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Lack of Tumor Necrosis Factor Alpha Induces Impaired Proliferation of Hepatitis B Virus-Specific Cytotoxic T Lymphocytes

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Recent studies have shown that tumor necrosis factor alpha (TNF-α) plays critical roles in not only viral clearance but also lymphoid tissue development and stem cell differentiation. In this study, we attempted to induce hepatitis B virus (HBV)-specific cytotoxic T lymphocytes (CTLs) by immunization of TNF-α knockout (TNF-$\alpha^{-/-}$) mice with HBsAg-encoding plasmid DNA. An immunization with the HBV plasmid failed to induce CTL responses in TNF-$\alpha^{-/-}$ mice, although CTLs were readily induced in wild-type mice by the same protocol. Weak CTL responses were produced in TNF-$\alpha^{-/-}$ mice after two sessions of immunization with the HBV plasmid; however, TNF-α was required to maintain the responses of these CTL lines to in vitro stimulation and, even then, the responses were lost after 3 weeks. Interestingly, a limiting dilution of a CTL line showed that HBV-specific CTL clones with high specific cytotoxicity were present in TNF-$\alpha^{-/-}$ mice, but these clones again failed to proliferate for more than 3 weeks. Furthermore, since exogenously added TNF-α enhanced the proliferation of a TNF-$\alpha^{-/-}$ clone but suppressed that of a TNF-$\alpha^{+/+}$ clone in vitro, TNF-α also has a direct effect on the proliferation of CTLs. In conclusion, TNF-α is essential rather than important for the proliferation of HBV-specific CTLs both in vivo and in vitro and this effect is not only due to the activation of dendritic cells but is also induced by the direct effect on CTLs.

Since major histocompatibility complex class I (MHC-I)-restricted cytotoxic T lymphocytes (CTLs) play a major role in viral clearance and immunopathology (8, 14, 26), it is important to understand the mechanisms of CTL induction in vivo. It is known that tumor necrosis factor alpha (TNF-α) is released by macrophages, $CD4^+$ and $CD8^+$ T cells, B cells, NK cells (45), and dendritic cells (DCs) (4). In particular, recent studies have shown that TNF-α is also released by $CD8^+$ CTLs (38) and contributes to CTL-mediated cytotoxicity, although its cytolytic activity is not as high as those of perforin and Fas ligand (Fas-L) (1, 9, 28, 32, 33). However, TNF-α is involved not only in cytotoxicity but also in viral gene regulation (17, 21, 22, 23). In hepatitis B virus (HBV) transgenic mice, HBV-specific CTLs abolish HBV gene expression and replication without killing hepatocytes (21, 23), mainly via up-regulation of TNF-α production.

Recently, gene knockout technology was used to establish TNF-α-deficient mice, and these were found to have impaired immune systems (44), suggesting that TNF-α may play an important role in the development of the immune system, including DC function (5, 34, 36, 39). Furthermore, TNF-α is a differentiation factor for several types of stem cells, such as hepatocytes (7, 37, 47), hematopoietic progenitor cells (6, 42), and embryonic stem cells (30). However, there have also been several reports that TNF-α is an inhibitory mediator for stem cell proliferation; these differences probably depend on the differentiation stage and on differences in TNF-α receptor expression (6).

Given this information, we speculated that TNF-α might be a potent synergistic factor for the differentiation or proliferation of CTLs and therefore tried, in the present study, to induce HBV-specific CTLs by DNA immunization (40) of TNF-$\alpha^{-/-}$ mice in order to evaluate the role of TNF-α.

We report here that, although it is quite difficult to prime and expand HBV-specific CTLs in TNF-$\alpha^{-/-}$ mice, HBV-specific CTL clones with high levels of cytotoxic activity are present in these mice. Our observations indicate that TNF-α is essential for sufficient proliferation of CTLs but not for their differentiation to mature functional CTLs.

MATERIALS AND METHODS

Mice. TNF-$\alpha^{-/-}$ mice were originally established from C57BL/6 J (*H-2*^{b}) mice (44) and then backcrossed on to B10.D2 mice (*H-2*^{d}). B10.D2 and AKR (*H-2*^{k}) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were bred and kept under standard pathogen-free conditions. The experiments were conducted according to institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

Cell lines. The *H-2*^{d} mastocytoma cell line P815 was obtained from the American Type Culture Collection (Manassas, Va.). P815 expressing HBV pre-S1, 2, and S (P815preS1) and the HBsAg-specific $CD8^+$ CTL clone 6C2 were generously provided by Francis V. Chisari (Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, Calif.) (2, 3).

Nucleic acid immunization. Plasmid DNA (40), which was generously provided by Francis V. Chisari, was used for immunization as follows: (i) one injection of 100 μg of plasmid DNA into each quadriceps muscle 5 days after injection of 50 μl of cardiotoxin (10 mM in phosphate-buffered saline) (Latoxan, Rosans, France) as described previously (12, 13) or (ii) two injections of 100 μg of plasmid DNA into each quadriceps muscle, each given 5 days after injection of 50 μl of cardiotoxin. The interval between the two plasmid DNA injections

