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Modeling the T-helper cell response in acute and chronic hepatitis B virus infection using T-cell receptor transgenic mice

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Abstract

Chronicity following hepatitis B virus (HBV) infection may be maintained by high levels of viral proteins circulating in the serum. To examine the characteristics of T cells capable of co-existing with the secreted hepatitis B e-antigen (HBeAg), T-cell receptor (TCR) transgenic (Tg) mice were produced. To insure that HBeAg-specific T cells would not be deleted in the presence of serum HBeAg, the TCR α and β-chain genes used to produce the TCR-Tg mice were derived from T-cell hybridomas from HBeAg-Tg mice. A TCR-Tg lineage (11/4–12) was produced that possessed a high frequency (~ 67%) of $CD4^+$ T cells that expressed a TCR-Tg specific for the HBeAg. As predicted, when 11/4–12 TCR-Tg mice were bred with HBeAg-Tg mice no deletion of the HBeAg-specific $CD4^+$ T cells occurred in the thymus or the spleen. Functional analysis of the TCR-Tg T cells revealed that the HBeAg-specific $CD4^+$ T cells escaped deletion in the thymus and periphery by virtue of low avidity. Regardless of their low avidity, HBeAg-specific TCR-Tg T cells could be activated by exogenous HBeAg as measured by cytokine production in vitro and T-helper cell function for anti-HBe antibody production in vitro and in vivo. Furthermore, activated TCR-Tg HBeAg-specific T cells polarized to the Th_1 subset were able to elicit liver injury when transferred into HBeAg or HBcAg-Tg recipients. Therefore, HBeAg-specific $CD4^+$ T cells that can survive deletion or anergy in the presence of circulating HBeAg nonetheless are capable of being activated and of mediating liver injury in vivo.

Keywords: Hepatitis B virus; T-cell receptor; Transgenic mice; Hepatitis B e-antigen

1. Introduction

The nucleoprotein of the hepatitis B virus (HBV) exists in two structural forms. The particulate nucleocapsid (HBcAg), which encapsulates

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the viral genome, and a monomeric secreted form (hepatitis B e-antigen, HBeAg). In order to examine HBeAg-specific $CD4^+$ T cells that can co-exist with circulating HBeAg and remain functional in vivo, we have produced mice transgenic (Tg) for T-cell receptors (TCRs) specific for HBeAg. First, T-cell hybridomas were produced by immunizing B10 wild-type (+/+) mice or HBeAg-Tg (B10 e/e) mice with HBeAg. The TCR-α/β genes derived from selected HBeAg-specific T-cell hybridomas were sequenced and inserted into T-cell expression shuttle vectors for use in the generation of TCR-α/β-Tg mice. The TCR-α/β-Tg lineage 11/4–12, derived from HBeAg-Tg (B10 e/e) mice, is the subject of this report. A second TCR-α/β-Tg lineage, 8/3–11, derived from B10 +/+ mice, is included as a comparative control. Fig. 1 is a schematic of the general experimental approach and a summary of the results.

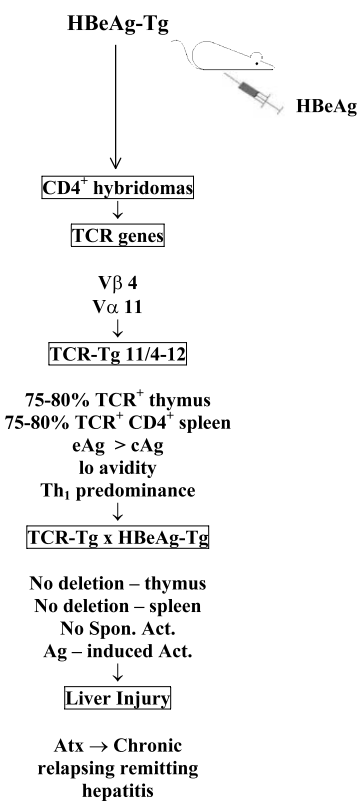

Fig. 1. A schematic of the experimental approach and summary of the results.

2. Materials and methods

2.1. Sequence determination of the V(D)J regions of the T-cell receptors derived from T-cell hybridomas

The Vα usage of each T-cell hybridoma was determined by RT-PCR using primers specific for each Vα family gene. Total RNA was isolated from 2×10^6 cells of each hybridoma using TRIZOL reagent (Gibco BRL, Grand Island, NY). Following extraction, 1 μg RNA was reverse transcribed to cDNA with M-MLV reverse transcriptase (Gibco BRL) and $pd(T)_{12-18}$ primer (Amersham Pharmacia Biotech, Piscataway, NJ). Aliquots of cDNA were subjected to 30 cycles of PCR under stringent primer annealing conditions with the reverse primer located at the constant region of the α-chain and each of the 13 forward primers specific for each Vα gene family (Solheim et al., 1993).

The Vβ usage was determined with a Vβ multiprobe ribonuclease protection assay (Gonzalez-Quintial and Theophilopoulos, 1992). Mixtures of radiolabled riboprobes specific for each Vβ gene family were hybridized with 10 μg of extracted RNA, digested with RNase and resolved on denaturing polyacrylamide gels. Protected RNA fragments were visualized on autoradiography and identified in comparison to the unprotected Vβ probes.

From the Vα and Vβ typing results, primers were designed to isolate the V(D)J and partial constant regions of the hybridomas. Following 30 thermal cycles, the PCR products were purified from agarose and cloned into the pSPORT plasmid vectors. Three individual colonies from each TCR-α- and β chain cDNA were sequenced using M13 forward and reverse primers.

2.2. Generation of TCR-α-chain and β-chain transgenes and Tg mice

After sequence analysis, the V(D)J regions of the TCRs were PCR amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using gene-specific primers. Restriction sites, intron sequence and splice donor/acceptors were also introduced

into the V(D)J fragments. The modified α-chain VJ fragment was inserted into a XhoI-NotI excised TCR α-chain shuttle vector which contains a rearranged TCR α-chain genomic DNA and the endogenous TCR α enhancer. The modified β-chain VDJ fragment was inserted into a ClaI-NotI excised TCR β-chain shuttle vector that contains a rearranged TCR β-chain genomic DNA and the endogenous β enhancer. The shuttle vectors (Ho et al., 1994) were kindly provided by Dr M.M. Davis, Stanford. To ensure that no mutation had been introduced, the V(D)J regions from each TCR construct was subcloned into pUC19 vector and re-sequenced. Prior to microinjection, the bacterial sequences were removed and the 15.4 kb α-chain and the 19.8 kb β-chain TCR DNA fragments were co-microinjected into fertilized mouse (C57 BL/10 (B10) embryos. Progeny mice were screened for the presence of the transgenes in PBL by PCR analysis using primers located on the V- and the CDR3 region for each TCR transgene. The expression of the TCR transgenes in peripheral blood lymphocytes (PBL) and lymphoid tissues was confirmed by immunofluorescence and RT PCR using monoclonal antibodies (mAbs) and oligo primers specific for the transgene TCRs. The Tg mice expressing the HBeAg (10 ng/ml) or the HBcAg (0.25 ng/mg of soluble liver protein) were produced at the Scripps Research Institute Transgenic Research Facility as previously described (Milich et al., 1990, 1994).

