表 1　B型慢性肝炎に対する抗原非特異的な免疫療法

免疫療法	臨床試験の種類	治療効果	文献
IFN-γ	非盲検試験	12/54 症例で線維化が軽減	5
IL-2	無作為化二重盲検比較試験	効果なし	6
IL-12	第 I/II 相臨床試験の改編	効果なし	7
GM-CSF	予備試験	不明	8
チモシン-α1	無作為化試験	中等度の効果	9
α-ガラクトシルセラミド	第 I/II 相臨床試験	効果なし	10
プロパゲニウム	無作為化比較試験	中等度の効果	11

B型慢性肝炎に対する抗原非特異的な免疫療法についてまとめた。

免疫療法の概念，さらには現在筆者らが行っている免疫療法について，ヒトの基礎，臨床データに基づき述べたい。

● B型慢性肝炎に対する免疫療法の従来の概念

免疫応答は「抗原非特異的」と「抗原特異的」の2種類に分類することができる。今までは，CHBではこの2種類の免疫応答がいずれも低下しており，この免疫の低下がCHBの病態の根本にあると考えられてきた。そのため，これらの免疫応答を賦活化するためのさまざまな免疫賦活薬を用いた免疫療法が試みられてきた。

1　HBVに非特異的な免疫療法

抗原非特異的に免疫を賦活するため，IFN-α，IFN-β，IFN-γ，インターロイキン（IL）-2，IL-12，顆粒球/マクロファージコロニー刺激因子（GM-CSF），チモシン-α，α-ガラクトシルセラミド，プロパゲニウムや漢方などが試みられてきた[3,5~11]。

IFN-α，IFN-βは，現在のCHB治療の主役のひとつであり，若年者では第一選択としてガイドラインで推奨されているが，前述のごとく，効果については満足できるものではない。さらには，IFN製剤によるウイルス排除機構については，いまだ不明な点も多い。

IFN-γ，IL-2とIL-12は，免疫賦活作用を有するサイトカインで，他の炎症性サイトカインの産生，細胞傷害性T細胞（CTL），TH1反応を誘導する。これらのサイトカインは健常人と比較してCHB患者では血中の濃度が低く，CHB患者ではこれらのサイトカインを補充し，免疫を賦活することが目的とされた。これらのサイトカインを用いた予備試験，臨床試験では，CHB患者の免疫を賦活することは可能であったが，HBVの排除，肝炎の鎮静化などの治療効果を示すことはできなかった。さらには一部では副作用もみられ，抗原非特異的な免疫賦活薬を用いた治療法を確立するまでには至らなかった（詳細は文献12を参照のこと）。

これらのサイトカインを用いても期待した効果が得られなかった理由として，これらのサイトカインによるHBV排除，肝炎鎮静化のメカニズムが明らかになっておらず，病態のエビデンスが欠如していた点があげられる。一時的な効果があった患者もみられたが，その効果の機序についても十分な説明が困難であった（**表1**）。

2　HBVに非特異的な細胞療法

リンパ球を活性化するため，リンパ球を取り出し，生体外で活性化する方法も行われた。CHB患者のリンパ球を生体外でanti-CD3モノクローナル抗体，IFN-γ，IL-12で抗原非特異的に刺激し，活性化したリンパ球をCHB患者に移入した。また，悪性リンパ腫のCHB患者を対象に，HBV既往感染者の骨髄移植が行われた。いずれの細胞療法においても，効果のみられる症例もあったが，セロコンバージョン率，HBV-DNA消失率についても核酸アナログ製剤やIFNを凌駕するものではなかった。また，誘導された免疫応答に関しても，長期間の持続の有無については明らかにされなかった[13,14]。

3　HBVに特異的な免疫療法

HBVに特異的な獲得免疫を賦活するために，HBV関連抗原を用いたワクチン治療が試みられてきた。

a　HBワクチン（HBs抗原）

HBワクチンは感染予防に使われるワクチンで，HBs抗原を含有している。HBワクチンに

表 2　B 型慢性肝炎に対する HB ワクチン療法

臨床試験		ワクチン	治療効果	文献
ワクチン単独療法	予備試験	HBs 抗原，6 回投与	効果なし	15
	予備試験	HBs 抗原，3 回投与	中等度の効果	16
	比較試験	HBs 抗原，5 回投与	特記する効果なし	17
	予備試験	HBs 抗原，12 回投与	中等度の効果	18
	予備試験	HBs 抗原，3 回投与	効果なし	19
	予備試験	HBs 抗原	効果なし	20
	予備試験	HBc 抗原エピトープ，4 回投与	効果なし	21
	予備試験	DNA ワクチン	免疫応答あり	22
	予備試験	HBs 抗原/抗体複合体ワクチン，6 回投与	有意な効果なし	23
HB ワクチンと核酸アナログの併用療法	予備試験	HBs 抗原ワクチン＋ラミブジン	相反する効果	26-29
	予備試験	HBs 抗原ワクチン＋クレブジン	効果あり	30
	無作為化比較試験	HBs 抗原ワクチン＋ラミブジン	効果なし	31

