated on SDS-PAGE and transferred to nitrocellulose membranes. Standard immunostaining was conducted using the ECL chemiluminescence technique. Anti-NF-κB (StressGen Biotechnologies), anti-phospho-IκB (StressGen Biotechnologies), and anti-phospho-STAT-1 (Cell Signaling Technology) Abs were used at 1/1000 to 1/2000 dilutions.

Bilirubin assay
Blood was drawn from SJL/J mice at various time points after bilirubin injections. Serum bilirubin concentration was measured using a bilirubin assay kit (Wako Diagnostics).

In vivo BrdU incorporation and isolation of mononuclear cells from the CNS
For labeling of proliferating cells in vivo, mice were injected i.p. with 50 μg/g body weight BrdU (BD Biosciences) every 2 h, for a total of 3 times. Mononuclear cells were isolated from the CNS as described previously (9). BrdU incorporation into cellular DNA was detected using a BrdU flow kit (BD Biosciences) according to the manufacturer's instructions.

Morphological techniques and histopathological studies
Animals were euthanized and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. The spinal cords were removed, dissected into lumbar, thoracic, and cervical segments, and embedded in Tissue-Tek. Serial transverse sections were cut on a Frigocut cryostat. The sections were stained with H&E to assess inflammation. The severity of inflammation in the lumbar, thoracic, and cervical spinal cord was graded on a scale of 0–4 as detailed previously (10), and the total inflammation score was obtained by adding these three scores. Luxol fast blue staining or x-ray film. Double-stranded mutated oligonucleotide (5'-AGTTGAGGCCATTTCCCCAGG-3') was used to verify the specificity of NF-κB binding to DNA.

Statistical analysis
Data are presented as means ± SEM. Data on the effect of various treatments on EAE were analyzed using two-way ANOVA with Fisher’s PLSD post hoc tests for multiple comparisons. Two-sample t tests were used to
Bilirubin inhibits T cell proliferation

To examine the potential immunomodulatory properties of bilirubin, we investigated the effect of bilirubin on the proliferative responses of naive SJL/J CD4+ T cells and PLP-specific CD4+ T cells following stimulation with Con A, anti-CD3 mAb with or without anti-CD28 mAb, or PLP139–151 as indicated. We first assessed the cytotoxicity of bilirubin to ensure that all the concentrations of bilirubin we used were nontoxic. Cultures of naive SJL/J CD4+ T cells or unstimulated PLP-immune CD4+ T cells incubated with bilirubin at concentrations up to 200 μM did not show reduced cell viability over 3 days as evaluated by colorimetric assays (Fig. 1A). Since in humans the normal plasma level of bilirubin is ~20 μM, and levels as high as 170 μM in neonates are still considered physiological (12, 13), we chose concentrations of bilirubin between 20 and 150 μM for the present study. As shown in Fig. 1, bilirubin significantly inhibited both polyclonal and Ag-specific T cell responses. Interestingly, the inhibitory effect of bilirubin was stronger on the PLP-specific CD4+ T cell proliferative response than on CD4+ T cell proliferation activated with Con A or anti-CD3 mAb (Fig. 1B). Comparable inhibitory effects were observed when CD4+ T cells were stimulated with anti-CD3 mAb alone or with anti-CD3 plus anti-CD28 mAbs (Fig. 1B).

The inhibitory effect of bilirubin was dose-dependent, with concentrations >50 μM significantly suppressing CD4+ T cell responses in all cases (Fig. 1C). To ascertain whether the immunosuppressive actions of bilirubin were of general importance for multiple cell types, similar experiments were performed on human PBMCs. The results showed that bilirubin also inhibited human T cell proliferation effectively (Fig. 1D).

Since several previous studies had demonstrated that reactive oxygen species (ROS) were able to promote the proliferation and growth of some cell types (14, 15), and bilirubin is an efficient free radical scavenger, we examined whether the antiproliferative actions of bilirubin observed above were attributable to its well-known antioxidant activity. The effects of several other powerful antioxidants on CD4+ T cell proliferation were examined, including GSH, another important endogenous antioxidant in mammals; α-tocopherol, also a potent lipophilic antioxidant; and conjugated bilirubin. Our results clearly showed that none of these antioxidants inhibited T cell responses to any degree, even at much higher concentrations (Fig. 1E). The data thus suggest that bilirubin possesses an important immunosuppressive effect in addition to its other biological functions.