2.3. rHBeAg and synthetic peptides

An *Escherichia coli* derived rHBeAg corresponding in sequence to serum-derived HBeAg encompassing the ten precore amino acids remaining after cleavage of the precursor and residues 1–149 of hepatitis B core antigen (ayw subtype) was provided by Florian Schödel (EVAX, Munich). The presence of the ten precore amino acids prevents particle formation, and the rHBeAg preparation is recognized efficiently by HBeAg-specific mAbs but displays little hepatitis B core antigenicity (Schödel et al., 1993). Peptides were synthesized by the simultaneous multiple peptide synthesis method. The following HBc/HBeAg-derived synthetic peptides representing Th-cell recognition sites were used and designated by amino acid position from the N terminus of HBcAg: 129–140, PPAYRPPNAPIL; and 120–140, VSFGVWIRTPPAYRPPNAPIL.

2.4. Serology

HBeAg was measured in diluted Tg mouse sera by a commercial ELISA (HBe enzyme immunoassay; Abbott Laboratories, Chicago, IL), and rHBeAg was used as a standard. Anti-HBc and anti-HBe IgG antibodies were measured in murine sera by an indirect solid-phase ELISA using rHBcAg or rHBeAg as the solid-phase ligands as described previously (Milich et al., 1988). The data are expressed as antibody titers representing the reciprocal of the highest dilution of sera required to yield an OD_{492} reading three times an equal dilution of preimmunization sera. IgG isotype-specific ELISAs were performed using IgG1-, IG2a, IgG2b, and IgG3-specific second Abs (Southern Biotechnology, Birmingham, AL).

2.5. Cytokine analysis

Spleen cells from either unprimed or primed TCR-Tg or wild-type mice were cultured (6×10^6/ml) with various concentrations of a series of antigens. Culture supernatants (SN) were harvested at 24 h for IL-2 determination, and at 48 h for IL-4 and IFN_γ determinations. Cytokines were measured by two-site ELISA using pairs of cytokine-specific mAb. One unlabeled mAb was absorbed to the microtiter plate well and used as a capture antibody and the other labeled mAb served as the probe. Alternatively a CELL–ELISA (Beech et al., 1997) was utilized. In this case, the cytokine-specific capture mAb was bound to the solid-phase of a cell culture well, and the last 24 h of the cell culture was conducted in the presence of the capture mAb. The CELL–ELISA is more sensitive than the SN-ELISA because the cytokines are directly bound by solid-phase mAb and are less likely to be absorbed by the cellular cytokine receptors.

2.6. Liver injury model

TCR-Tg or wild-type mice served as donors of spleen cells for adoptive transfer into HBeAg-Tg or HBcAg-Tg recipients. Unprimed spleen cells from donor mice were cultured in two cycles (5 days/cycle) with HBeAg (5 μg/ml) in the presence of IL-12 (2.0 ng/ml) and anti-IL-4 (mAb 11B11) in order to polarize the activated T cells toward the Th_1 subset. Virtually 100% of the T cells were $CD4^+$ after the second culture cycle. Activated Th_1 cells (20×10^6) were transferred into sublethally irradiated (400 R) HBeAg-Tg or HBcAg-Tg recipients. Hepatocellular injury was monitored biochemically by measuring serum alanine aminotransferase (ALT) activity.

2.7. Flow cytometry

Single cell suspensions of thymus or spleen were prepared. Before staining cells were incubated with an anti-Fc mAb (2.4G2) to block non-specific Fc receptor uptake. For staining with directly-labeled antibodies, 1×10^6 cells were incubated with antibodies at 4 °C for 15 min. Cells were washed three times and analyzed with a FACScan (Becton Dickinson). Gates were set only on viable cells and usually $> 10^4$ cells were analyzed using LYSIS II (Becton Dickinson). The murine antibodies used for two and three color staining were as follows: anti-TCR Vβ4 (KT4), anti-TCR Vβ11 (RR3-15), anti-TCR Vα11 (RR8-1), anti-CD4 (H129.19), and anti-CD8a (53–6.7) (Pharmingen, Palo Alto, CA).

3. Results

3.1. Establishment of the TCR-Tg lineage 11/4–12

A T-cell hybridoma was derived from HBeAg-Tg mice immunized with HBeAg. After micro-injection of the T-cell hybridoma-derived V_α and V_β-chain gene constructs, putative founder mice were screened for the presence of the transgenes by PCR analysis of DNA extracted from PBL. Mice positive for both TCR transgenes were examined for TCR expression by FACS analysis (Fig. 2). In TCR-Tg lineage 11/4–12, 87.1% of splenic $CD4^+$ T cells express $V_\beta4$ as compared to 7.7% of control $CD4^+$ spleen cells; and $V_\alpha11$ is expressed on 73.4% of $CD4^+$ T cells in lineage 11/4–12 as compared to 4.7% in control mice. Splenic T cells expressing both $V_\beta4$ and $V_\alpha11$ represented 67% of the $CD4^+$ population in the 11/4–12 TCR-Tg lineage as compared to 0.3% in control mice (Fig. 2).