B 型慢性肝炎に対する HBs 抗原含有ワクチンを用いたワクチン単独療法と，HB ワクチンと核酸アナログ製剤の併用療法についてまとめた。

より誘導される HBs 抗体は中和抗体であり，HBV の排除に重要な役割を果たす。この HBs 抗体の誘導を目的とした HBs 抗原ワクチン治療は，1981 年に Dienstag らによって初めて行われた[15]。その後，HBs 抗原を用いた CHB に対するワクチン療法は，それぞれの施設により，投与量，ワクチンの組成や投与回数などの違いはあるものの，種々の施設で行われてきた[16~20]（**表 2**）。さらには，より強力な免疫応答を誘導するため，さまざまなアジュバントも試みられた[21~23]。HBs 抗原を用いたワクチン療法の最大の利点はその安全性であり，このワクチン療法による重大な副作用は，現在まで報告されていない。一部の患者ではセロコンバージョンを起こすことが可能であり，ワクチン療法により HBV 特異的な免疫応答を誘導することが可能であった。しかしながら，HBV ウイルス量を減少させる効果が不十分であり，HBs 抗原を用いたワクチン単独療法は CHB に対する最良の治療法とは言い難い結果であった。

b　HB core ワクチン

HBV core 蛋白上に存在する T cell epitope を用いたワクチン（CY-1899）の報告もあるが，パイロット試験であり，臨床的な改善効果はみられなかった[24]。しかし，単一エピトープではなく，複数のエピトープを組み合わせることで，より強力で臨床効果のあるエピトープワクチンになる可能性は残されている。

c　HBV-DNA ワクチン

DNA ワクチンは目的とする抗原をベクターに埋め込み投与することで，生体が多量の目的抗原を作製するため，従来のペプチドワクチンより強力な免疫誘導を起こすと考えられている[25]。しかし，外来 DNA を組み込むため安全性が問題視されている。

d　HB ワクチンと核酸アナログの併用療法

HB ワクチンの単独療法では HBV ウイルス量を低下させる効果が小さかったため，HB ワクチンと核酸アナログとの併用療法が試みられた。まず，核酸アナログ製剤で HBV-DNA 量を低下させ，ウイルス量が低下してからワクチン療法を行い，HBV 特異的な免疫応答を誘導することが目的であった[26~30]。しかしながら，多施設によるコントロール試験では十分な治療効果を確認できなかった[31]。

4　HBV に特異的な細胞療法

樹状細胞はプロフェッショナルな抗原提示細胞であり，抗原特異的な獲得免疫を誘導する。筆者らは，この樹状細胞をアジュバントとして用いた樹状細胞ワクチン療法を行った。樹状細胞を分離培養後，HBs 抗原でパルスし，樹状細胞ワクチンを作製し，CHB 患者に投与した。5

表 3　B 型慢性肝炎における免疫異常
必ずしも低下していない

- HBV 抗原に非特異的な免疫応答
  - 炎症性サイトカイン　➡亢進，低下いずれもあり
  - 抗炎症性サイトカイン➡亢進，低下いずれもあり
- HBV 抗原に特異的な免疫応答
  - HBc 抗原 ➡
    - HBc 抗体：すべての病期で検出
    - 細胞性免疫：すべての患者でみられる
  - HBs 抗原 ➡
    - HBs 抗体：存在するが，通常の測定法では検出不可能
    - 細胞性免疫：みられるが，とても弱い
  - HBe 抗原 ➡ HBe 抗体：非活動期の患者の多くで陽性

症例に施行したが，副作用はみられず，安全性を確認した。2 症例では，HBs 抗体の出現を確認したが，HBV-DNA の消失，肝炎の鎮静化はみられなかった[32]。CHB に対する樹状細胞ワクチン療法も期待した臨床効果が得られなかった。さらに，筆者らは CHB の治療だけではなく，樹状細胞ワクチンによる感染予防の予備試験を行った。通常の HB ワクチンでは中和抗体が誘導できない non-responder 6 例に，樹状細胞ワクチンを投与した。6 例全例に中和抗体の誘導が可能であり，HBV 感染予防における活用に期待ができるものであった[33,34]。

● 従来の免疫療法からの教訓と明らかになりつつある CHB の病態

CHB に対する免疫療法が始まり，約 30 年が経とうとしているが，CHB に対する免疫療法の最適のレジメンはいまだ確立されていない。この原因は，いままでの免疫治療の概念と実際の病態との間にギャップがあったからである。今までは，CHB では HBV 特異的，非特異的のいずれの免疫応答も低下していると考えられてきた。しかし実際には，CHB 患者では肝内へのリンパ球浸潤が著明であり，自然免疫が活性化し，リンパ球が多量の炎症性サイトカインを産生することが示すように，これらの免疫応答は必ずしも低下しておらず，一部ではむしろ亢進していることがわかってきた[35]。さらには，活性化した HBV-specific CD8（＋）cells こそが肝障害の原因であることも証明された[36]。

実際 CHB 患者においては，HBV に特異的な免疫応答は必ずしも低下していないのである。たとえば HB コア抗原に対する抗体（HBc 抗体）は CHB 患者の全病期を通じて血中にみられ，血中の HBV-DNA 量と並行して変動する。また，HBe 抗原に対する抗体である HBe 抗体は，ウイルス量の低下した非活動期の CHB 患者に多くみられる。S 抗原に対する抗体である HBs 抗体は，現行の測定法では測定できないが，中和されているために検出できないだけであって[37]，理論上はすべての CHB 患者でみられる（表 3）。これらのことを加味すると，HBV に特異的な免疫と非特異的な免疫が一様に低下しているという旧来の仮説に基づく免疫治療は，エビデンスに基づいておらず，科学的とはいえない。CHB 患者の生体内では，むしろ HBV にきわめて特異的な免疫が反応し，作動している。

今まで，中和抗体である HBs 抗体がウイルス排除の中心であると考えられてきた。近年，HBc 抗原に対する免疫応答，特に HBc 抗原に特異的な CTL が HBV の排除において重要であることが明らかになった[36,38]。血中に HBc 抗体が存在していても，肝臓内の HBc 抗原に特異的な CTL が肝臓内で減少していれば，HBV-DNA の複製や肝臓の炎症をコントロールすることができず，他方，HBc 抗原に特異的な CTL が肝内に豊富に存在すれば，HBV-DNA の複製，肝臓の炎症ともにコントロールされていた。加えて，血中 HBV-DNA 量が低く，肝炎も鎮静化にある状態の CHB 患者では，HBc 抗原特異的な CTL が有意に多く，HBc 抗原に対する反応も大きかった。つまり，肝臓内の HBV-DNA の排除と肝炎の抑制に HBc 抗原に特異的な CTL が深く関与していることが明らかになった。