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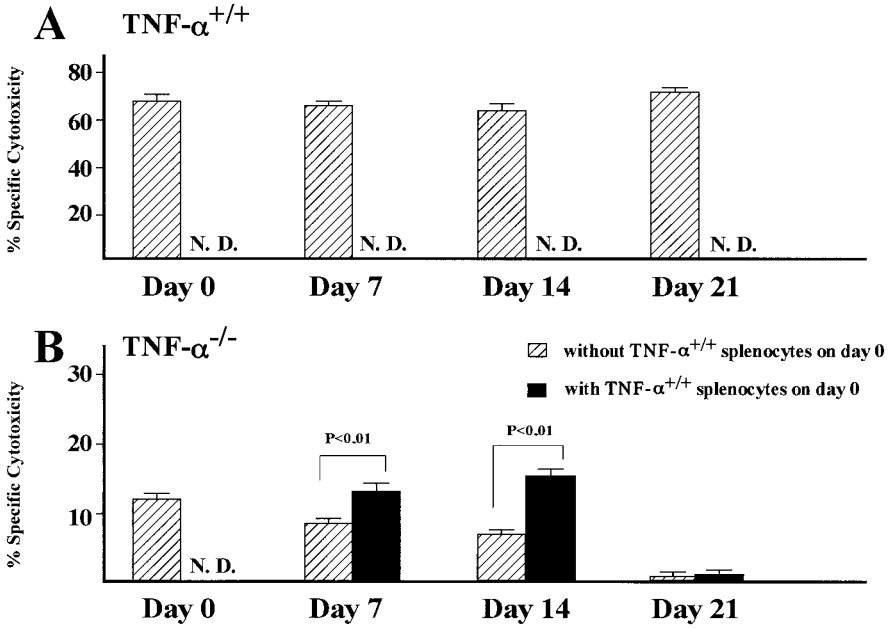

FIG. 1. Cytotoxic activity of HBsAg-specific CTLs induced in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. (A) TNF-$\alpha^{+/+}$ mice; (B) TNF-$\alpha^{-/-}$ mice. Mice were immunized by two cycles of injection of plasmid into the bilateral quadriceps muscles 5 days after cardiotoxin injection. Splenocytes prepared 2 weeks after the final immunization were incubated with or without MMC-treated TNF-$\alpha^{+/+}$ splenocytes on day 0. For the assay, effector cells were incubated for 4 h with Eu-labeled target cells at an effector-to-target cell ratio of 20, and the percent specific cytotoxicity was calculated by subtracting the percent cytotoxicity for P815 cells (HBsAg negative) from that for P815preS1 cells (HBsAg positive). Spontaneous release was always less than 20% of the total. Each data point represents the mean of triplicate samples. N.D., not done.

was 2 weeks. Spleen cells were prepared from immunized mice 2 weeks after the final immunization.

Establishment of HBsAg-specific CTLs. The immunized spleen cells (4×10^6/well) were cultured in 24-well plates with mitomycin C (MMC)-treated P815preS1 (2×10^5/well) in complete RPMI 1640 (Sigma-Aldrich, St. Louis, Mo.) containing 10% heat-inactivated fetal calf serum (Sigma-Aldrich) and 5% EL4 supernatant as a source of T-cell growth factor. In some experiments, immunized TNF-$\alpha^{-/-}$ spleen cells (2×10^6/well) were cultured with or without MMC-treated TNF-$\alpha^{+/+}$ spleen cells (2×10^6/well) on day 0 in culture. In all experiments, the immunized spleen cell lines (and clones) (see below) were restimulated with MMC-treated TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ spleen cells (2×10^6/well) and P815preS1 (2×10^5/well) every 7 days. To establish CTL clones, CTL lines were plated at a rate of 0.3 cells/well in 96-well round-bottom plates (Corning, Corning, N.Y.) in medium with MMC-treated TNF-$\alpha^{+/+}$ splenocytes (4×10^5/well) and P815preS1 (1×10^4/well). After several weeks of repeated stimulation (see above), growing cells were tested for HBsAg-specific cytotoxicity as described below.

Cytotoxicity assay. The cytolytic activity of HBV-specific CTLs was assessed by using a europium (Eu) release assay as described previously (29). Target cells (P815, P815preS1, or P815 plus HBsAg peptide [S28-39; Kurabo Industries, Osaka, Japan]) were labeled with Eu diethylenetriaminepentaacetate (80 mmol/liter; Wako Pure Chemical Industries Ltd, Osaka, Japan). Labeled targets (5×10^3 cells) 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 4 h at 37°C. Then 20 μl of the culture supernatant was mixed with 100 μl of enhancing solution (Wallac Oy, Tuerku, Finland), and the released Eu was measured by using a time-resolved fluorometer (1230 Arcus; Wallac Oy). The percentage of Eu release was determined as follows: ([experimental release − spontaneous release]/[maximal release − spontaneous release]) × 100. The maximal release was measured after lysis with Triton X-100; spontaneous release was 10 to 20% of the maximal release.

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated by using an RNAqueous isolation kit (Ambion Inc., Austin, Tex.) and reverse transcribed into cDNA by using an Omniscript reverse transcriptase kit (QIAGEN GmbH, Hilden, Germany). To test whether the cells were capable of producing TNF-α, CTLs were stimulated for 6 h with plate-bound anti-CD3ε antibody (BD Pharmingen, San Diego, Calif.) before RNA isolation (25, 38). Sense and antisense primers were based on CLONTECH Amplimer sets (CLONTECH Laboratories, Inc., Palo Alto, Calif.) for mouse TNF-α and glyceraldehyde-3-phosphate dehydrogenase, and the mouse TNF RI and RII PCR Primer Pair (R&D systems, Minneapolis, Minn., and Ambion Inc., respectively) was used for mouse TNF receptor I and II.

Preparation of $CD8^+$ T cells, $CD4^+$ T cells, and $CD11c^+$ DCs. The cell separation from the spleen was performed by using CD8a magnetic microbeads for $CD8^+$ cells, CD4 magnetic microbeads for $CD4^+$ T cells, and CD11c magnetic microbeads for $CD11c^+$ DCs, respectively (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The magnetically labeled cells were purified at a level of more than 95% by using the VarioMACS system (Miltenyi Biotec GmbH).