High levels of bilirubin induce apoptosis in reactive T cells

Inhibition of T cell proliferation after stimulation with bilirubin treatment could be due to either cellular unresponsiveness or depletion of responder cells (16). We next determined whether
incubated with 150 g/ml anti-CD3 mAb for 72 h in the presence of different concentrations of bilirubin. T cell apoptosis and death were detected by annexin V/PI staining and analyzed by flow cytometry. Results from one representative out of four independent experiments are shown.

Bilirubin could induce responder T cell deletion. Purified SJL/J CD4+ T cells were stimulated with anti-CD3 mAb for 72 h in the presence of different concentrations of bilirubin. Annexin V/PI staining was used to detect T cell apoptosis and death. The results demonstrated that incubation of activated CD4+ T cells with bilirubin at a high concentration of 200 μM resulted in higher percentages of apoptotic cells (Fig. 2). However, bilirubin at lower concentrations did not strongly induce responder CD4+ T cell apoptosis. The level of apoptotic cells changed 15% in cultures incubated with 150 μM bilirubin, although the proliferative response was reduced by >65% (Figs. 2 and 1C). Bilirubin at the concentrations of 100 or 50 μM did not increase apoptosis in the activated CD4+ T cells (data not shown), but at the same concentrations it still significantly inhibited the proliferative response (Fig. 1C). These results demonstrate that although cell death may contribute to immunologic tolerance induced by bilirubin, bilirubin also causes anergy in reactive T cells, especially at physiological concentrations. Similar results were observed for the effects of bilirubin on the Ag-specific CD4+ T cell responses activated with PLP139–151 (data not shown).

**Bilirubin does not induce immune deviation or expansion of several regulatory T (Treg) cell types**

To further elucidate the mechanisms underlying T cell suppression by bilirubin, we then investigated whether bilirubin had effects on Th cell differentiation. Cytokine secretion was measured in PLP-specific CD4+ T cells after stimulation with 50 μg/ml PLP139–151 for 48 h in the presence of APCs. As shown in Fig. 3A, the production of Th1 cytokines, including IL-2 and IFN-γ, was strongly suppressed by bilirubin in a dose-dependent manner. The production of Th2 cytokines, including IL-4 and IL-10, was also decreased by bilirubin treatment, suggesting that bilirubin does not lead to a skewing of immune responses from a Th1 cell to a Th2 cell response. The reduced production of IL-10 by activated PLP-specific T cells after treatment also indicates that bilirubin does not induce IL-10-producing Treg 1 cells.

We next asked whether bilirubin favors the development of CD4+Foxp3+ Treg cells, which is another crucial mechanism to maintain immunologic tolerance (17). Flow cytometric analyses showed that bilirubin did not promote generation of CD4+Foxp3+ Treg cells (Fig. 3B). The percentage of PLP-immune CD4+ T cells expressing Foxp3 following PLP139–151 stimulation with bilirubin treatment was not increased when compared with control treatments. Consistent with the results, ELISA study showed that bilirubin treatment did not increase TGF-β1 production in PLP-specific CD4+ T cells after TCR stimulation (Fig. 3C).

**Bilirubin suppresses costimulatory molecule activities**

T cell activation requires two distinct signals. One signal is delivered through the interaction of the Ag-specific TCR with MHC molecules expressed on APCs, while the other is received from interactions with costimulatory molecules. T cells that receive a TCR stimulus without adequate costimulation are rendered anergic (18, 19). We then asked whether bilirubin induces anergy in reactive T cells by down-regulating costimulator activity. PLP-immune spleen cells were activated with PLP139–151 in the presence or absence of bilirubin. CD28 and CTLA-4 expression on CD4+ T cells, as well as B7-1 and B7-2 expression on macrophages (CD11b+) and DCs (CD11c+), was measured by flow cytometry after 48 h of culture. As shown in Fig. 4A, bilirubin at 150 μM inhibited the activation-induced expression of CD28 on CD4+ T cells by 51.4 ± 10.2% (p < 0.01). B7-1 activity in macrophages and DCs was reduced by bilirubin treatment by 71.1 ± 17.2% (p < 0.05) and 54.6 ± 11.1% (p < 0.05), respectively. B7-2...
activity in macrophages and DCs was reduced by bilirubin treatment by 55.3 ± 9.9% (p < 0.05) and 62.2 ± 14.2% (p < 0.05), respectively. However, bilirubin did not alter the expression of CTLA-4 by activated CD4+ T cells (Fig. 4A), which indicates that the effect of bilirubin on the costimulator activity is specific. The data also provide evidence that our findings are not a general phenomenon related to suppression of protein synthesis after bilirubin treatment. However, we observed that such effects of bilirubin at low concentrations are not strong. For example, the costimulatory molecule activities following stimulation with 50 μM bilirubin treatment were not significantly different from controls, although the means were slightly lower (data not shown).