● CHB の病態のエビデンスに基づいた免疫療法（混合ワクチン）の概念

1　HBc 抗原と HBs 抗原の混合ワクチンの概念

前項で述べたように，現在，HBV の病態と

HBV に特異的な免疫の役割が明らかになりつつある。肝内の HBV は肝内の HBc 抗原に特異的な CTL によってコントロールされており，血中の遊離 HBV 粒子は HBs 抗体によってコントロールされている。これらのことから，HBV の排除には HBc 抗原特異的な CTL，HBs 抗体，さらには，それらを誘導するためのヘルパー T 細胞が必要となる。つまり，HBc 抗原に特異的な CTL，HBs 抗体，HBc 抗原・HBs 抗原に特異的なヘルパー T 細胞を誘導することが，理想の免疫療法といえる。それらを誘導するには，HBs 抗原と HBc 抗原の両抗原を含む混合ワクチンが必要となる。

2　HBc 抗原と HBs 抗原の混合ワクチンを用いた第 I 相臨床試験

HBs 抗原は予防ワクチンとして過去 30 年にわたり臨床で使用されており，安全性も確認されている。しかしながら，ヒトに使用可能な HBc 抗原は市販されておらず，市場では入手できない。筆者らは，ヒトに使用できる GMP グレードの HBc 抗原を入手することができた[39]。その後，倫理委員会の承認を経て，18 人の無治療歴の CHB 患者を対象に，HBs 抗原と HBc 抗原を含んだ混合ワクチンによる第 I 相臨床試験を開始した[40]。より効果的に免疫を誘導する投与経路を見いだすため，筋肉注射だけでなく，点鼻（経鼻）にて混合ワクチンの投与を行った。

この臨床試験では，すべての CHB 患者において，全身もしくは鼻咽喉に関する副作用はみられず，安全に投与することができた。HBV-DNA 量については，9/18 症例において測定感度以下（<500 copies/mL）となり，8/18 症例では低下し，残りの 1 症例については変化がみられなかった。また，すべての症例で，混合ワクチン投与後に，末梢血単核球の HBs 抗原と HBc 抗原刺激による *in vitro* での IFN-γ の産生が増加した。これは今までの免疫療法と比較しても高い治療効果を示すことができた。今後の第 II/III 相臨床試験では，より詳細な免疫学的な解析も行い，混合ワクチンにより HBc 抗原と HBs 抗原に特異的な免疫の誘導が可能であったかについても確認する予定である。

● 免疫療法の今後の展開

免疫療法は補充代替治療ではなく，目的をもって免疫の賦活，抑制を行うべきものである。病態を考慮し，そのエビデンスに基づいた免疫療法でなければ，核酸アナログ製剤，IFN 製剤よりも強力な治療効果を示すことは困難である。HBV による急性肝炎では，一時的に HBV ウイルスが増加し，多数の肝細胞が破壊される。しかしながら，多くの症例では HBV が排除され，さらには肝炎も消失し，治癒に至る。また，CHB の一部の患者では治療しなくても HBV が消失し，セロコンバージョンが起こり，肝炎の進行が止まる。このような免疫応答を，今までの免疫療法では，成し遂げることができなかった。筆者らの臨床試験は病態のエビデンスに基づいたものであり，予備の段階であるが，今までの免疫療法より高い治療効果を示すことができた。

しかし，筆者らが挑戦している“HBV 特異的な免疫応答の誘導”とて必ずしも免疫療法の到達目標ではないかもしれない。筆者らは 2 つの抗原を用いた免疫療法を行っているが，理想の免疫療法とは，さらに多くの抗原を用いて，複数の投与経路で，複数のアジュバントを用いる必要があるのかもしれない。CHB に対する免疫療法が確立されるのは，多施設臨床試験にて，無治療症例，治療歴のある症例，いずれにおいても良好な結果を示してからである。さらには，免疫療法により誘導された免疫応答が長期間にわたり持続するか否かについても明らかにする必要がある。CHB の病態についても明らかになりつつある現在，効果的な免疫療法のプロトコールを示す準備ができた。あとは多施設臨床試験にてエビデンスを示すだけである。

最後に，心にとどめておかなければならないことは，CHB に対する免疫療法の目的は，HBV に特異的な免疫の誘導ではない。HBV の持続感染によって破壊されてしまった肝組織の組織環境を改善し，不幸な転帰，合併症を低下させ，予後を改善することである。

● まとめ

今までのCHBの免疫療法は，免疫の賦活のみを目的としており，病態を考慮することに欠けていた。現在は，CHBの病態も明らかになりつつあり，病態のエビデンスに基づいた免疫療法を設計することが可能となってきている。筆者らは病態のエビデンスに基づき，HBs抗原とHBc抗原の2つの抗原を用いた免疫療法を行っており，今後第II/III相の臨床試験を計画中である。また世界各国において，抗原を用いたワクチン療法だけでなく，細胞療法，樹状細胞ワクチン，DNAワクチンなどさまざまな免疫療法が開発中である。しかしながら，実際に免疫療法として認知，確立されるのは，臨床試験でその治療効果が明確に示されてからである。

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Antiviral Therapy 2010 15:887–895 (doi: 10.3851/IMP1637)

Original article

Immune modulator and antiviral potential of dendritic cells pulsed with both hepatitis B surface antigen and core antigen for treating chronic HBV infection

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Background: Commercially available prophylactic vaccines containing hepatitis B surface antigen (HBsAg), which are used to prevent HBV infections, are not as effective as a therapeutic immune modulator for treating patients with chronic hepatitis B (CHB). In this study, the immunogenicity of dendritic cells (DC) loaded with both HBsAg and hepatitis B core antigen (HBcAg) was tested in HBV transgenic mice (TM; 1.2HB-BS10) *in vivo* and in patients with CHB *in vitro*.