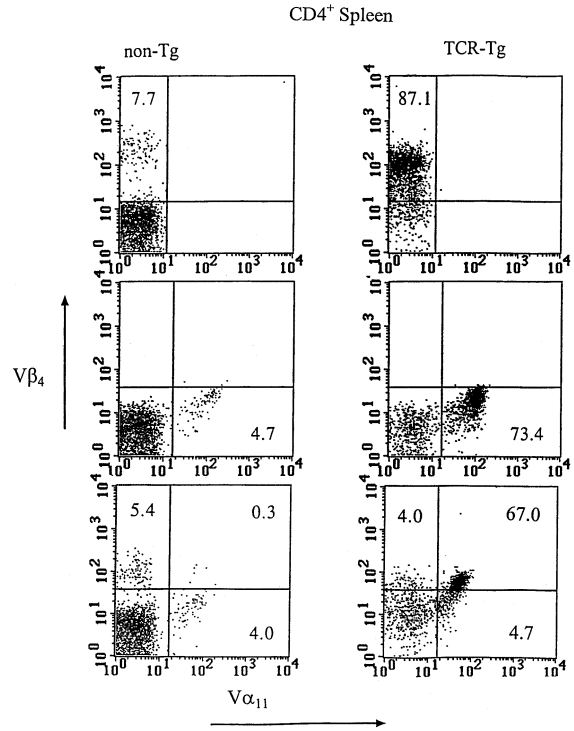

Fig. 2. One and two-color FACS analysis of non-Tg and 11/4–12 TCR-Tg splenic $CD4^+$ T cells. Purified $CD4^+$ splenic T cells were stained with anti-$V_\beta4$ (top panels); anti-$V_\alpha11$ (middle panels); or both anti-$V_\beta4$ and anti-$V_\alpha11$ (bottom panels) (Chen et al., 2000).

TCR-Tg mice were also derived from micro-injection of V_α/V_β-chain genes derived from another T-cell hybridoma, which originated from B10 +/+ mice immunized with HBeAg (lineage 8/3–11). In the case of 8/3–11 TCR-Tg mice, only 11.5% of splenic $CD4^+$ T cells express the Tg $V_\beta11$ chain as compared to 4.5% in control mice (data not shown).

3.2. Functional analysis of HBeAg-specific CD4+ T cells in TCR-Tg lineages 11/4–12 and 8/3–11

To ascertain the functional ability of the CD4+ T cells expressing the TCR transgenes to respond to antigen, unprimed spleen cells from 11/4–12 and 8/3–11 TCR-Tg mice were cultured for 3 days either with HBcAg (particulate form), HBeAg (monomeric form) or peptide 120–140 and antigen-specific IL-2 production was determined. As shown in Fig. 3, naive spleen cells from non-TCR-Tg mice did not respond to the antigen panel establishing that the endogenous HBe/HBcAg-specific T-cell repertoire requires in vivo expansion by priming in order to be detected. In contrast, naive splenic T cells from both TCR-Tg lineages produced IL-2 in response to culture with the HBeAg. However, significant differences existed between the two TCR-Tg lineages. Splenic T cells from 11/4–12 TCR-Tg mice 'recognized' HBeAg preferentially to the particulate HBcAg and were not activated by peptide 120–140. Splenic T cells from 8/3–11 TCR-Tg recognized HBcAg preferentially to HBeAg and were activated by peptide 120–140. Also note that 8/3–11 T cells produced higher levels of IL-2 and responded to lower doses of in vitro antigen than 11/4–12 T cells even though the frequency of HBe/HBcAg-specific CD4+ T cells expressing a TCR-Tg is much lower in 8/3–11 mice (11.5%) than in 11/4–12 mice (67%).

In addition to IL-2, production of IFN_γ and IL-4 by naive spleen cells from 11/4–12 and 8/3–11 TCR-Tg mice was monitored by CELL–ELISA over a 4 day culture period. As shown in Fig. 4, CD4+ T cells from 8/3–11 TCR-Tg mice produced increasing amounts of all three cytokines upon culture with the HBcAg beginning at day 1 for IL-2 and day 2 for IFN_γ and IL-4. Maximum cytokine levels were present in 4-day cultures. In contrast, CD4+ T cells from 11/4–12 TCR-Tg mice cultured with HBeAg produced maximum levels of IL-2 on day 2, IFN_γ on day 3 and produced very little IL-4 at all time points. Cytokine levels were decreasing by day 4 in the 11/4–12 TCR-Tg splenic cultures indicating that in vitro T-cell activation was less sustainable in 11/4–12 as opposed to 8/3–11 TCR-Tg mice.

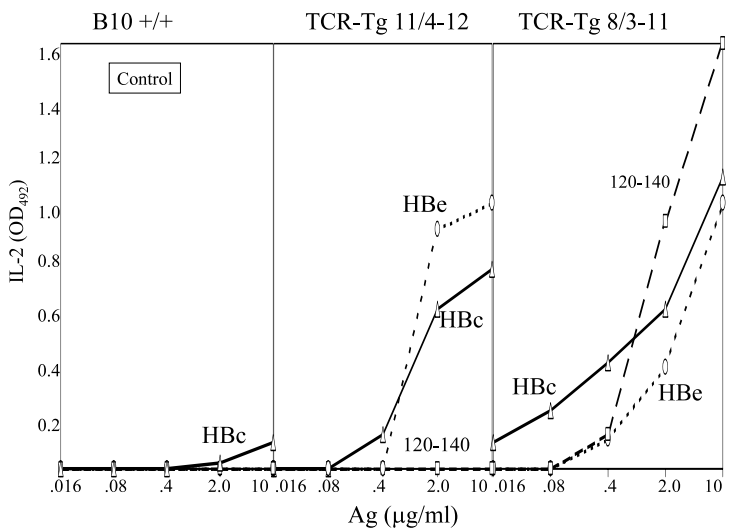

Fig. 3. Antigen-specific IL-2 production by naive splenic T cells from non-Tg (B10 +/+), TCR-Tg 11/4–12 and TCR-Tg 8/3–11 mice. Unprimed spleen cells were cultured with concentration of the indicated antigens in vitro for 3 days and SNs were collected and assayed for IL-2 by ELISA. Comparative IL-2 levels are expressed in OD units. This is one of four assays and is representative (Chen et al., 2000).