Precursor frequency of HBsAg-specific $CD8^+$ cells. Since immunodominant HBsAg-specific CTLs in mice are known to be restricted by *H-2L*^{d} of MHC-I and since the shortest optimal peptide is the HBsAg peptide (S28-39) (2), peptide-loaded recombinant soluble dimeric mouse *H-2L*^{d}–immunoglobulin (di-*H-2L*^{d}–Ig [mouse IgG1]; BD PharMingen) was prepared by mixing di-*H-2L*^{d}–Ig for 48 h at 4°C with a 160 M excess of HBsAg peptide (S28-39). The peptide-loaded di-*H-2L*^{d}–Ig (4 μg) was then added to $CD8^+$ cells prepared from immunized TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ splenocytes or restimulated splenocytes (MMC-treated TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ splenocytes were used as feeder cells, respectively). After incubation for 1 h at 4°C, the cells were stained by fluorescein isothiocyanate-conjugated anti-mouse IgG1 and phycoerythrin (PE)-conjugated anti-mouse CD8a (BD PharMingen). The proportion of HBsAg-specific cells was measured by flow cytometric analysis on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Effect of TNF-α on CTL proliferation in vitro. To study the effects of TNF-α on the proliferation of CTL clones, 0.01 to 0.10 ng of recombinant mouse TNF-α (Genzyme/Techne, Framingham, Mass.)/ml or 5 μg of TN3 19.12/ml, a hamster monoclonal antibody (MAb) specific for mouse TNF-α (generously provided by Robert D. Schreiber, Department of Pathology, Washington University School of Medicine, St Louis, Mo.), was added to the culture. Purified hamster IgG (Jackson ImmunoResearch, West Grove, Pa.) was used as a control antibody. To block the release of TNF-α from the cell membrane, matrix metalloproteinase (MMP) inhibitor (KB8301; BD PharMingen) was added at a concentration of 10 μM. KB8301, chemically referred to as [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphthyl) alanine-*N*-methylamide, is a hydroxamic acid inhibitor of MMPs and blocks the cleavages of TNF-α and

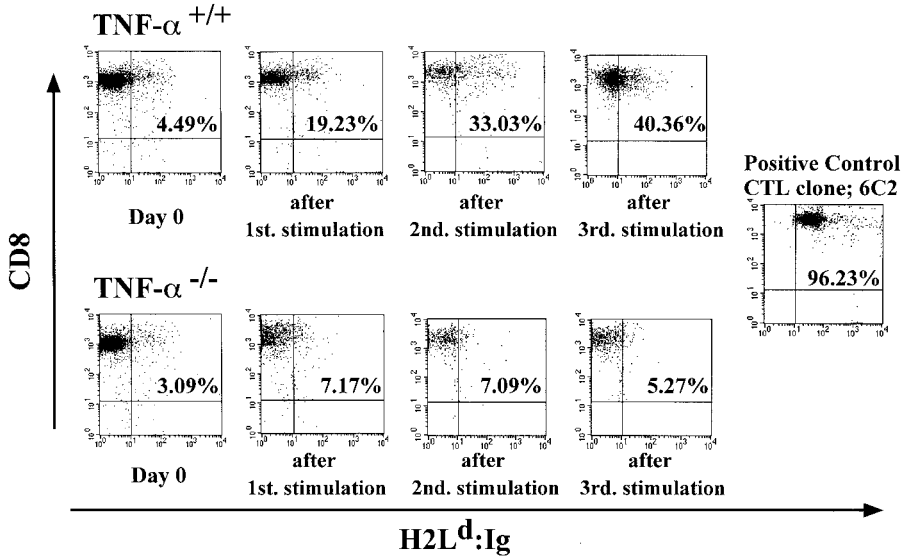

FIG. 2. Flow cytometric analysis of the frequency of HBsAg-specific $CD8^+$ cells in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Mice were immunized by two cycles of injection of plasmid into the bilateral quadriceps muscles 5 days after cardiotoxin injection, and splenocytes were prepared 2 weeks after the final injection. In order to evaluate differences in HBsAg-specific cells in immunized splenocytes, the $CD8^+$ *H-2L*^{d}–Ig^+ fraction (upper right) was used as the HBsAg-specific range. The HBsAg-specific range of the TNF-$\alpha^{+/+}$ CTL line gradually increased by more than 40% after the third stimulation in vitro. On the other hand, the HBsAg-specific range of the TNF-$\alpha^{-/-}$ CTL line did not expand after stimulation in vitro. The HBsAg-specific range of the TNF-$\alpha^{+/+}$ HBsAg-specific CTL clone 6C2 (established after immunization with recombinant vaccinia virus) was more than 95%. Similar results were obtained in three separate experiments. In this experiment, immunized TNF-$\alpha^{-/-}$ splenocytes were restimulated with MMC-treated TNF-$\alpha^{-/-}$ splenocytes and P815preS1 and TNF-$\alpha^{+/+}$ spleen cells were restimulated with MMC-treated TNF-$\alpha^{+/+}$ splenocytes and P815preS1 every 7 days, respectively.

Fas-L, resulting in decreased levels of the soluble forms of these molecules. Since the MMP inhibitor stock solution was prepared in dimethyl sulfoxide (Sigma Chemical Co.) and then diluted 1,000-fold in culture medium for use, 0.1% dimethyl sulfoxide was added to control cultures. The CTL clones (2×10^5/well) were stimulated with MMC-treated TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ splenocytes and P815preS1 every 7 days. The number of CTLs was counted, and the proliferative ratio was calculated as the number of CTL 1 week after the last stimulation/number of CTL before the last stimulation (2×10^5/well).