Methods: Spleen DC from HBV TM were cultured with a vaccine containing both HBsAg and HBcAg to produce HBsAg/HBcAg-pulsed DC. HBV TM were immunized twice at an interval of 4 weeks with HBsAg/HBcAg-pulsed DC and other immune modulators. Antibody titres to HBsAg (anti-HBs) were measured in sera. Antigen-specific T-cells and cytotoxic T-lymphocytes (CTLs) in the spleen and liver were detected by lymphoproliferative and ELISPOT assays, respectively. HBsAg/HBcAg-pulsed human blood DC were cultured with autologous T-cells from CHB patients to assess their antigen-specific immune modulatory capacities.

Results: Significantly higher levels of anti-HBs, HBsAg-specific and HBcAg-specific T-cells and CTLs were detected in the spleen and liver of HBV TM immunized with HBsAg/HBcAg-pulsed DC compared with those immunized with other vaccine formulations ($P<0.05$). HBsAg/HBcAg-pulsed human blood DC also induced HBsAg- and HBcAg-specific proliferation of autologous T-cells from CHB patients.

Conclusions: The immune modulatory capacities of HBsAg/HBcAg-pulsed DC in HBV TM *in vivo*, and in patients with CHB *in vitro*, inspire optimism about a clinical trial with this cell-based vaccine in patients with CHB.

Introduction

Important insights about epidemiology, virology, immunology and pathogenesis of HBV have been documented during the past four decades. However, no curative therapy against chronic hepatitis B (CHB) has been developed. Clinical trials with different antiviral drugs (type-I interferon [IFN] and nucleoside/nucleotide analogues) have inspired considerable optimism about their use in CHB patients on the basis of intermediate outcomes [1]. However, a systemic review of a National Institutes of Health Consensus Development Conference that analysed all randomized clinical trials (RCTs) with antiviral drugs in CHB patients from 1989 to 2008 showed that drug treatment did not improve ultimate clinical outcomes or all intermediate outcomes in any RCT [2]. Low and moderate quality RCTs suggested improvement of some intermediate parameters by using antiviral drugs in CHB patients [2]. However, these drugs are costly, might require prolonged use, and are associated with several side effects, including the emergence of treatment-induced mutant HBV. These findings indicate that alternative therapeutic approaches should be developed to treat CHB.

The concept of immune therapy as an alternative therapeutic approach for treating CHB patients has emerged for several reasons. First, patients with CHB exhibit distorted immune responses to various HBV-related antigens [3]. In addition, sustained control of HBV replication and liver damage in CHB patients is usually associated with restoration of host immunity [4]. However, the therapeutic efficacy of polyclonal immune

modulators, such as immune IFN, growth factors and cytokines, was not satisfactory in CHB patients [5]. Subsequently, therapeutic vaccines containing hepatitis B surface antigen (HBsAg) have been used since the early 1990s. However, it is unlikely that the present vaccines will be able to stand the test of time [6].

We found some limitations to the current therapeutic vaccines for CHB patients. First, HBsAg is administered to CHB patients with the assumption that it will be internalized, processed and presented by antigen-presenting cells to induce HBsAg-specific immune responses [7]. However, because the phenotypes and functions of dendritic cells (DC), the most potent antigen-presenting cells, are distorted in CHB [8], it is unlikely that the DC of CHB patients would be able to properly process and present HBsAg for restoration of HBV-specific immunity. Second, vaccine therapies in CHB patients have been accomplished with HBsAg-based vaccines only. However, both HBsAg and hepatitis B core antigen (HBcAg)-specific immune responses are essential for sustained control of HBV replication and containment of liver damage [3]. Third, almost all patients with CHB harbour considerable levels of HBsAg; thus, μg levels of HBsAg in commercial vaccines might not be sufficient to overcome the immune tolerance state of these patients. In addition, clonal deletion of HBV-specific T-cells and the exhaustion of antiviral cytotoxic T-lymphocytes (CTLs) by high doses of antigen might also have a role in the minimal effect of HBsAg-based vaccine therapy [9]. Taken together, these factors indicate that a better regimen of immune therapy against HBV might be created by delivering HBV-related antigens with adequately activated antigen-presenting DC in CHB patients.

The immune modulator effects of HBsAg-pulsed DC have been evaluated in HBV transgenic mice (TM) by other investigators, as well as our group [10,11]. Jiang *et al.* [10] have shown that immunization with peptide-pulsed DC could elicit antiviral immunity in HBV TM. However, they used a murine model of HBV that expressed only HBsAg; therefore, the clinical implications of their study for translation research in patients with CHB are limited [10]. We found that HBsAg-pulsed DC induced antibody to HBsAg (anti-HBs) production, but not HBsAg-specific cellular immunity, in HBV TM [11].

Availability of a human consumable vaccine containing both HBsAg and HBcAg led us to perform the present study. First, we loaded spleen DC from normal C57BL/6J mice with this vaccine to optimize culture conditions. Then, we assessed the specificity and immune modulatory functions of HBsAg/HBcAg-pulsed DC *in vitro*. Subsequently, a preclinical study was conducted in HBV TM with HBsAg/HBcAg-pulsed DC and several other immune modulators. Finally, we extended this study to evaluate whether HBsAg/HBcAg-pulsed human blood DC from CHB patients were capable of activating autologous immunocytes in an antigen-specific manner *in vitro*. The research potential of this study will be discussed for developing antigen-specific immune therapy against chronic HBV infection in humans.

Methods

Mice

HBV TM (official designation 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 base pairs (bp) of HBV DNA into the fertilized eggs of C57BL/6 mice. The HBV TM expressed HBV DNA and messenger RNA of 3.5, 2.1 and 0.8 kbp of HBV in the liver. HBV DNA were also detected in the liver and sera of HBV TM. HBsAg was found in the sera of all HBV TM [12]. Male C57BL/6J mice that were 8 weeks old were purchased from Nihon Clea Co. (Tokyo, Japan). Mice were housed in polycarbonate cages in our laboratory facilities and maintained in a temperature- and humidity-controlled room (23 ±1°C) with a 12-h light/dark cycle. All mice received humane care, and the study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Ehime University (Ehime, Japan).