Since bilirubin can suppress costimulatory activity and induce anergy, we then investigated whether bilirubin renders the treated T cells incapable of re-responding to the Ag. PLP-immune T cells were stimulated for 72 h with PLP139–151 in the presence of APCs with or without bilirubin treatment. After two rounds of stimulation under the same conditions, the cells were washed, counted, and the same number of control or bilirubin-treated PLP-specific CD4+ T cells were rechallenged with PLP139–151 for 48 h in the absence of bilirubin. Cell proliferation was determined by [3H]thymidine incorporation, and IFN-γ in the supernatants was measured by ELISA. All results are representative of three independent experiments. #, p < 0.05; ##, p < 0.01 vs controls.

**FIGURE 4.** Bilirubin suppresses inducible expression of costimulatory molecules. A, PLP-immune spleen cells were activated with 50 μg/ml PLP139–151 in the presence or absence of 150 μM bilirubin. After 48 h of culture, CD28 and CTLA-4 expression was measured by flow cytometry with gating on live CD4+ T cells. B7-1 and B7-2 expression was analyzed with gating on live macrophages (CD11b+) or DCs (CD11c+). Histograms and quantifications (mean fluorescence intensity, MFI) are shown. B, PLP-specific CD4+ T cells were stimulated for 72 h with 50 μg/ml PLP139–151 in the presence of APCs with or without 150 μM bilirubin treatment. After two rounds of stimulation under the same conditions, the cells were washed, counted, and the same number of control or bilirubin-treated PLP-specific CD4+ T cell lines were rechallenged with PLP139–151 for 48 h in the absence of bilirubin. Cell proliferation was determined by [3H]thymidine incorporation, and IFN-γ in the supernatants was measured by ELISA. All results are representative of three independent experiments. #, p < 0.05; ##, p < 0.01 vs controls.

Bilirubin inhibits NF-κB activation

Bilirubin appears able to act directly on T cells since proliferation of purified CD4+ T cells in response to anti-CD3 plus anti-CD28 mAb costimulation, which stimulates T cells in an APC-independent manner, was also significantly suppressed (Fig. 1B). To further understand the potential mechanisms through which bilirubin regulates T cell immune response, we examined the impact of bilirubin treatment on NF-κB, which is a key transcription factor involved in TCR-mediated signaling (20). Purified mouse CD4+ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 24 h with or without 150 μM bilirubin. Nuclear, cytoplasmic, and total cell protein extracts were subsequently prepared for Western blot assays and EMSA. Western blot studies showed that the level of NF-κB in the nucleus was dramatically decreased in the bilirubin-treated CD4+ T cells (Fig. 5A). The decreased level of NF-κB in the nucleus of bilirubin-treated CD4+ T cells was not due to an overall suppression of NF-κB in these cells, since no significant difference was observed for total cellular NF-κB level after bilirubin treatment (Fig. 5A). The results suggest that bilirubin interferes with NF-κB nuclear translocation following activation.

In resting cells, NF-κB dimers are in the cytoplasm in an inactive state, bound to IκB. Upon T cell activation, NF-κB is translocated into the nucleus after disassociating from phosphorylated IκB (16, 21). Since bilirubin has widespread inhibitory effects on protein phosphorylation (14, 22), we hypothesized that bilirubin might interfere with IκB degradation by inhibiting its phosphorylation. Our results demonstrated that this is indeed the case. As shown in Fig. 5B, bilirubin markedly suppressed activation-induced IκB phosphorylation. Consequently, the DNA binding activity of NF-κB was significantly inhibited by bilirubin treatment (Fig. 5C). Furthermore, our in vitro experiments suggested that bilirubin could also directly interfere with the binding of NF-κB to DNA, while conjugated bilirubin was devoid of this activity (Fig. 5D).