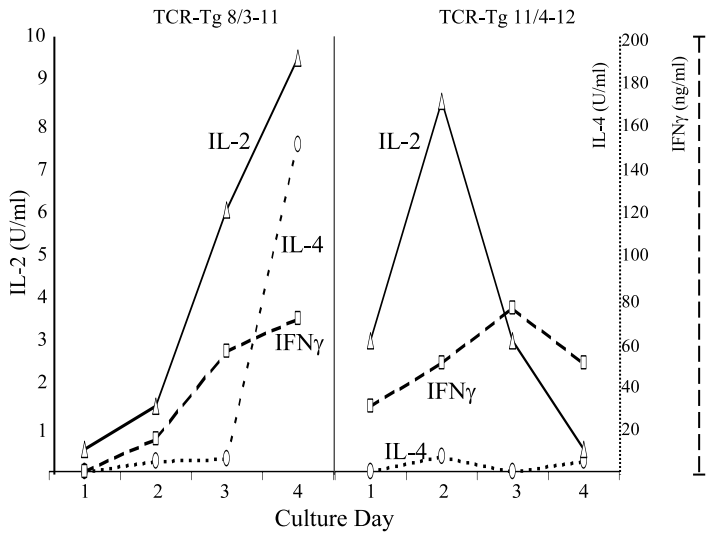

Fig. 4. Kinetics of cytokine production by TCR-Tg 11/4–12 and TCR-Tg 8/3–11 naive, splenic T cells cultured with antigen. Unprimed TCR-Tg 8/3–11 spleen cells were cultured with HBcAg (1.0 μg/ml) and TCR-Tg 11/4–12 spleen cells were cultured with HBeAg (5.0 μg/ml) for from 1 to 4 days. Amounts of IL-2, IFN_γ and IL-4 produced each day were determined by CELL–ELISA. Spleen cells were transferred to CELL–ELISA plates during the last 24 h of culture to measure contemporaneous rather than accumulated cytokine production (Chen et al., 2000).

3.3. T cells expressing the 11/4–12 Tg TCR are not deleted in the thymus or the periphery in TCR-Tg 11/4–12 × HBeAg-Tg 'double' Tg mice

Since the TCR genes used to generate 11/4–12 TCR Tg mice originated from immunization of HBeAg-Tg mice, it was predicted that the Tg TCR-bearing $CD4^+$ cells would not be deleted in the presence of HBeAg, at least at the HBeAg concentration occurring in the original HBeAg-Tg mouse (i.e. 10 ng/ml). To test this prediction, TCR-Tg 11/4–12 mice were bred with HBeAg-Tg mice and the frequencies of the Tg V_α11-chain or the Tg V_β4-chain among $CD4^+$ T cells in the thymus or the spleen in non-Tg, single TCR-Tg, or double TCR-Tg × HBeAg-Tg mice were compared (Fig. 5). In the normal thymus, the vast majority (95.6%) of T cells are $CD4^+/CD8^+$ double-positive cells. Note that in the TCR-Tg 11/4–12 thymus 73.6% of T cells are double-positive and 18.2% of thymic T cells are $CD4^+$ single-positive (Fig. 5A). The skewing towards the more mature $CD4^+$ population in the thymus is typical in TCR-Tg mice expressing a MHC class II-restricted TCR. In Fig. 5B, the frequencies of $CD4^+$ T cells expressing the TCR-Tg V_α11-chain in the thymus are shown in non-Tg, TCR-Tg (11/4–12), TCR-Tg × HBeAg-Tg and double-Tg mice given Zn^+ to induce the HBeAg-specific MT promoter, which increases the HBeAg concentration in the serum approximately 7-fold to ~70 ng/ml (Milich et al., 1990). No significant decreases in the frequency of the Tg V_α11-chain occurred in the thymus of 11/4–12 TCR-Tg mice also expressing the HBeAg in the serum at levels of 10 ng/ml ($-$Zn) or 70 ng/ml ($+$Zn) (Fig. 5B). Similarly, the presence of HBeAg in the serum did not deplete the $CD4^+$ T cells in the spleen carrying the TCR-Tg V_β4-chain (Fig. 5C). It is clear from this FACS analysis that the HBeAg-specific, $CD4^+$ T cells bearing the 11/4–12 Tg TCR are not negatively selected in the thymus or physically depleted in the periphery by exposure to circulating HBeAg.

3.4. T cells bearing the 11/4–12 and 8/3–11 transgenic TCR are not functionally altered by exposure to HBeAg in the serum of double Tg mice