Patients with CHB

Peripheral blood mononuclear cells and DC were isolated from five patients with clinical, biochemical and histological evidence of CHB. They were attending Ehime University Hospital (Ehime, Japan) for regular follow-up. The mean ±SD age of the patients was 35 ±12 years and the level of alanine aminotransferase was 127 ±32 IU/l (normal range 5–48 IU/l). Liver biopsy revealed a moderate degree of activity of hepatitis and moderate levels of fibrosis. Patients had not taken any antiviral or immune modulator drugs during the previous 6 months. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Ehime University Hospital human research committee.

Detection of HBV-related markers

Levels of HBsAg and anti-HBs in sera were estimated using a chemiluminescence enzyme immunoassay (Special Reference Laboratory, Tokyo, Japan) and were expressed as IU/ml and mIU/ml, respectively [13]. Antibodies to HBcAg in sera were estimated by the passive hemagglutination method (Tokyo Institute of Immunology, Tokyo, Japan). HBV DNA in the sera of HBV TM was assessed by the PCR method (Special Reference Laboratory).

Isolation of T-cells, B-cells and DC

The methodologies for isolating spleen cells, T-cells, B-cells and DC are described in detail elsewhere [14]. In brief, spleens were removed aseptically, cut into pieces and incubated at 37°C in 5% CO_2 for 30 min in RPMI 1640 (Nipro, Osaka, Japan) supplemented with 1 µg/ml collagenase (type IV; Sigma Aldrich Corporation, St Louis, MO, USA), and a single-cell suspension of spleen was produced. T-cells were purified from single-cell suspensions of spleen by a negative-selection column method (Mouse Pan T Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ T-cells were purified from T-cells by a column method (Mouse CD8 Isolation Kit; Miltenyi Biotec).

To isolate spleen DC, single-cell suspension of spleen was centrifuged at 10,000×*g* for 30 min on a dense albumin column (specific gravity 1.082) at 4°C and then cultured on a plastic surface for 90 min at 37°C. The adherent cells were cultured for an additional 18 h in culture medium containing RPMI 1640 plus 10% fetal calf serum (Filtron PTY Ltd, Brooklyn, Australia). Macrophages were discarded from DC populations by two additional adherent steps on plastic dishes at 37°C.

Liver non-parenchymal cells (NPC) were isolated as described previously [15]. In brief, liver tissues were homogenized, suspended in 35% percoll (Sigma Aldrich Corporation), and centrifuged to get liver NPC. Liver NPC were suspended in RPMI 1640 plus 10% fetal calf serum.

Human blood DC were enriched from peripheral blood of CHB patients by culturing them with granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 for 6 days, as described previously [16].

Preliminary experiments to optimize culture conditions for preparing immunogenic antigen-pulsed DC

Normal C57BL/6J mice were immunized with HBsAg (10 µg; Tokyo Institute of Immunology), HBcAg (10 µg; Tokyo Institute of Immunology), HBsAg/HBcAg (10 µg Center for Genetic Engineering and Biotechnology [CIGB], Havana, Cuba) and pyruvate dehydrogenase complex (PDC; 10 µg; Sigma Aldrich Corporation) [17] in phosphate-buffered saline (PBS) twice at an interval of 4 weeks. Lymphocytes from immunized mice were cultured with antigen-pulsed DC from non-immunized mice to evaluate if antigen-pulsed DC could induce proliferation of antigen-specific lymphocytes *in vitro*.

Preparation of antigen-pulsed DC for immunization of HBV TM

HBsAg, HBcAg and HBsAg/HBcAg, used for the assessment of therapeutic efficacy of antigen-pulsed DC in HBV TM, were provided by the CIGB [18]. *Pichia-pastoris*-derived recombinant HBsAg was used. HBcAg were derived from *Escherichia coli* purified recombinant full-length HBcAg (GenBank accession number X02763). HBsAg/HBcAg consisted of equal amounts of HBsAg and HBcAg. HBsAg was produced as a 22 nm particle to >95% purity at the CIGB production facilities as a component of the commercial anti-HBV vaccine, Heberbiovac-HB1. HBcAg was purified from *E. coli* strain W3110, which had been transformed previously with a plasmid containing the entire core antigen gene under the control of a tryptophan promoter. The resulting HBcAg had a purity >95% [18].

Murine antigen-pulsed DC were prepared based on data from preliminary studies and also according to our previous report [19]. Briefly, spleen DC were cultured with PDC (10 µg), HBsAg (10 µg), HBcAg (10 µg) and HBsAg/HBcAg vaccine (HBsAg 5 µg and HBcAg 5 µg) in culture medium for 48 h. DC were recovered from the cultures and washed 5× with PBS. The viability of DC was assessed by the trypan blue exclusion test. The T-cell stimulatory capacity of antigen-pulsed DC were confirmed by lymphoproliferative assays. Human blood DC was cultured with HBsAg/HBcAg vaccine (HBsAg 5 µg and HBcAg 5 µg) or PDC (10 µg) for 48 h to prepare antigen-pulsed DC, as described elsewhere [16].