**Bilirubin inhibits the activation-induced expression of class II MHC molecules in APCs**

It is noteworthy that the inhibitory effects of bilirubin were strongest on Ag-specific CD4+ T cell proliferative responses (Fig. 1B),
Figure 5. Bilirubin inhibits NF-κB activation. A. Purified SJL/J mouse CD4+ T cells were stimulated with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 mAbs in the presence or absence of 150 μM bilirubin. After 24 h of culture, nuclear, cytoplasmic, and total cell protein extracts were prepared for Western blot assays and EMSA. The protein levels of NF-κB in nuclear, cytoplasmic, and total cell extracts were measured by Western blot assays with anti-IκB Ab. For this study, the cells were pretreated with bilirubin for 1 h before stimulation. C. Nuclear NF-κB DNA binding activity was examined by EMSA after 24 h of culture. D. To investigate whether bilirubin can directly inhibit the binding of NF-κB to DNA, CD4+ T cells were stimulated with anti-CD3/anti-CD28 mAbs for 24 h without bilirubin treatment. Nuclear extracts were prepared and then incubated with bilirubin vs conjugated bilirubin. EMSA was then performed as in C. All results are representative of three independent experiments.

Figure 6. Bilirubin inhibits activation-induced class II MHC expression in APCs. A. PLP-immune spleen cells were activated with 50 μg/ml PLP<sub>139–151</sub> in the presence or absence of 150 μM bilirubin. After 48 h of culture, class II MHC expression was analyzed by flow cytometry using anti-I-A mAb in macrophages and DCs, the important APCs for T cell reactivity. During the immune responses, the expression of class II MHC was significantly increased in these APCs (Fig. 6A). Treatment with 150 μM bilirubin significantly suppressed activation-induced up-regulation of this molecule in both macrophages (p < 0.01) and DCs (p < 0.01) (Fig. 6A). Addition of IFN-γ (50 U/ml) 24 h after bilirubin treatment did not overcome the suppression mediated by bilirubin (Fig. 6A). The data suggest that the inhibitory effect of bilirubin on inducible expression of class II MHC was not simply due to the decreased production of IFN-γ after the treatment. Therefore, high levels of bilirubin can also affect TCR signaling.

CIITA is a cell-specific, cytokine-inducible, and differentiation-specific transcription factor that is the master regulator of MHC II gene expression (23, 24). To assess whether the suppressive effect of bilirubin on MHC II expression was due to reduced CIITA transcription, specific mRNA was first analyzed in purified macrophages by RT-PCR. As shown in Fig. 6B, bilirubin significantly inhibited CIITA induction in macrophages at the mRNA level. Because IFN-γ-induced CIITA gene activation is phospho-STAT-1-dependent (23, 25), the effect of bilirubin on activation-induced STAT-1 phosphorylation was also evaluated. Western blot assays demonstrated that bilirubin also suppressed STAT-1 phosphorylation (Fig. 6C). Similar results were observed with DCs (data not shown).

Bilirubin suppresses EAE in SJL/J mice

To determine whether bilirubin also exerts immunomodulatory effects in vivo, we explored the therapeutic effect of bilirubin in EAE, a T cell-mediated autoimmune inflammatory disease of the CNS (26). Chronic EAE was induced in female SJL/J mice. From 6 to 21 DAI, animals were treated with bilirubin vs other potent antioxidants (GSH or α-tocopherol) twice daily at increasing doses until maximal effects were achieved, but without notable side effects. Most of the control mice developed severe EAE starting at 9–10 DAI. Symptoms peaked at 13–18 DAI. The animals temporarily recovered around 35 DAI, after which relapsing-remitting signs developed. Treatment with bilirubin successfully prevented the development of chronic EAE (Table I). The highest dose of bilirubin, 100 mg/kg twice daily, delayed the onset of EAE (p < 0.01) and substantially ameliorated the clinical signs (p < 0.01).

The therapeutic effect of bilirubin was long-lasting. Disease did not rebound after treatment ceased. Additionally, the second, more chronic phase was also significantly suppressed. To “mimic” realistic therapeutic circumstances, bilirubin treatment was started at 9–10 DAI. Symptoms peaked at 13–18 DAI. The animals temporarily recovered around 35 DAI, after which relapsing-remitting signs developed. Treatment with bilirubin significantly prevented the development of chronic EAE (Table I). The highest dose of bilirubin, 100 mg/kg twice daily, delayed the onset of EAE (p < 0.01) and substantially ameliorated the clinical signs (p < 0.01). The therapeutic effect of bilirubin was long-lasting. Disease did not rebound after treatment ceased. Additionally, the second, more chronic phase was also significantly suppressed. To “mimic” realistic therapeutic circumstances, bilirubin treatment was started at onset of symptoms. As shown in Table I, bilirubin at this stage effectively halted disease progression. Moreover, bilirubin treatment during the first clinical episode decreased the relapse of chronic EAE. To assess bilirubin administration efficiency, we measured mouse serum bilirubin levels after a single 100 mg/kg bilirubin injection. Basal serum bilirubin concentrations in the untreated mice were 5.8 ± 2.4 μM. Upon bilirubin administration, serum bilirubin peaked at 57.6 ± 12.6 μM at 30 min, decreased to 30.7 ± 7.8 μM after 4 h, and returned to basal levels after 8 h. The
Concentration of bilirubin in the CNS was also increased after these treatments as detected by immunohistochemical studies (Fig. 7A). No significant therapeutic effect was observed at a low dose of bilirubin, 25 mg/kg twice daily, whereas 50 mg/kg twice daily showed an intermediate effect. We did not observe significant effect of GSH treatment on EAE even with higher doses (Table I). alpha-tocopherol at the highest dose only slightly reduced the symptoms of EAE, and its maximal effect was much weaker than that observed with bilirubin treatment (Table I).