Analysis of surface markers on $CD11c^+$ DCs from TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. The surface molecule expression of splenic DCs was assessed by flow cytometric analysis. To evaluate the expression intensities of MHC and costimulatory molecules, mouse or rat biotin-conjugated anti-mouse MHC-I (*H-2L*^{d}), MHC-II (*I-A*^{d}/*I-E*^{d}), PE-labeled streptavidin (BD PharMingen), rat PE-conjugated anti-mouse CD80 and CD86 (Cedarlane, Ontario, Canada) were used for staining.

MLR. To measure DC function in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice, the allostimulatory capacity of purified $CD11c^+$ DCs was assessed by using the mixed lymphocyte reaction (MLR). Allogeneic murine $CD4^+$ T cells were obtained from AKR mice splenocytes (*H-2*^{k}). MMC-treated DCs were plated at 1×10^2 to 2×10^4 cells/well in 96-well flat-bottom culture plates, then the $CD4^+$ T cells were added (2×10^5/well), and the cells were cultured for 5 days at 37°C in 5% CO_2. Cell proliferation was assessed by using the 5-bromo-2′-deoxyuridine labeling and detection kit III (Roche Diagnostics Co., Indianapolis, Ind.), with the absorbance measured with a microtiter plate reader.

Statistics. Values are expressed as means ± standard deviations (SD). The differences between the experimental and control groups were analyzed by the Kruskal-Wallis test followed by Scheffé's F test. *P* values of <0.05 were considered significantly different.

RESULTS

CTL responses in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Two methods of immunization with plasmid DNA were used to prime HBsAg-specific CTLs in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. A single injection into the bilateral quadriceps muscles induced high specific CTL responses (65.4% specific lysis at an effector/target cell ratio of 20) in TNF-$\alpha^{+/+}$ mice, but not in TNF-$\alpha^{-/-}$ mice (data not shown), while the same approach, but with two injections, induced specific CTL responses in both TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice (specific lysis of 69.3 and 12.2%, respectively, on day 0 in vitro at an effector/target cell ratio of 20) (Fig. 1). In case of two injections, the TNF-$\alpha^{+/+}$ CTL lines maintained their high cytotoxic activity (Fig. 1), whereas the TNF-$\alpha^{-/-}$ CTL lines showed initially a relatively low cytolytic activity, which fell below 10% within a week. The addition of MMC-treated TNF-$\alpha^{+/+}$ spleen cells to the cultures on day 0 allowed the TNF-$\alpha^{-/-}$ CTL lines to maintain their specific cytotoxicity at a level greater than 10% for 2 weeks, but cytotoxicity was again lost by day 21 (Fig. 1).

Frequency of HBsAg-specific $CD8^+$ cells in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. HBsAg-specific $CD8^+$ cell counts in splenocytes from immunized TNF-$\alpha^{+/+}$ mice were higher than those in splenocytes from immunized TNF-$\alpha^{-/-}$ mice (Fig. 2). By the stimulations in vitro, the counts markedly increased in TNF-$\alpha^{+/+}$ CTL lines but not in TNF-$\alpha^{-/-}$ CTL lines. Five days after the third stimulation in vitro, the counts expanded in the TNF-$\alpha^{+/+}$ line (40.36%) but not in the TNF-$\alpha^{-/-}$ line (5.27%). The HBsAg-specific range of TNF-$\alpha^{+/+}$ HBsAg-specific CTL clone 6C2 was more than 95% (Fig. 2).

HBsAg-specific CTL clones with high cytolytic activity are present in TNF-$\alpha^{-/-}$ mice. Limiting dilution was used to obtain some TNF-$\alpha^{-/-}$ CTL clones; these were HBsAg specific (Fig. 3A) and the specific peptide was S28-39 (data not shown). Interestingly, their cytolytic activity was higher than that of TNF-$\alpha^{-/-}$ CTL lines but lower than that of TNF-$\alpha^{+/+}$ CTL clones (Fig. 1 and 3A). RT-PCR revealed that, although nei-

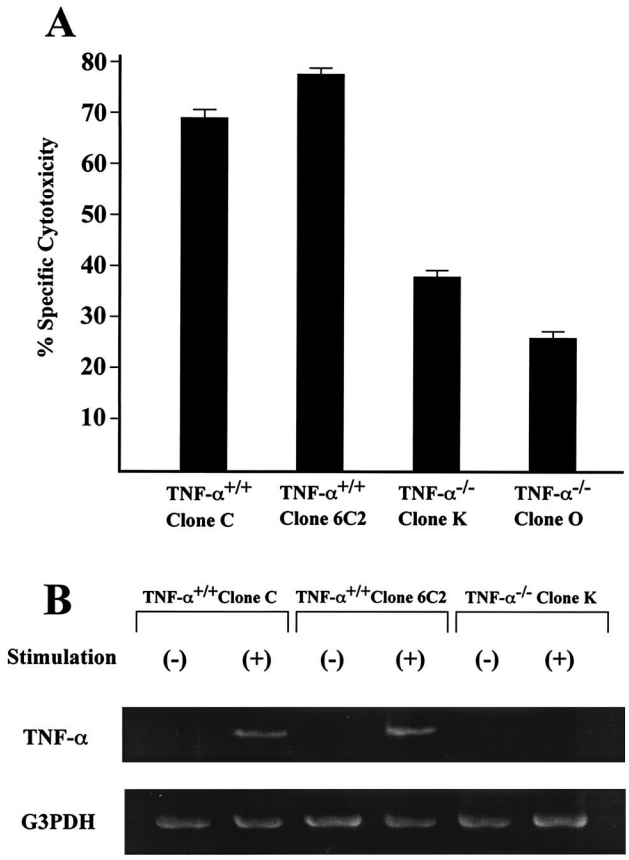

FIG. 3. Generation of HBsAg-specific TNF-$\alpha^{-/-}$ CTL clones. Immunized splenocytes were incubated with MMC-treated TNF-$\alpha^{+/+}$ splenocytes on day 0 and stimulated once a week with MMC-treated TNF-$\alpha^{+/+}$ splenocytes (feeder cells) and P815preS1 cells (specific stimulator). After the second stimulation, the primed splenocytes were cloned at a rate of 1 cell/well. (A) Results of a cytotoxicity assay performed as described in the legend to Fig. 1, at an effector-to-target cell ratio of 20. Each data point represents the mean of triplicate samples. (B) Expression of TNF-α mRNA confirmed by RT-PCR. To avoid detecting TNF-α mRNA expression in feeder and P815preS1 cells, the CTL clones were stimulated with plate-bound anti-CD3ε antibody. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