Because $CD4^+$ T cells expressing the 11/4–12 Tg

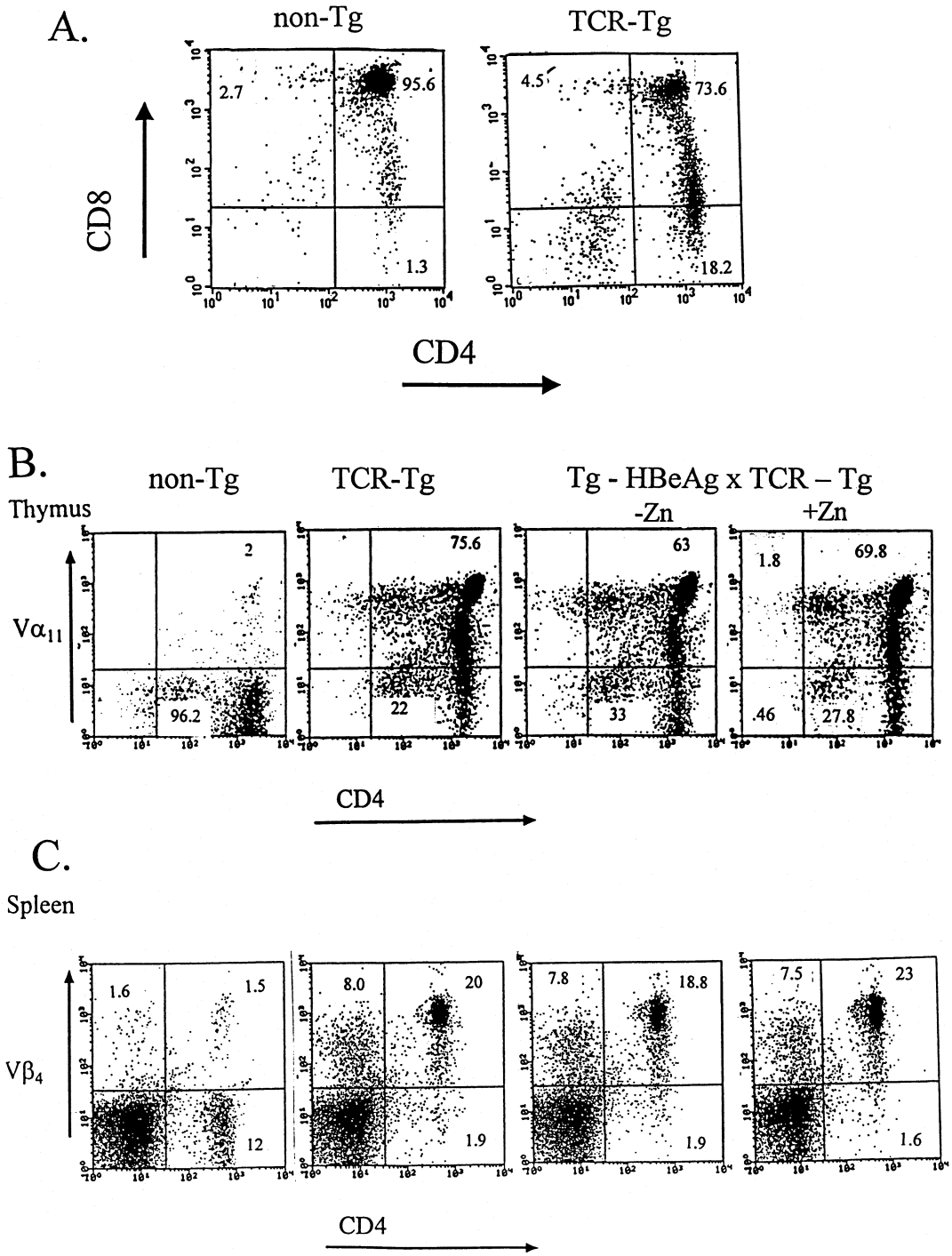

Fig. 5. Two-color FACS analysis of thymic and splenic T cells derived from non-Tg mice, TCR-Tg 11/4–12 mice, HBeAg-Tg × TCR-Tg 11/4–12 (double Tg mice) and double-Tg mice given Zinc sulfate (Zn, 25 mM) in the drinking water. Panel A: thymic cells from non-Tg and TCR-Tg 11/4–12 mice were double-stained with anti-CD8 and anti-CD4. Panel B: thymic cells from non-Tg, TCR-Tg and double-Tg mice minus and plus Zn treatment were double stained with anti-$V_{\alpha}11$ and anti-CD4. Panel C: splenic cells from non-Tg, TCR-Tg, and double-Tg mice minus and plus Zn treatment were double-stained with anti-$V_{\beta}4$ and anti-CD4 (Chen et al., 2000).

TCR were not physically deleted by HBeAg in the thymus or the periphery, the next issue was to determine if exposure to circulating HBeAg altered the functional capacity of these T cells. For this purpose, naive splenic T cells from TCR-Tg 11/4–12 or double-Tg mice were cultured in vitro with HBeAg and HBcAg and T-cell activation was monitored by IL-2 production after 2 days in culture (Fig. 6). Splenic T cells derived from naive single TCR-Tg mice or TCR × HBeAg double-Tg mice produced equivalent amounts of IL-2 upon exposure to HBe/HBcAg in vitro. T cells from double –Tg mice required exposure to HBeAg in vitro to elicit IL-2 production indicating that the T cells were not spontaneously activated by endogenous HBeAg in vivo. Similarly, FACS analysis revealed that the $CD4^+$ T cells bearing the Tg TCR derived from both single and double-Tg mice possessed a resting phenotype as opposed to an activated or memory phenotype prior to in vitro culture (data not shown). Therefore the presence of endogenous HBeAg in the serum of TCR 11/4–12 double-Tg mice neither suppresses nor enhances T-cell activation in vivo.

3.5. T cells bearing the transgenic 11/4–12 TCR mediate anti-HBe antibody production in vitro

To determine if 11/4–12 TCR-Tg and 8/3–11 TCR-Tg T cells could function as T-helper cells for HBcAg and HBeAg-specific B cells and mediate antibody production, 7 day spleen cultures in the presence or absence of antigens were monitored for anti-HBC and anti-HBe antibodies in the SN (Fig. 7). Control cultures from both strains not containing antigen yielded no antibody production (data not shown). In the presence of (A) HBcAg or (B) HBeAg (0.0016–5.0 μg/ml) spleen cells from 11/4–12 and 8/3–11 TCR-Tg mice produced anti-HBC and anti-HBe antibodies detectable in the SN. Antibodies were first detectable on day 3 of culture and continued to accumulate in the SN throughout the 7-day culture. It is of interest that the Th cells of 8/3–11 TCR-Tg mice elicted higher levels of anti-HBC antibodies (Fig. 7A) and the Th cells of 11/4–12 TCR-Tg mice mediated higher levels of anti-HBe antibodies (Fig. 7B). This most likely reflects the differing fine specificity profiles of these two TCR-

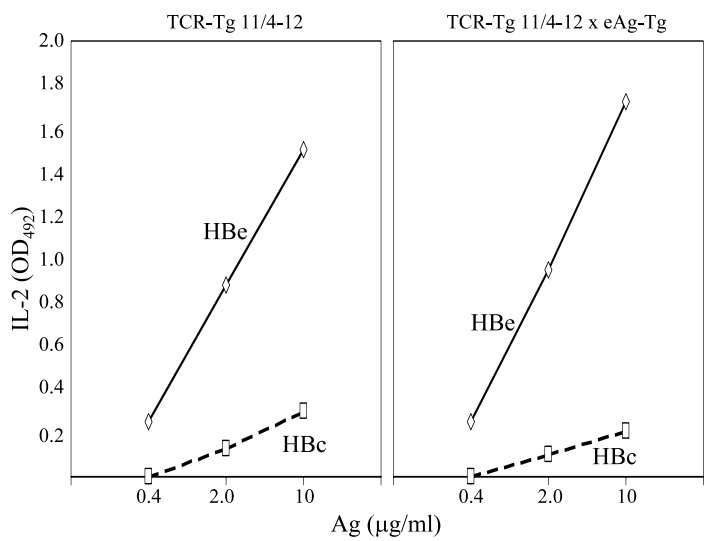

Fig. 6. Comparative antigen-specific IL-2 production by naive splenic T cells derived from TCR-Tg 11/4–12 and double-Tg mice. Spleen cells from TCR-Tg 11/4–12 and double-Tg mice were cultured with various concentrations of HBeAg or HBcAg for 2 days at which time SNs were collected in order to measure IL-2 levels by ELISA. Comparative IL-2 levels are expressed in OD units (Chen et al., 2000).