Pathological studies were performed in animals that were treated with vehicle solution and also in animals treated with bilirubin or alpha-tocopherol at the highest doses starting before the onset of symptoms. All control-treated mice at the peak of EAE developed severe inflammation in spinal cord lesions with an average inflammatory grade in the lesions was also much milder, correlating well with the alleviated EAE symptoms (1.8 ± 1.10) (p < 0.05). However, the inflammatory grade in alpha-tocopherol-treated mice was only slightly decreased when compared with control animals (2.8 ± 0.7) (p < 0.05). Overall, bilirubin delayed the onset and alleviated the severity of EAE much more efficiently than treatment with alpha-tocopherol, although many previous studies, including ours, have demonstrated that they are similarly potent antioxidants (10, 27). Therefore, the results herein suggest that bilirubin’s efficacy in vivo is not solely due to its antioxidant effect.

To define the mechanisms of the in vivo effects of bilirubin, mononuclear cells were isolated from the spleens and the spinal cords of bilirubin-, alpha-tocopherol-, or control-treated mice on 12 DAI. Purified spleen CD4+ T cells were restimulated with PLP139-151 in the presence of APCs for 48–72 h to determine their proliferation and cytokine production. As shown in Fig. 7B, the proliferation capacity of CD4+ T cells derived from bilirubin-treated animals was clearly reduced when compared with control-treated mice (p < 0.01). Also, IL-2, IFN-γ, and IL-10 production was significantly decreased (p < 0.01) IL-4 levels were generally low. However, CD4+ T cells derived from alpha-tocopherol-treated animals were comparable to controls in both proliferative capacity and cytokine production when rechallenged with PLP139-151 (Fig. 7B). Similarly, bilirubin but not alpha-tocopherol treatment interfered with the activation of CD4+ T cells in the CNS (Fig. 7C). Bilirubin significantly reduced the frequency of CNS-infiltrating CD4+ T cells undergoing cell cycle progression (BrDU+) and that of CNS-infiltrating CD4+ T cells expressing IL-2 or IFN-γ when compared with vehicle treatment (Fig. 7C). Additionally, PLP-immune T cells isolated from bilirubin-treated mice were much less efficient than control PLP-immune T cells in transferring EAE to naive mice (Table I). In contrast, treatment with alpha-tocopherol did not reduce the efficacy of PLP-immune T cells in inducing passive EAE but resulted in the usual form of adoptively transferred EAE, although it could still inhibit active EAE (Table I). All of these data indicate that bilirubin, in addition to its established antioxidant activity, also functions as a strong immunomodulator in vivo.