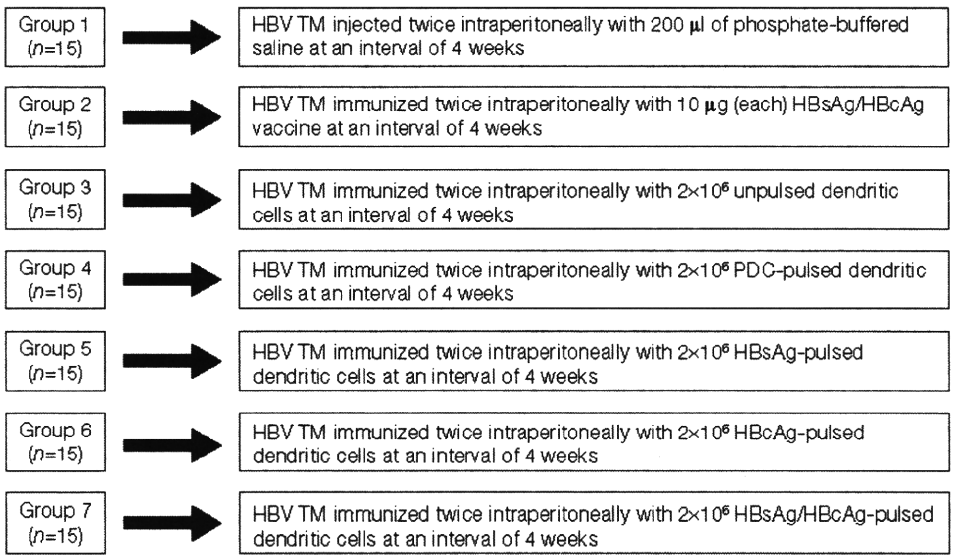
Immunization schedule

The immunization schedule is shown in Figure 1. Seven groups of HBV TM with comparable levels of HBsAg in the sera were used for this preclinical study. One group of HBV TM was injected with PBS (n=15). A second and third group of HBV TM received the HBsAg/HBcAg vaccine (10 µg; n=15) and 2×10^6 unpulsed DC (n=15), respectively. A fourth, fifth and sixth group of HBV TM received 2×10^6 PDC-pulsed DC (n=5), 2×10^6 HBsAg-pulsed DC (n=15) and 2×10^6 HBcAg-pulsed DC (n=15). Finally, a seventh group of HBV TM received HBsAg/HBcAg-pulsed DC (n=15). All vaccinations were done by the intraperitoneal route, twice at an interval of 4 weeks. HBV TM were bled from the tail vein at different intervals to assess different immunological parameters. The mice were finally euthanized to estimate vaccine-induced cellular immune responses in the spleen and liver.

Lymphoproliferative assays

As described previously, murine lymphocytes, murine liver NPC and human peripheral blood mononuclear cells were cultured in the absence or presence of different immune modulators for 120 h to evaluate antigen-specific cellular immune responses [14–17,19,20]. All cultures were performed in 96-well U-bottom plates (Corning Inc., New York, NY, USA). [^{3}H]-thymidine (1.0 µCi/ml; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile PBS, added to the cultures for the last 16 h and harvested automatically by a multiple cell harvester (Labo Mash;

Figure 1. Immunization schedule for assessment of antigen-specific humoral and cellular immune responses in HBV TM



HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; PDC, pyruvate dehydrogenase complex; TM, transgenic mice.

Futaba Medical, Osaka, Japan) onto a filter paper (LM 101–10; Futaba Medical). The levels of incorporation of [^{3}H]-thymidine were determined in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA) from the level of blastogenesis. Triplicate cultures were assayed routinely and the results were expressed as cpm. The stimulation index was calculated as the ratio of cpm obtained in the presence of antigen or antigen-pulsed DC to that obtained without antigen or in presence of only DC or irrelevant antigen-pulsed DC. A stimulation index >3.0 was considered significant.

ELISPOT assays

Different types of immunocytes (1×10^5) were stimulated with antigen in presence of mitomycin-C-treated spleen adherent cells in an IFN-γ-coated ELISPOT plate (Mabtech, Nacka Strand, Sweden) for 24 h. After removing the cells, biotinylated antibody (2A5-biotin) was added into the wells. After 2 h incubation, the plates were further incubated with streptavidin–alkaline phosphatase for 1 h. After washing the plates, the substrate solution, BCIP/NBT, was added. The reaction was stopped by washing the plates extensively with tap water. The numbers of spot-forming units were counted using an ELISPOT reader (KS ELISPOT; Carl Zeiss, Thornwood, NY, USA). Different types of immunocytes were stimulated with concanavalin A as a positive control.

Statistical analyses

Data are shown as mean ±SD. Means were compared using the Student's *t*-test. For differences determined by the F-test, the Student's *t*-test was adjusted for unequal variances (Mann–Whitney U test). $P<0.05$ was considered statistically significant.

Results

Features of antigen-pulsed DC and their functional capacities

We first isolated DC from normal C57BL/6 mice spleen and human blood. Murine spleen DC expressed major histocompatibility complex (MHC) class II antigen and CD86 antigens. Human monocyte-derived DC expressed human leukocyte antigen DR and CD86 antigens. A functional study showed that both murine spleen DC and human blood DC induced proliferation of allogenic T-cells in a dose-dependent manner (SMFA *et al.*, data not shown).

Antigen-pulsed DC from normal C57BL/6 mice produced significantly higher levels of IL-12 (HBsAg-pulsed DC 154.3 ±12.3 pg/ml and HBcAg-pulsed DC 213.2 ±23.6 pg/ml; $n=3$) compared with unpulsed DC (35.3 ±9.6 pg/ml; $n=3$; $P<0.05$). Also, HBsAg-pulsed and HBcAg-pulsed DC induced proliferation of lymphocytes from HBsAg- and HBcAg-immunized normal C57BL/6J mice (SMFA *et al.*, data not shown). After

optimizing culture conditions for preparing immunogenic HBsAg-pulsed DC and HBcAg-pulsed DC, we prepared HBsAg/HBcAg-pulsed DC from normal C57BL/6J mice. HBsAg/HBcAg-pulsed DC expressed significantly higher levels of MHC class II and CD86 compared to unpulsed DC ($P<0.05$). Also, HBsAg/HBcAg-pulsed DC produced significantly higher levels of IL-12 and IFN-γ compared with unpulsed DC ($P<0.05$). As shown in Table 1, HBsAg/HBcAg-pulsed DC did not induce significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 1.0), but induced vigorous proliferation of lymphocytes from HBsAg/HBcAg-immunized normal C57BL/6J mice (stimulation index 17.3 ±3.2; $n=3$). By contrast, PDC-pulsed DC induced significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 9.6 ±2.2; $n=3$). However, PDC-pulsed DC did not induce proliferation of HBsAg/HBcAg-immunized normal C57BL/6 mice (stimulation index 1.0). After assessment of immunogenecity of antigen-pulsed DC in normal C57BL/6 mice *in vitro*, we next evaluated immunogenecity of antigen-pulsed DC in HBV TM *in vivo*.