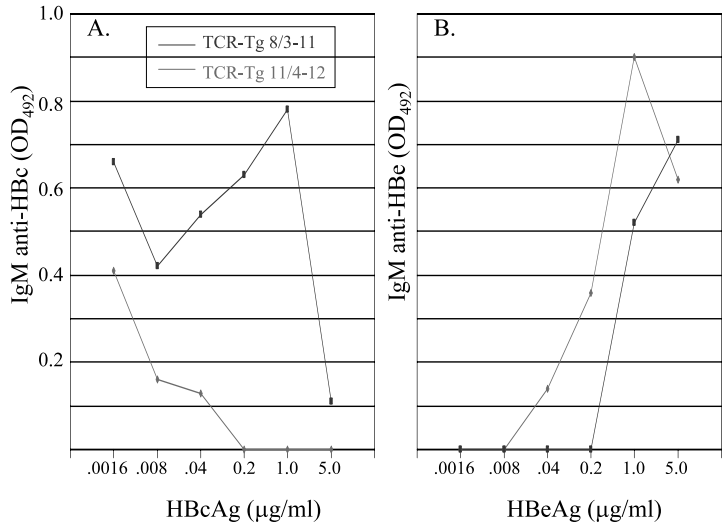

Fig. 7. In vitro anti-HBc and anti-HBe antibody production in splenic cultures from naive TCR-Tg 11/4–12 and TCR-Tg 8/3–11 mice. Spleen cells were cultured for 7 days with from 0.0016 to 5.0 μg/ml of (A) HBcAg and (B) HBeAg and SN was harvested on day 7 for the measurement of IgM anti-HBc and anti-HBe antibodies by ELISA. Undiluted SN was used in the ELISA and comparative antibody levels are expressed as OD units.

Tg lineages. Although the high frequency of HBeAg-specific and HBcAg $CD4^+$ T cells present in 11/4–12 TCR-Tg and 8/3–11 TCR-Tg mice was sufficient to mediate primary anti-HBC and anti-HBe antibody responses in vitro, it is notable that the antibody responses consisted exclusively of IgM antibodies, no IgG antibodies were detected. Furthermore, 11/4–12 TCR-Tg mice immunized with 10 μg of HBeAg in saline produced anti-HBe antibody in vivo (data not shown). Although HBeAg-immunized 11/4–12 TCR-Tg mice were competent to produce IgG anti-HBe antibodies, the levels of anti-HBe were $\approx$100-fold less as compared to HBeAg-immunized wild-type B10 +/+ mice. Therefore, production of high-titer IgG anti-HBe antibodies in vivo was dependent on factors other than HBeAg-specific Th-cell frequency.

3.6. Adoptive transfer of 11/4–12 TCR-Tg T cells polarized to the Th1 subset can mediate liver injury in HBe/HBcAg-expressing-Tg recipients

Because 11/4–12 TCR-Tg T cells were not deleted or functionally altered by exposure to endogenous HBeAg yet could be activated in vivo by exogenous HBeAg, it was of interest to determine the potential of these T cells to mediate liver injury upon adoptive transfer into HBeAg or HBcAg-expressing Tg recipients. For this purpose, T cells derived from B10 +/+ mice or 11/4–12 TCR-Tg mice were cultured for two 'cycles' with HBeAg in the presence of IL-12 and anti-IL-4 in order to activate and polarize the HBeAg-specific $CD4^+$ T cells toward the Th_1 subset. These polarized Th_1 cells secrete low levels of IL-2 and high levels of IFNγ and no IL-4 upon further culture with HBeAg (data not shown). Activated HBeAg-specific Th_1 cells were then transferred into sublethally irradiated HBeAg or HBcAg-expressing Tg recipients. Serum samples were collected on a daily or weekly basis and serum ALT levels were determined as a measure of liver injury. Normal ALT levels in our mouse colony ranged between 20–60 U/L with occasional elevations at single time points, therefore, ALT values of $\geqslant$80 U/L at multiple time points were considered significant elevations.

As shown in Fig. 8, adoptive transfer of in vitro activated Th_1 cells from 11/4–12 TCR-Tg mice into both HBeAg-Tg and HBcAg-Tg recipients resulted in relatively mild relapsing and remitting chronic liver injury, whereas, adoptive transfer of Th_1 cells

cultured in vitro with HBeAg from non-Tg control mice elicited no liver injury. However, there were differences between HBeAg-Tg and HBcAg-Tg recipients of 11/4–12 TCR-Tg Th_1 cells. Only 50% of HBeAg-Tg recipients demonstrated chronic liver injury as compared to 100% of HBcAg-Tg recipients, and the onset of liver injury appeared sooner in the HBcAg-recipients (Fig. 8). The kinetics of liver injury in both groups was unique in each recipient and quite variable from mouse to mouse. At the termination of adoptive transfer experiments (6 months), liver sections of recipient mice were prepared for histological examination. The H&E stained liver sections were read in a blinded fashion by a pathologist (S.N.T.). In summary, the HBcAg-Tg recipients demonstrated a greater degree of focal necrosis and pleiomorphic hepatocytes than the HBeAg-Tg recipients and portal inflammation was minimal in both groups. This histology is consistent with chronic lobular hepatitis, which does not usually progress to cirrhosis.

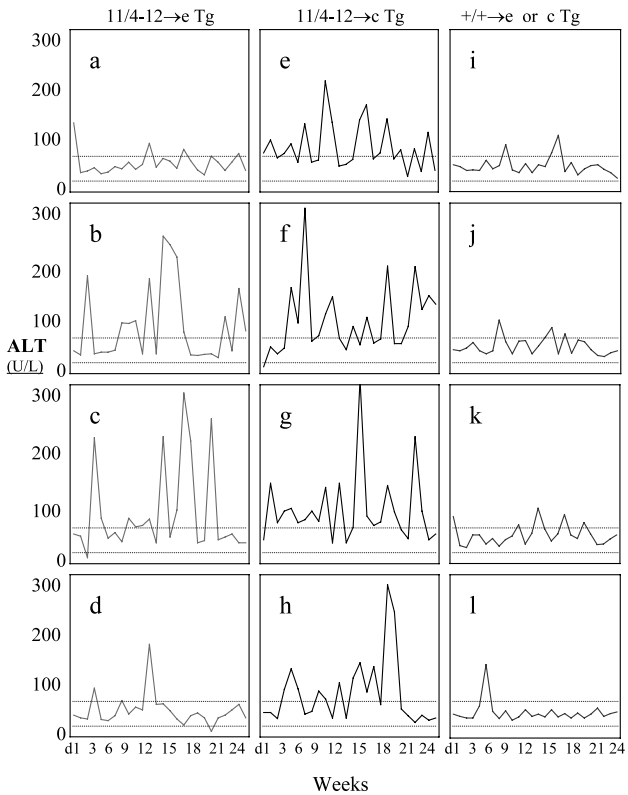

Fig. 8. Adoptive transfer of 11/4–12 TCR-Tg Th_1 cells mediates liver injury in HBe/HBcAg-expressing Tg recipients. Unprimed, donor T cells derived from either B10 +/+ mice or from 11/4–2 TCR-Tg mice were cultured with HBeAg (5 μg/ml) in the presence of IL-12 and anti-IL-4 for two cycles of 5 days each. In vitro activated and Th_1-polarized 11/4–12 TCR-Tg T cells (20×10^6) were then transferred into either HBeAg-Tg recipients (a–d) or HBcAg-Tg recipients (e–h). Donor cells from control (+/+) mice were transferred into HBeAg-Tg (i, j) or HBcAg-Tg (k, l) recipients. The recipients were sublethally irradiated (400 R) at the time of adoptive transfer. Hepatocellular injury was monitored by measuring serum ALT levels on a weekly basis. The normal range of serum ALT values in our mouse colony is 20–60 U/L depicted by the horizontal bars. Each graph represents an individual mouse (Chen et al., 2000).