Finally, we assessed whether bilirubin represents an important endogenous factor in animals to defend against this autoimmune disease. In comparison to chronic EAE, acute EAE in Lewis rats offers the advantage of a predictable time of onset and uniform severity of disease. Most rats spontaneously recover completely. Therefore, it is commonly used for evaluating the effects of new therapies, especially when the treatments are supposed to deteriorate the disease. First, we examined bilirubin system activity in EAE. Serum bilirubin levels in naive and untreated EAE Lewis rats were under the detection limit. However, immunohistochemical study showed that, during EAE, both HO-1 and BVR were
FIGURE 7. Effect of bilirubin treatment on EAE. A. The effects of bilirubin vs GSH or α-tocopherol treatments on chronic and adoptive transfer EAE in SJL/J mice are detailed in Table I. The concentration of bilirubin in the spinal cords was detected by immunohistochemical study with specific bilirubin Ab. Results from one representative donor out of four are shown. Scale bar = 30 μm. B. Spleens were removed on 12 DAI from EAE mice with vehicle, bilirubin (2 × 100 mg/kg/day), or α-tocopherol (2 × 200 mg/kg/day) treatments starting from before the onset of symptoms. PLP-immune CD4+ T cells were restimulated with 50 μg/ml PLP139–151 for 72 or 48 h to determine their proliferation and cytokine production, respectively. Results from one representative out of four independent experiments are shown. *, p < 0.01 vs control. C. Mononuclear cells were isolated from the spinal cords of bilirubin, α-tocopherol, or control-treated mice on 12 DAI. For detection of proliferating T cells in vivo, animals were given i.p. injections of BrdU as described in Materials and Methods. Percentages of CNS CD4+ T cells expressing intracellular BrdU (cell cycle progression), IL-2, IFN-γ, and IL-10 were analyzed by flow cytometry. #, p < 0.05; **, p < 0.01 vs control. D. Acute EAE was induced in Lewis rats. The expression of HO-1 and BVR in the spinal cords on 12 DAI was detected by immunohistochemical study with specific Abs. Scale bars for both HO-1 and BVR staining = 30 μm. Groups of animals were treated with vehicle solution, ZnPP, and TCPOBOP, respectively, as indicated. Data were analyzed using two-way ANOVA with Fisher’s PLSD post hoc tests for multiple comparisons. #, p < 0.05; *, p < 0.01 vs control. E. Inflammation (H&E staining) and bilirubin concentrations in the spinal cord lesions on 12 DAI were compared among control-, ZnPP-, and TCPOBOP-treated EAE rats. Results from one representative donor out of three are shown. Scale bar for H&E staining = 50 μm. Scale bar for bilirubin immunohistochemistry = 30 μm.
The Journal of Immunology

Discuss the potential cytotoxicity of administered bilirubin in the CNS, lumbosacral spinal cord sections were obtained from normal mice, control EAE mice, and bilirubin-treated mice without EAE symptoms. Animals were euthanized for histological studies when control animals reached the peak of illness (around 15 DAI). Axonal pathology was examined by immunohistochemical studies with anti-β-APP Ab. Myelin structure was shown by Luxol fast blue staining. Results from one representative donor out of three are shown. Scale bar for β-APP immunohistochemical staining = 30 μm; Scale bar for Luxol fast blue staining = 20 μm.

strongly induced in the spinal cord lesions, and the concentration of bilirubin was subsequently increased (Fig. 7, D and E). As our previous study demonstrated, bilirubin system activity substantially increased as EAE progressed and reached its maximum at the peak of clinical sign (28, 29). Even after cessation of clinical disease, a high level of bilirubin system expression was still found in slowly resolving lesions (28, 29), which suggests that this endogenous inducible system may contribute to animals’ recovery from EAE. We then treated acute EAE in Lewis rats with ZnPP, a specific inhibitor of the bilirubin-producing enzyme HO-1. ZnPP markedly exacerbated acute EAE ($F(1, 126) = 186.19, p < 0.01$, Fig. 7D). In contrast to controls, many ZnPP -treated rats never recovered from disease and developed tetraplegia before euthanasia, and some died of severe EAE. Since inhibition of HO-1 can also affect the production of another immunomodulator, carbon monoxide (5, 30), we further tried to treat acute EAE with TCPOBOP, a potent activator of bilirubin clearance. Similar therapeutic effects were observed ($F(1, 126) = 175.28, p < 0.01$, Fig. 7D). Interestingly, immunohistochemical analyses showed that both treatments decreased bilirubin concentrations in the spinal cord lesions, even though the inflammatory infiltrates were extensive (Fig. 7E). To exclude any toxicity of ZnPP and TCPOBOP, we administered ZnPP or TCPOBOP daily on the same schedule to normal Lewis rats. No adverse reactions were observed.

Bilirubin treatment does not damage neural cells or myelin in the CNS

Since bilirubin is potentially neurotoxic, we examined the possible CNS cytotoxicity of administered bilirubin, even though the bilirubin-treated mice appeared normal in behavior. Histological studies were performed in lumbosacral spinal cord of normal mice, control mice, and high dosage bilirubin-treated mice without EAE symptoms. Morphological examination and quantitation of positively stained neurons (MAP2+), oligodendrocytes (CNPase+), and astrocytes (GFAP+) in spinal cord sections from bilirubin-treated animals revealed no significant differences from normal animals (data not shown). Additionally, in tissues obtained from bilirubin-treated mice, immunohistochemical studies with anti-β-APP Ab did not show axonal pathology (Fig. 8), and Luxol fast blue staining showed intact myelin structure (Fig. 8). Similar studies were performed in the medulla, the pons, and the cerebrum. No obvious neural cell damage at these higher levels of the CNS was observed in any of these examined animals (data not shown). In conclusion, bilirubin treatment did not cause neural cell damage in the CNS.