HBsAg and anti-HBs in HBV TM immunized with antigens and antigen-pulsed DC

When the levels of HBsAg were estimated 4 weeks after the second immunization, the levels of HBsAg in the sera were decreased or became undetectable in HBV TM immunized with HBsAg-pulsed DC and HBsAg/HBcAg-pulsed DC. However, there was no significant alteration in HBsAg levels in HBV TM immunized with other formulations (Figure 2A).

Anti-HBs were not detected in PBS-injected HBV TM ($n=15$). In addition, anti-HBs were not detected in HBV TM injected twice with vaccine containing HBsAg/HBcAg ($n=15$) or unpulsed DC ($n=15$), PDC-pulsed DC ($n=5$) or HBcAg-pulsed DC ($n=15$). However, anti-HBs were detected in all HBV TM after two injections with HBsAg/HBcAg-pulsed DC (Figure 2B). Anti-HBs were also detected in HBV TM immunized with HBsAg-pulsed DC, but the levels of anti-HBs in these HBV TM were significantly lower than those in HBV TM immunized with HBsAg/HBcAg-pulsed DC (Figure 2B).

Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC

We checked antigen-specific cellular immune responses in different groups of HBV TM. Lymphocytes from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to both HBsAg and HBcAg (Figure 3). However, lymphocytes from PBS-injected HBV TM, HBsAg/HBcAg vaccine-immunized HBV TM, unpulsed DC-injected HBV TM and PDC-pulsed DC-immunized HBV TM did not proliferate because of stimulation with HBsAg or HBcAg. Lymphocytes from HBV TM immunized with HBsAg-pulsed DC and HBcAg-pulsed DC proliferated in response to respective antigens only, but not to both HBsAg and HBcAg (Figure 3).

These data revealed that immunization with HBsAg/HBcAg-pulsed DC was capable of inducing HBsAg-specific humoral, HBsAg-specific cellular and HBcAg-specific cellular immune responses in HBV TM. Next, we checked if antigen-specific CTLs were produced in the spleen as a result of immunization of HBV TM with HBsAg/HBcAg-pulsed DC.

Detection of IFN-γ producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC

Spleen T-cells from HBV TM injected with HBsAg/HBcAg-pulsed DC, but not those from HBV TM immunized with HBsAg/HBcAg vaccine, unpulsed DC or PBS, or PDC-pulsed DC, produced significant numbers of IFN-γ-secreting T-cells in response to stimulation with HBsAg and HBcAg in the ELISPOT assay (Table 2). Also, abundant numbers of IFN-γ-secreting T-cells were detected in ELISPOT assays when spleen T-cells were stimulated with concanavalin A (positive control; SMFA *et al.* data not shown).

Antigen-specific immunocytes in the liver resulting from immunization with HBsAg/HBcAg-pulsed DC

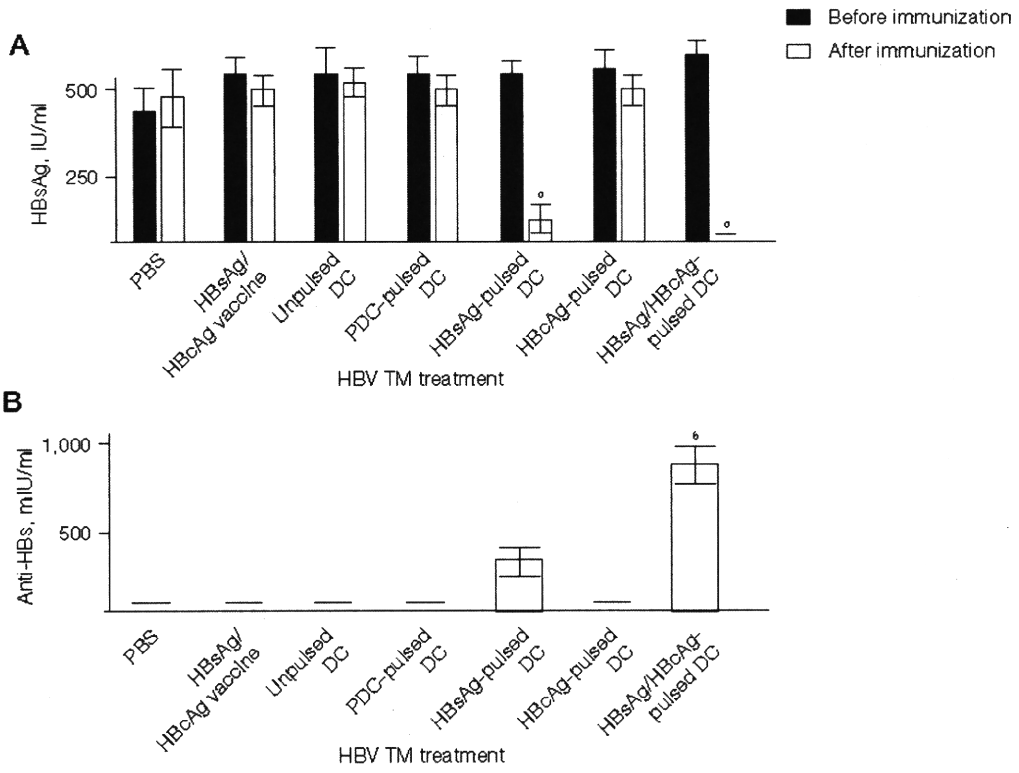
Although antigen-specific lymphocytes and CTLs were detected in the spleen, it was important to assess if antigen-specific lymphocytes and CTLs were induced

Table 1. Antigen-specific proliferative capacities of antigen-pulsed dendritic cells