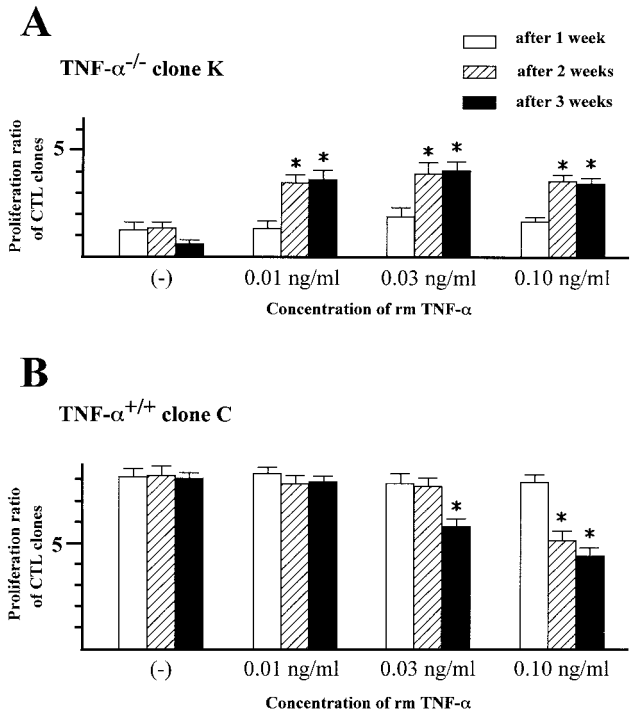

FIG. 4. Effect of exogenous sTNF-α on the proliferation of TNF-$\alpha^{-/-}$ (A) and TNF-$\alpha^{+/+}$ (B) CTL clones. At each weekly stimulation with irradiated TNF-$\alpha^{+/+}$ splenocytes (feeder cells) and P815preS1 (specific stimulator), HBsAg-specific TNF-$\alpha^{-/-}$ CTL clones were incubated with sTNF-α at concentrations of 0.01 to 0.1 ng/ml and the number of CTLs was counted 1 week after stimulation. The proliferative ratio was calculated as the number of CTLs 1 week after stimulation/the number of CTLs before stimulation. Each bar and error bar represents the mean and the SD, respectively, of results for triplicate samples. *, $P < 0.001$ versus the control group; rmTNF-α, recombinant mouse TNF-α; (−), no TNF-α was used.

ther set of clones expressed TNF-α mRNA in the absence of stimulation, TNF-$\alpha^{+/+}$ CTL clones, but not TNF-$\alpha^{-/-}$ CTL clones, did so after stimulation (Fig. 3B). In contrast, TNF receptors I and II were detected in both types of CTLs after stimulation (data not shown).

Exogenously added TNF-α enhances the proliferation of TNF-$\alpha^{-/-}$ CTL clones but suppresses that of TNF-$\alpha^{+/+}$ CTL clones. In order to evaluate the effect of TNF-α on the proliferation of HBsAg-specific CTL, we added exogenous TNF-α to the cultures at concentrations of 0.01, 0.03, and 0.1 ng/ml. As shown in Fig. 4A, although the proliferation ratio of TNF-$\alpha^{-/-}$ CTL clones was lower than that of TNF-$\alpha^{+/+}$ CTL clones in the absence of added TNF-α, it was increased by exogenous TNF-α, with a maximal increase seen at the concentration of 0.03 ng/ml. In contrast, the proliferation ratio of TNF-$\alpha^{+/+}$ CTL clones was not increased by TNF-α at any concentration tested but was markedly suppressed by the higher concentrations of TNF-α (Fig. 4B).

Effect of TNF-$\alpha^{-/-}$ feeder cells, neutralizing anti-TNF-α MAb, or MMP inhibitor on the proliferation of TNF-$\alpha^{+/+}$ HBsAg-specific CTL clones. TNF-$\alpha^{+/+}$ HBsAg-specific CTL clones 6C2 and C (established after immunization with HBV plasmid) were used. When MMC-treated TNF-$\alpha^{-/-}$ splenocytes were added as feeder cells, the proliferation of both clones was significantly lower than when the feeder cells were MMC-treated TNF-$\alpha^{+/+}$ splenocytes (Fig. 5A). However, proliferation was not completely zero in the presence of TNF-$\alpha^{-/-}$ feeder cells, probably as a result of TNF-α released from CTLs. When neutralizing anti-TNF-α MAb was added to cultures using either set of MMC-treated splenocytes as feeder cells, the proliferation of these clones was significantly suppressed compared to that of the control group (Fig. 5B). MMP inhibitor, which completely blocks the formation of the soluble form of TNF-α (sTNF-α), dramatically inhibited the proliferation of TNF-$\alpha^{+/+}$ CTLs in vitro, irrespective of the MMC-treated feeder cells used (Fig. 5C).