4. Discussion

The functional studies of 11/4–12 TCR-Tg mice suggest that T cells expressing the Tg HBeAg-specific TCR survive in the presence of HBeAg in double-Tg mice by virtue of low avidity. The evidence for the low avidity of 11/4–12 TCR-Tg $CD4^+$ T cells is functionally defined and includes: (1) a right-shifted dose response curve after primary activation in vitro; (2) the transient nature of T-cell activation in vitro; (3) lack of clonal deletion in the thymus and the periphery in the context of HBeAg concentrations of 10–70 ng/ml in the serum; (4) lack of anergy of peripheral $CD4^+$ T cells; (5) altered fine specificity; and (6) inefficient activation in vivo demonstrated by low levels of anti-HBe antibodies produced after immunization. Presumably, HBeAg-specific T cells demonstrating higher avidities than 11/4–12 TCR-Tg T cells would be either deleted in the thymus or anergized in the periphery upon exposure to serum HBeAg. For example, HBeAg-Tg mice on an $H\text{-}2^s$ background are totally tolerant at the level of $CD4^+$ T cells in the context of a 10 ng/ml serum concentration of HBeAg (Milich et al., 1991, 1994).

Studies in HBV chronically infected patients suggest that HBeAg-specific $CD4^+$ T cells are present even in the context of relatively high levels of circulating HBeAg (Ferrari et al., 1990; Jung et al., 1995; Maruyama et al., 1993a; Tsai et al., 1992). We suggest that the Tg HBeAg-specific $CD4^+$ T-cell population existing in 11/4–12 TCR-Tg mice

may represent a monoclonal model for the HBeAg-specific CD4$^+$ T-cell repertoire present in long-term chronic carriers of HBV. Similar to TCR-Tg HBeAg-specific CD4$^+$ T cells in double-Tg mice, HBeAg-specific CD4$^+$ T cells in chronically infected patients must be able to co-exist with serum HBeAg. In 11/4–12 TCR-double Tg mice, the Tg T cells are quiescent in vivo and are neither spontaneously activated nor inactivated by deletion or anergy. However, exposure to exogenous HBeAg in vitro or in vivo is capable of activating 11/4–12 TCR-Tg CD4$^+$ T cells derived from TCR-single or double Tg mice sufficiently to elicit cytokine production and T-helper cell function for anti-HBe antibody production in vitro and in vivo. It is notable that the minimum HBeAg concentrations necessary to activate 11/4–12 TCR-Tg CD4$^+$ T cells in vitro or in vivo are greater than the endogenous HBeAg concentrations (i.e., 10–70 ng/ml) examined. This suggests that the well recognized fluctuations in HBeAg concentration during natural HBV infection may variably effect HBeAg-specific CD4$^+$ T-cell activation. It has been previously proposed that increases of HBeAg concentration in the serum and accumulation of HBcAg intracellularly triggers HBe/HBcAg-specific CD4$^+$ T-cell activation and at least partially may account for the cyclic pattern of liver cell injury often observed in chronically infected patients (Maruyama et al., 1993b). Similarly, the ability to detect Th-cell sensitization to the HBe/HBcAgs in the PBL of chronic HBV patients coincides with periods of liver cell injury (Tsai et al., 1992). These findings support the contention that the HBe/HBcAg-specific CD4$^+$ T-cell repertoire remaining in long-term chronically infected patients may be of low avidity. This would explain the limited ability to detect HBe/HBcAg-specific T-cell proliferation or cytokine production in the PBL of chronic HBV patients (Ferrari et al., 1990; Jung et al., 1995; Tsai et al., 1992).

The preferential recognition of the monomeric HBeAg over the particulate HBcAg by 11/4–12 TCR-Tg T cells is an important observation. This finding establishes that CD4$^+$ T cells with this unusual fine specificity do exist. CD4$^+$ T cells that recognize the HBeAg preferentially to the HBcAg may represent at least one type of effector cell capable of selecting the HBeAg-negative mutant of the HBV (Raimondo et al., 1990). Generally, it has been observed that the HBeAg and HBcAg are fully cross-reactive at the level of CD4$^+$ T-cell recognition due to the shared amino acid sequence between these two antigens with the exception of the ten residual precore amino acids present on serum HBeAg (Ferrari et al., 1991; Milich et al., 1988). The 11/4–12 TCR-Tg T cells do not recognize the precore sequence (data not shown). The HBcAg is recognized preferentially to the HBeAg by HBe/HBcAg-specific CD4$^+$ T cells presumably because the particulate structure confers an advantage in terms of antigen uptake by antigen presenting cells (APC) (Milich et al., 1997). It appears that the epitope recognized by 11/4–12 TCR-Tg T cells is generated more efficiently by the presentation and processing of the HBeAg monomer as opposed to the particulate HBcAg.

It was somewhat surprising that 11/4–12 TCR-Tg T cells, which are of sufficiently low avidity to escape deletion in the thymus and in the periphery, nevertheless can under the appropriate conditions mediate liver injury as demonstrated by the adoptive transfer experiments. First, it was unexpected that low avidity CD4$^+$ T cells would be competent to mediate liver injury because hepatocytes express such limited amounts of MHC class II molecules. Secondly, it was of interest that the liver injury elicited was of a chronic relapsing and remitting nature, which is often observed in chronic HBV patients and in many autoimmune disorders as well. The low avidity of the 11/4–12 TCR Tg CD4$^+$ T cells may contribute to the relapsing and remitting course of liver injury. The partial or transient activation observed for these low avidity T cells may permit their continued survival in the liver due to less efficient down regulation via apoptosis or other regulatory mechanisms. An absence of sustained T-cell activation has been attributed to low TCR density (Viola and Lanzavecchia, 1996). Similarly, low avidity TCR binding resulting in fewer engagements of the TCR with peptide-MHC complexes may also deter sustained T-cell activation. We are currently investigating whether 11/4–12 TCR-Tg CD4$^+$ T cells directly cause liver injury or perhaps mediate it through the activation of endogenous HBe/

HBcAg-specific $CD8^+$ CTL. It is also possible that the $CD4^+$ T cells mediate liver injury directly through a FAS-FASL mechanism since FAS + hepatocytes are very sensitive to apoptosis (Galle et al., 1995). Alternatively, proinflammatory cytokines produced by $CD4^+$, Th_1-like cells such as IFNγ and TNFα may mediate liver injury. The rather mild liver injury observed in this model most likely reflects limiting antigen concentrations in the Tg recipients rather than limited effector cell function.