Discussion

In the present study, we analyzed the immunomodulatory effects of bilirubin, a bile pigment that is abundant in mammalian serum. Our results clearly showed that bilirubin represents a molecule of immunologic importance. Bilirubin has a powerful suppressive effect on both polyclonal and Ag-specific T cell responses. Bilirubin suppresses CD4+ T cell reactivity through multiple mechanisms, including inhibition of TCR signaling, down-regulation of co-stimulatory activity, suppression of immune transcription factor activation, and induction of reactive T cell apoptosis when used at high concentrations. By inhibiting activation-induced expression of class II MHC molecules in APCs, it may affect Ag presentation to CD4+ T cells and therefore impair subsequent T cell response. In reactive CD4+ T cells, bilirubin can cause anergy via its inhibitory effects on costimulatory signaling. The effect of bilirubin on the expression of the key costimulators is specific. It significantly suppresses the up-regulation of CD28, B7-1, and B7-2 upon activation, but it does not alter the expression of CTLA-4 by reactive CD4+ T cells. The data also provide evidence that our findings are not a general phenomenon related to reduction of protein synthesis after bilirubin treatment. Additionally, bilirubin can directly interfere with immune transcription factor (NF-κB) activation, as proliferation of purified CD4+ T cells in response to anti-CD3 plus anti-CD28 mAb costimulation, which stimulates T cells in an APC-independent manner, was also significantly suppressed. Furthermore, in vitro data suggest that bilirubin may also directly block the binding of NF-κB to DNA.

Bilirubin has widespread inhibitory effects on protein phosphorylation (14, 22), which may contribute to its neurotoxicity. It has been shown that bilirubin inhibits protein kinase C and cAMP-dependent, cGMP-dependent, and Ca2+-calmodulin-dependent protein kinases at concentrations ranging from 20 to 125 μM (22, 31). These kinases initiate and regulate various signal transduction processes, including those involved in the immune response (16, 23). Our results demonstrate that suppression of protein phosphorylation also represents an important mechanism by which bilirubin exerts its powerful immunosuppressive effects. Bilirubin can strongly inhibit inducible STAT-1 and IκB phosphorylation, which are the prerequisites for CIITA and NF-κB activation, respectively (21, 25). Through these effects, bilirubin suppresses class II MHC expression and T cell reactivity. The mechanisms by which bilirubin down-regulates activation-induced CD28, B7-1, and B7-2 expression were not thoroughly explored in this study, partially because the regulation of expression of these molecules is not well established. It is reasonable to hypothesize that similar mechanisms may be involved. Additionally, as regards the effect of bilirubin on surface receptors, its direct interaction with cell membranes should also be taken into account, since bilirubin can be associated intimately with cell membranes due to its lipophilic nature (32, 33). Bilirubin may bind to cell membranes and thus interfere by a hitherto unknown mechanism with various receptors involved in the immune reactivity of these cells. For example, Vetticka et al.’s data suggest that binding of bilirubin to the macrophage membrane changed the lipid environment of the plasma membrane, which in turn significantly influenced the expression of some Fc receptors (8, 34). The action of bilirubin in this respect should be further analyzed and will be the topic of a different study. Only free unconjugated bilirubin can easily enter cells by
passive diffusion. One may speculate that this lipophilic property is essential to the effects of bilirubin on both membrane-associated molecules and immune transcription factors. Indeed, consistent with all of the above findings, our results demonstrate that water-soluble conjugated bilirubin has no detectable immunomodulatory activity, although it remains a powerful antioxidant (35, 36). However, it is noteworthy that most of the effects of bilirubin described above were only marginal at low concentrations (e.g., at 50 μM), even though at the same concentrations, it still significantly inhibited T cell proliferation. It seems that the potent immunosuppressive effect of bilirubin is a consequence of combined activities. Other unidentified mechanisms may also be involved. Further study is required to define a broader spectrum of bilirubin’s immunomodulatory actions.