Comparison of DC functions in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. Since TNF-α can affect DC function, CD11c^{+} TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ DCs were also analyzed by MLR and flow cytometry. Because HBV-specific CTL clones are *H-2L*^{d} restricted, expression of *H-2L*^{d} as the MHC-I molecule of the DC was analyzed. Since the maturity of DCs is generally esti-

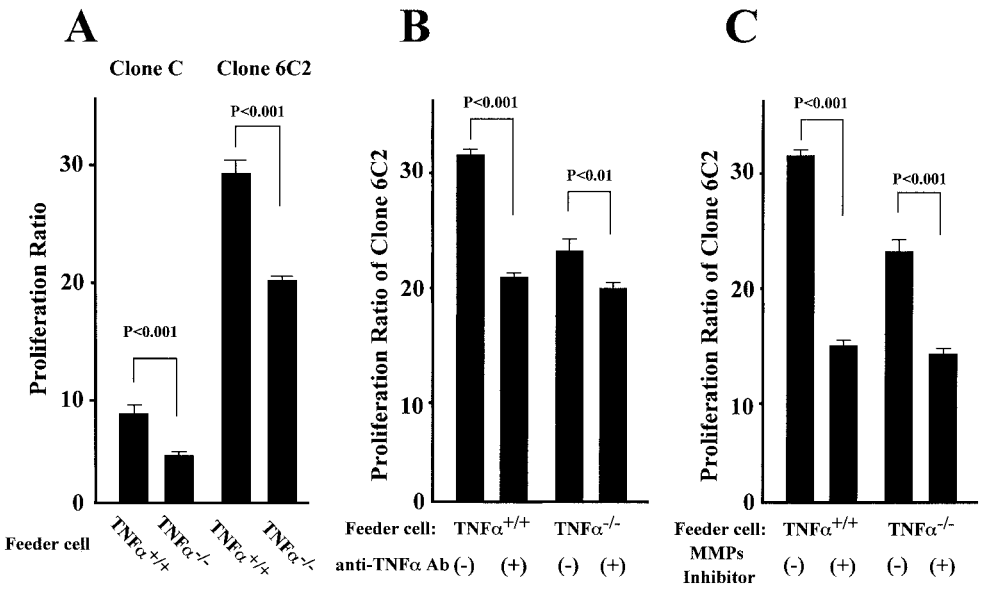

FIG. 5. Effect of TNF-α on the proliferation of TNF-$\alpha^{+/+}$ CTL clones. (A) The TNF-$\alpha^{+/+}$ CTL clones C and 6C2 were incubated with MMC-treated TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ splenocytes (feeder cells) and P815preS1 cells (specific stimulator). Then 1 week after the second weekly stimulation, the number of CTLs was counted and the proliferative ratio was calculated as the number of CTLs 1 week after the last stimulation/the number of CTLs before the last stimulation. (B) The TNF-$\alpha^{+/+}$ CTL clone 6C2 was stimulated twice at weekly intervals with MMC-treated TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ splenocytes (feeder cells) and P815preS1 cells (specific stimulator) in the presence of 5 μg of anti-TNF-α antibody (Ab) or hamster IgG/ml. Then 1 week later, the number of CTLs was counted and the proliferative ratio was calculated as described above. Purified hamster IgG was used as a control antibody. (C) Clone 6C2 was stimulated once with MMC-treated TNF-$\alpha^{+/+}$ feeder cells and P815preS1 cells alone or in the presence of MMP inhibitor (10 μM). Then 1 week later, the number of CTLs was counted and the proliferative ratio was measured as described above. MMP inhibitor was added on day 0. Each bar and error bar represent the mean and the SD, respectively, of results for triplicate samples. Statistically significantly differences between groups are indicated.

mated by the expression of MHC-II and costimulatory molecules, such as CD80 and CD86, we also analyzed these cell surface molecules. As shown in Fig. 6A, flow cytometric analysis revealed no difference between TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ DCs in terms of the expression of the MHC-I molecule (*H-2L^d*), the MHC-II molecules (*I-A*/*I-E*), CD80, or CD86. In the MLR, the two sets of DCs showed virtually the same capacity to stimulate allogeneic T cell proliferation (Fig. 6B).

DISCUSSION

Viral clearance has been widely thought to be mediated by the destruction of infected cells by CTLs. However, CTLs also secrete antiviral cytokines such as gamma interferon (IFN-γ) and TNF-α. As it has been reported that both IFN-γ and TNF-α released from HBV-specific CTLs can abolish HBV replication in hepatocytes in HBV transgenic mice (2, 3, 10, 17) and deliver noncytopathic antiviral signals to hepatocytes to degrade the cytoplasmic transcript and the nucleocapsid particles of HBV (19, 20, 23), IFN-γ and TNF-α are thought to be important immune mediators in host defense against HBV infection. The antiviral effect of TNF-α has been also demonstrated both in vitro and in vivo in some viral infections (15, 48). The important role of IFN-γ in the viral clearance by CTL has been revealed by using IFN-γ knockout CTL (31). Although it has been known that TNF-α plays a partial role in hepatocellular injury (1) and a critical role in viral clearance in viral hepatitis (21, 23), the effect of TNF-α on CTLs, which play a leading role in viral clearance, is unclear. To address this issue, we set out to induce HBV-specific CTLs in TNF-$\alpha^{-/-}$ mice by DNA immunization.

We expected that it might be difficult to induce HBV-specific CTLs in TNF-$\alpha^{-/-}$ mice because of both the lack of TNF-α itself and the presence of immune system abnormalities due to the absence of TNF-α. Although it has been reported that TNF-deficient mice immunized with the OVA peptide SIINFKL in the adjuvant TiterMax can generate antigen-specific CTLs (35), there is no information about whether CTLs can recognize endogenously processed antigen. Although CTL clones derived from knockout mice, such as perforin-deficient, Fas-L-deficient, and IFN-γ-deficient mice, have been established to explore the pathogenesis of CTL-induced cytotoxicity (18, 31, 38, 46), TNF-$\alpha^{-/-}$ CTL clones, specific for antigen which has been endogenously processed after particle capture by DCs, have never been established to investigate the role of TNF-α in CTL-induced cytotoxicity.