The development of HBe/HBcAg-specific TCR-Tg mice should enable us to obtain a greater understanding of the role of T-cell tolerance in the complex interactions between the immune response to the HBV nucleocapsid antigens, liver injury, and HBV clearance. We are currently examining additional HBe/HBcAg-specific TCR-Tg lineages possessing an array of specificities and avidities in order to obtain a more complete view of the HBe/HBcAg-specific $CD4^+$ T-cell repertoire in the context of constant exposure to the HBe/HBcAgs much like what occurs in chronic HBV infection.

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References

Beech, J.T., Bainbridge, T., Thompson, S.J., 1997. Incorporation of cells into an ELISA system enhances antigen-driven lymphokine detection. J. Immunol. Meth. 205, 163–168.

Chen, M., Sällberg, M., Thung, S.N., Hughes, J., Jones, J., Milich, D.R., 2000. Non-deletional T-cell receptor transgenic mice: a model for the $CD4^+$ T-cell repertoire in chronic hepatitis B virus infection. J. Virol. 74, 7587–7599.

Ferrari, C., Penna, A., Bertoletti, A., Valli, A., Degli Antoni, A.M., Giuberti, T., Cavalli, A., Petit, M.A., Fiaccadori, F., 1990. Cellular immune response to hepatitis B virus encoded antigens in acute and chronic hepatitis B virus infection. J. Immunol. 145, 3442–3449.

Ferrari, C., Bertoletti, A., Penna, A., Cavalli, A., Missale, G., Pilli, M., Fowler, P., Giuberti, T., Chisari, F.V., Fiaccadori, F., 1991. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. J. Clin. Invest. 88, 214–222.

Galle, P.R., Hofmann, W.J., Walczak, H., Schaller, H., Otto, G., Stremmel, W., Krammer, P.H., Runkel, L., 1995. Involvement of the CD95 (APO-1/Fas)receptor and ligand in liver damage. J. Exp. Med. 182, 1223–1230.

Gonzalez-Quintial, R., Theophilopoulos, A.N., 1992. Vβ gene repertoires in aging mice. J. Immunol. 149, 230–236.

Ho, W.Y., Cooke, M.P., Coodnow, C.C., Davis, M.M., 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive $CD4^+$ T cells. J. Exp. Med 179, 1539–1549.

Jung, M.C., Diepolder, H.M., Spengler, U., Wierenga, E.A., Zachoval, R., Hoffmann, R.M., Eichenlaub, D., Frosner, G., Will, H., Pape, G.R., 1995. Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific $CD4^+$ T cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J. Virol. 69, 3358–3368.

Maruyama, T., McLachlan, A., Iino, S., Koike, K., Kurokawa, K., Milich, D.R., 1993a. The serology of chronic hepatitis B infection revisited. J. Clin. Invest. 91, 2586–2595.

Maruyama, T., Iino, S., Koike, K., Yasuda, K., Milich, D.R., 1993b. Serology of acute exacerbation in chronic hepatitis B virus infection. Gastroenterology 105, 1141–1151.

Milich, D.R., McLachlan, A., Stahl, S., Wingfield, P., Thornton, G.B., Hughes, J.L., Jones, J., 1988. Comparative immunogenicity of hepatitis B virus core and E antigens. J. Immunol. 141, 3617–3624.

Milich, D.R., Jones, J.E., Hughes, J.L., Price, J., Raney, A.K., McLachlan, A., 1990. Is a function of the secreted hepatitis B E antigen to induce immunologic tolerance in utero. Proc. Natl. Acad. Sci. USA 87, 6599–6603.

Milich, D.R., McLachlan, A., Raney, A.K., Houghten, F., Thornton, G.B., Maruyama, T., Hughes, J.L., Jones, J.E., 1991. Autoantibody production in HBeAg transgenic mice elicited with a self T cell peptide and inhibited with non-self peptides. Proc. Natl. Acad. Sci. USA 88, 4348–4352.

Milich, D.R., Jones, J.E., Hughes, J.L., Maruyama, T., Price, J., Melhado, I., Jirik, F., 1994. Extrathymic expression of the intracellular hepatitis B core antigen results in T cell tolerance in transgenic mice. J. Immunol. 152, 455–466.

Milich, D.R., Chen, M., Schödel, F., Peterson, D.L., Jones, J.E., Hughes, J.L., 1997. Role of B cells in antigen presentation of the hepatitis B core. Proc. Natl. Acad. Sci. USA 94, 14648–14653.

Raimondo, G., Schneider, R., Stemler, M., Smedile, V., Rodino, G., Will, H., 1990. A new hepatitis B virus variant

in a chronic carrier with multiple episodes of viral reactivation and acute hepatitis. Virology 179, 64–68.

Schödel, F., Peterson, D., Zheng, J., Jones, J.E., Hughes, J.L., Milich, D.R., 1993. Structure of hepatitis B virus core and E-antigen: a single precore amino acid prevents nucleocapsid assembly. J. Biol. Chem. 268, 1332–1337.

Solheim, J.C., Alexander-Miller, M.A., Martinko, J.M., Connolly, J.M., 1993. Biased T cell receptor usage by Ld-restricted, turn-peptide-specific cytotoxic T lymphocyte clones. J. Immunol. 150, 800–811.

Tsai, S.-L., Chen, P.J., Lai, M.Y., Yang, P.M., Sung, J.-L., Huang, J.-H., Hwang, L.H., Chang, T.-H., Chen, D.S., 1992. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. J. Clin. Invest. 89, 87–96.

Viola, A., Lanzavecchia, A., 1996. T cell activation determined by T cell receptor number and tunable thresholds. Science 273, 104–106.