Our data indicate that bilirubin directly suppresses T cell responses, rather than via the induction of active immunosuppression or immune deviation from a Th1 toward a Th2-like response. Bilirubin did not increase the generation of CD4+ Foxp3+ Treg cells. The reduced production of IL-10 by reactive CD4+ T cells after treatment also suggests that bilirubin did not induce IL-10-producing Treg 1 cells. While bilirubin could strongly inhibit Th1 cell response, the therapeutic effect was not associated with a shift to Th2 cell polarization. Our results are consistent with previous findings. As mentioned earlier, high levels of bilirubin can impair the formation of various AbS in infants (6). In recent years, it has also been demonstrated that bilirubin can protect against Th2-associated diseases. For example, Ohru et al. reported a case of significant relief of asthma symptoms during jaundice (37). All these data suggest that bilirubin may not enhance Th2 cell immune response.

Furthermore, we demonstrate that bilirubin also acts as an immunosuppressant in vivo and inhibits EAE. Bilirubin prevented EAE effectively, and it was also active in the therapeutic setting. It halted disease progression even when administered after the onset of clinical EAE. More importantly, in contrast to the traditional immunosuppressive agents such as dexamethasone (10), the therapeutic effect of bilirubin was long-lasting after treatment ceased, and it improved the long-term course of chronic EAE successfully. Analyses of animals with EAE showed that the clinical protection afforded by bilirubin was associated with changes in the PLP-specific autoimmune response. Studies of CD4+ T cell activation in the spleens and the CNS demonstrated a clear reduction of proliferation in the bilirubin-treated animals, accompanied by significantly decreased production of both Th1 and Th2 cytokines, including IL-2, IFN-γ, and IL-10. Moreover, bilirubin treatment impaired the ability of PLP-immune T cells to induce EAE by adoptive transfer. In contrast, α-tocopherol did not cause a change in T cell immune function although it still inhibited clinical EAE as an antioxidant. Our results therefore provide strong evidence that bilirubin functions as an immunomodulatory agent both in vitro and in vivo. Additionally, bilirubin proves to be an important endogenous factor with which animals defend against EAE. Bilirubin system activity substantially increased in the spinal cord lesions of EAE from the onset to the peak of illness, and its expression correlated well with recovery from disease. Treatment of EAE with ZnP or TCPOBOP, which inhibit the production of bilirubin and activate its clearance, respectively, was fatal to most of the treated animals. The severity of clinical signs was exacerbated severely at the peak period of disease. However, contrary to our expectation, neither ZnP nor TCPOBOP advanced the onset of EAE. Later, we found that this was because bilirubin system could not be strongly induced in SJL/J mice or Lewis rats at the early stages of EAE. Additionally, we note that the basal serum bilirubin levels of SJL/J mice and Lewis rats are much lower than those of some other strains of mice and rats (38, 39), and serum bilirubin concentrations in them were not significantly up-regulated after induction of EAE. All these factors may contribute to their susceptibility to this autoimmune disease.

Interestingly, our data indicate that the strong immunosuppressive effect of bilirubin cannot be attributed to its antioxidant activity. Previous studies have demonstrated that ROS play an important role in the proliferation of some cell types, such as airway and vascular muscle cells (14, 40). In certain circumstances, ROS can induce proinflammatory cytokine expression (41). However, our results suggest that free radicals do not play an essential role in CD4+ T cell proliferative responses, as several other powerful antioxidants could not mimic the effect of bilirubin to any degree, including conjugated bilirubin, GSH, and α-tocopherol, another potent lipophilic antioxidant. In recent decades, the recognition of bilirubin has been transformed from that of a waste product without beneficial effects to that of a biologically important antioxidant with a wide range of protective actions. While most interest in bilirubin as a potential therapeutic agent lies in its antioxidant properties, increasing evidence suggests that bilirubin may also possesses other physiological functions, including its immunomodulatory effects. In recent years, bilirubin has been found to play a protective role in a variety of immune-related disorders. For example, Arriaga et al. demonstrated that unconjugated bilirubin inhibited complement-mediated intravascular hemolysis (42). Wang et al. showed that bilirubin could suppress the expression of some proapoptotic and proinflammatory genes and prolong the survival of islet allografts (39). Additionally, in traditional Oriental medicine animal biles have proved useful in the treatment of bronchitis, asthma, and hypersensitivity. However, currently, the activities of bilirubin in this respect are not well understood. The present study is among the first to systematically explore the immunological properties of bilirubin. Our results demonstrate that bilirubin is an important immunomodulator of functional significance, and these data underline the therapeutic potential of this molecule in multiple sclerosis and other immune diseases.

Acknowledgments
We thank Tariq Aziz for valuable assistance with human PBMC proliferation assays.

Disclosures
The authors have no financial conflicts of interest.

References