We found that a single cycle of plasmid DNA injection induced HBsAg-specific CTL responses in TNF-$\alpha^{+/+}$ mice, but not in TNF-$\alpha^{-/-}$ mice. At this time, we thought this lack of induction in TNF-$\alpha^{-/-}$ mice might be explained in two ways, namely a lack of CTL differentiation from immature precursor cells or impaired CTL proliferation. In fact, it has been reported that T cells derived from mice deficient in the TNF-α receptor also proliferate poorly to alloantigen (24). We therefore used two cycles of injection to enhance the intensity of immunization and obtained high CTL responses in TNF-$\alpha^{+/+}$ mice and weak responses in TNF-$\alpha^{-/-}$ mice; however, the CTL responses in TNF-$\alpha^{-/-}$ mice were lost within a week (Fig.

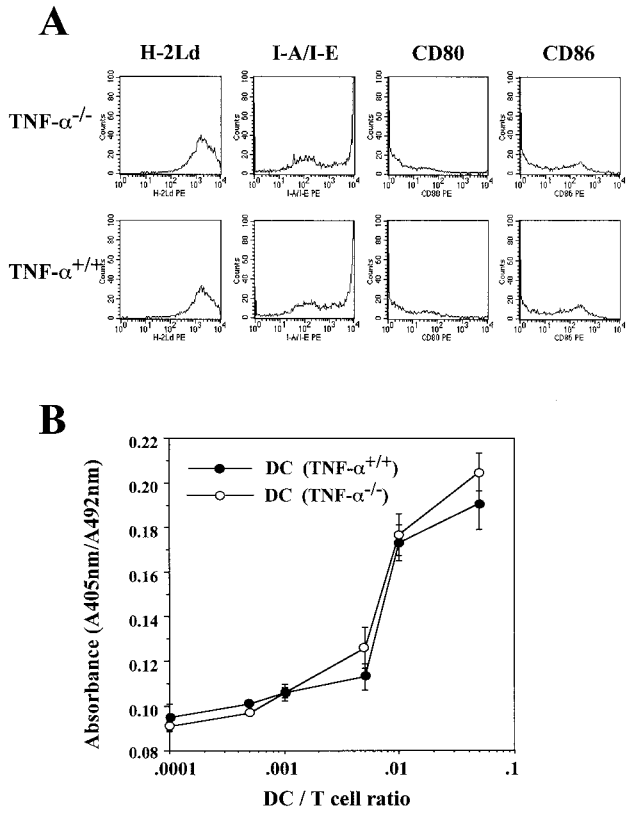

FIG. 6. Comparison of DC function in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice. $CD11c^+$ cell separation was carried out by using the VarioMACS system. (A) Flow cytometric analysis of the expression of the MHC-I molecule *H-2L*^{d} (restriction element for HBsAg-specific CTL), the MHC-II molecule *I-A/I-E*, CD80, and CD86 in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ DCs. All samples were assayed in duplicate. (B) The allostimulatory capacity of $CD11c^+$ DCs from TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice was assessed by the MLR as described in Materials and Methods with allogeneic murine $CD4^+$ T cells from AKR mice (*H-2*^{k}). Each data point and error bar represent the mean and SD, respectively, of results for triplicate samples.

1). Since immunized splenocytes from TNF-$\alpha^{-/-}$ mice cannot produce TNF-α, we added irradiated TNF-$\alpha^{+/+}$ splenocytes to TNF-$\alpha^{-/-}$ cultures on day 0 and managed to maintain the CTL responses for 3 weeks (Fig. 1). To assess quantitatively the role of TNF-α in CTL proliferation, we used peptide-loaded di-*H-2L*^{d}–Ig protein to estimate the number of $CD8^+$ HBsAg-specific lymphocytes in immunized splenocytes and found that it was decreased in TNF-$\alpha^{-/-}$ mice compared to TNF-$\alpha^{+/+}$ mice (Fig. 2).

As shown in Fig. 3, it was possible to produce TNF-$\alpha^{-/-}$ CTL clones, which showed higher specific cytotoxicity than that of the TNF-$\alpha^{-/-}$ CTL lines, but which was still lower than that of TNF-$\alpha^{+/+}$ CTL clones. Since TNF-α is one of the cytolytic mediators (1, 32, 33), we think that the cytotoxicity of the TNF-$\alpha^{-/-}$ CTL clones may be relatively low. In addition, the shortest optimal peptide for the TNF-$\alpha^{-/-}$ clones, as well as that for the TNF-$\alpha^{+/+}$ clone 6C2 described previously (2, 3), was HBsAg peptide 28-39 (data not shown). These data suggested that CTL differentiation can be induced in the absence of TNF-α, but lack of TNF-α may result in impaired proliferation of HBsAg-specific CTLs.

The increase in the in vitro proliferation of TNF-$\alpha^{-/-}$ clones seen when exogenous TNF-α was added (Fig. 4A) showed that TNF-α plays an important role in the proliferation of HBsAg-specific CTLs. Likewise, it has been reported that TNF-α enhanced the proliferation of T cells induced by malignant tumor cells such as human ovarian and breast carcinoma cells (18, 27). Furthermore, the in vitro proliferation of TNF-$\alpha^{+/+}$ CTL clones was significantly lower when the cells were cocultured with TNF-$\alpha^{-/-}$ feeder cells than when TNF-$\alpha^{+/+}$ feeder cells were used (Fig. 5A). In addition, when neutralizing anti-TNF-α MAb was added, the proliferation of TNF-$\alpha^{+/+}$ CTL clones was significantly suppressed (Fig. 5B).

The MMP enzyme family is classified into subgroups of collagenases, gelatinases, stomelysins, and membrane-type MMPs which digest various components of the extracellular matrix during physiological and pathological remodeling. Recent studies have indicated that TNF-α and Fas-L released from the cell membrane are mediated by certain MMPs (11, 16). We therefore tested the effect of MMP inhibitor on the proliferation of TNF-$\alpha^{+/+}$ CTL clones to determine the role of sTNF-α and found that it dramatically inhibited proliferation (Fig. 5C), suggesting the importance of sTNF-α in CTL proliferation. MMP inhibitor also actually blocks the cleavage of Fas-L. However, since the addition of TNF-α (sTNF-α) increases the proliferation of the TNF-$\alpha^{-/-}$ clone K (Fig. 4A), sTNF-α is thought to be required for the proliferation of HBsAg-specific CTLs. Thus, we suggest that sTNF-α but not membranous TNF-α is essential for CTL proliferation. The in vitro proliferation of TNF-$\alpha^{+/+}$ CTL clones was significantly lower when the cells were cocultured with TNF-$\alpha^{-/-}$ feeder cells than when TNF-$\alpha^{+/+}$ feeder cells were used (Fig. 5A). Furthermore, when neutralizing anti-TNF-α MAb or MMP inhibitor was added, the proliferation of TNF-$\alpha^{+/+}$ CTL clones was significantly suppressed when either TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ feeder cells were used (Fig. 5B). Interestingly, the proliferation of clone 6C2 was suppressed down to a similar level with either TNF-$\alpha^{+/+}$ or TNF-$\alpha^{-/-}$ feeder cells when anti-TNF-α MAb or MMP was added in the culture (Fig. 5A and B). These results suggested that TNF-α produced by CTLs partially stimulates proliferation by a paracrine and/or autocrine mechanism.

Interestingly, although TNF-α has been shown to be a specific inhibitor of early human hematopoiesis (41), it is a potent synergistic factor for the proliferation of primitive human hematopoietic progenitor cells and hepatocytes (6, 42, 47). Fas-L, one of the TNF family members, also exhibits the capacity to reverse signal, to transduce a signal inward upon binding specific receptors (43). Thus, TNF-α is a very interesting and important cytokine with apparently contradictory functions. In the present study, exogenous TNF-α enhanced the proliferation of TNF-$\alpha^{-/-}$ CTL clones (Fig. 4A) but suppressed that of TNF-$\alpha^{+/+}$ CTL clones (Fig. 4B), suggesting that TNF-α is essential for CTL proliferation, but its overproduction suppresses CTLs.

Since TNF-α plays an important role in the development of the immune system (44), we suspected that TNF-$\alpha^{-/-}$ DCs may function abnormally. DCs are known to be major antigen-presenting cells and are therefore thought to play an important role in the induction of CTLs. Thus, we needed to evaluate unstimulated DC function both in TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$

mice. However, as shown in Fig. 6, DCs from TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice did not differ in their expression of MHC-I and -II molecules, CD80, or CD86 or in their allostimulatory capacity. As described above, the presence of CTLs with cytotoxic activity in TNF-$\alpha^{-/-}$ mice indicated that CTLs can differentiate from immature precursor cells in the absence of TNF-α. This fact also indicated that DCs from TNF-$\alpha^{-/-}$ mice were not functionally suppressed in vivo, at least in terms of antigen presentation. It has been reported that TNF-α may promote DC migration from nonlymphoid tissues to secondary lymphoid tissues (42). So although TNF-$\alpha^{-/-}$ DCs have normal allostimulatory capacity and maturity, the lack of recruitment of these cells to lymphoid tissues may be a factor responsible for the difficulty of CTL induction.

To analyze the role of TNF-α produced by DCs in the proliferation of CTL, we measured the proliferation ratio of wild-type CTL clone C with MMC-treated TNF-$\alpha^{+/+}$ DC plus TNF-$\alpha^{+/+}$ T cells (A), TNF-$\alpha^{-/-}$ DC plus TNF-$\alpha^{-/-}$ T cells (B), and TNF-$\alpha^{+/+}$ DC plus TNF-$\alpha^{-/-}$ T cells (C) 5 days after the second stimulation in vitro. The proliferation ratio of C was significantly lower than that of A, and that of B was between those of A and C (data not shown). This result indicated that TNF production by DC is necessary but not sufficient for proliferation of CTL.

Despite these observations, we would like to emphasize that TNF-α promotes the proliferation of CTLs not only through the stimulation of DCs but also by the direct effect on CTLs. In our stimulation in vitro, the cultures did not contain DCs that can present HBV antigen except for the experiment shown in Fig. 1, and we used P815preS1 as antigen-presenting stimulator cells. As shown in Fig. 4, although the condition of the culture was the same in the experiments shown in panels A and B, exogenously added TNF-α enhanced the proliferation of TNF-$\alpha^{-/-}$ clone but suppressed that of the TNF-$\alpha^{+/+}$ clone. In fact, TNF-α was produced by the CTL clone by itself under the conditions for the experiment shown in panel B but not under those for the experiment shown in panel A. However, in the presence of a sufficiently high dose of TNF-α (0.03 and 0.10 ng/ml; at these concentrations, the proliferation of the TNF-$\alpha^{+/+}$ clone was suppressed), the conditions of cultures were thought to be the same, including indirectly produced cytokines by feeder cells. Thus, we suggest that TNF-α can directly promote the proliferation of CTLs.

We conclude that TNF-α plays an essential role in the sufficient proliferation, but not in the differentiation, of HBsAg-specific CTLs both in vivo and in vitro and that this effect is not only due to the activation of DCs but also induced by the direct effect on CTLs.

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