Lymphocyte	DC	Stimulation index
HBsAg/HBcAg-immunized mice	PDC-pulsed	1.0
HBsAg/HBcAg-immunized mice	HBsAg/HBcAg-pulsed	17.3 ±3.2
PDC-immunized mice	HBsAg/HBcAg-pulsed	1.0
PDC-immunized mice	PDC-pulsed	9.6 ±2.2

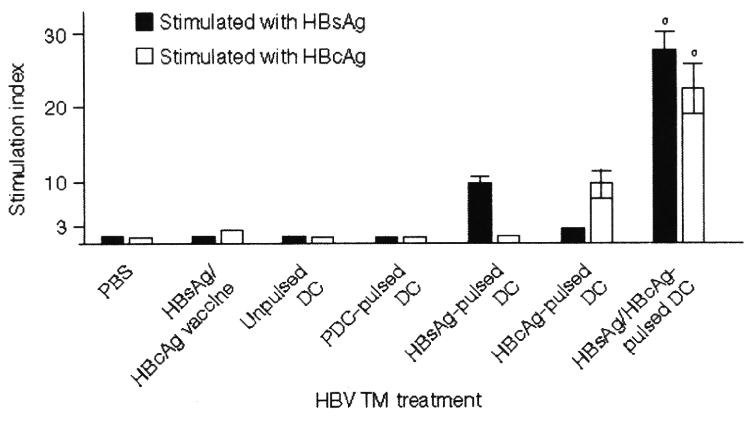
Normal C57BL/6J mice were immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg) or pyruvate dehydrogenase complex (PDC)-pulsed dendritic cells (DC), as described in *Methods*. Mice were euthanized 4 weeks after the second immunization, and spleen cells were injected with different stimulants. The levels of blastogenesis in cultures containing T-cells and irreverent antigen-pulsed DC were regarded as a stimulation index of 1.0. Data for stimulation indices are mean ±SD of three separate experiments.

Figure 2. Levels of HBsAg and anti-HBs in HBV TM before and after immunization with vaccines and antigen-pulsed DC



(A) HBV transgenic mice (TM) were immunized with different immunization regimens, as described in Figure 1. The levels of hepatitis B surface antigen (HBsAg) in the sera of different groups of mice before immunization and 4 weeks after second immunization are shown. (B) Antibody to HBsAg (anti-HBs) was not detected in HBV TM 4 weeks after injection with phosphate-buffered saline (PBS), vaccine containing HBsAg/hepatitis B core Antigen (HBcAg), unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBcAg-pulsed DC. However, anti-HBs was detected in all HBV TM 1 month after immunization with HBsAg/HBcAg-pulsed DC. Data are mean ±SD of the levels of anti-HBs in sera. *P<0.05 compared with the levels of HBsAg before immunization. $^{\delta}P$<0.05 compared with other groups.

Figure 3. Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC



Hepatitis B surface antigen (HBsAg)- and hepatitis B core antigen (HBcAg)-specific proliferation was observed in T-cells from HBV transgenic mice (TM) immunized with HBsAg/HBcAg-pulsed dendritic cells (DC); however, proliferation did not result from immunization with other vaccine or DC formulations. Data are mean ±SD of levels of proliferation. *P<0.05 compared with other groups. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

in the liver of HBV TM as a result of vaccination with HBsAg/HBcAg-pulsed DC. Liver NPC from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to stimulation with HBsAg (stimulation index 12.8 ±3.2; $n=5$) and HBcAg (stimulation index 16.4 ±4.5; $n=5$). However, liver NPC from HBV TM injected with PBS, HBsAg/HBcAg vaccine, unpulsed DC and PDC-pulsed DC did not proliferate in response to HBsAg or HBcAg (stimulation index <3.0). In addition, significantly higher numbers of IFN-γ-secreting $CD8^+$ T-cells were detected among liver NPC only from HBV TM immunized with HBsAg/HBcAg-pulsed DC, but not from other HBV TM (Figure 4). However, we did not observe an increase of alanine aminotransferase (before vaccination 29 ±4 IU/l versus 4 weeks after vaccination 27 ±3 IU/l) or evidence of liver damage in liver biopsy specimens in any HBV TM immunized with HBsAg/HBcAg-pulsed DC (SMFA *et al.*, data not shown).

Induction of antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulsed DC *in vitro*

To assess a clinical implication of this study regarding immunogenecity of HBsAg/HBcAg-pulsed DC in HBV TM, we performed an *in vitro* study using lymphocytes from patients with CHB. Autologous T-cells from patients with CHB were cultured with HBsAg/HBcAg-pulsed DC, unpulsed DC, PDC-pulsed DC or HBsAg/HBcAg vaccine. T-cells from CHB patients did not exhibit significant proliferation in response to unpulsed DC or PDC-pulsed DC. However, T-cells from CHB patients proliferated in the presence of HBsAg/HBcAg-pulsed autologous DC (Figure 5). Low levels of proliferation of autologous T-cells from one of five patients with CHB were also detected when these were cultured with HBsAg/HBcAg (Figure 5).

Antiviral capacity of HBsAg/HBcAg-pulsed DC in HBV TM

All HBV TM expressed HBsAg; however, free HBV DNA could be detected in some but not all HBV TM. To assess the antiviral capacity of HBsAg/HBcAg-pulsed DC, we immunized five HBV TM with detectable levels of HBV DNA using HBsAg/HBcAg-pulsed DC, unpulsed DC or PDC-pulsed DC. Levels of HBV DNA in the sera were decreased in all HBV TM as a result of immunization with HBsAg/HBcAg-pulsed DC. HBV TM expressed a mean ±SD level of 354 ±14 copies/ml of HBV DNA in the sera before vaccination. At 4 weeks after two vaccinations with HBsAg/HBcAg-pulsed DC, HBV DNA could not be detected in any HBV TM (level of detection; 200 copies/ml). However, no significant changes of HBV DNA levels were seen in HBV TM immunized with two injections of HBsAg/HBcAg vaccine, unpulsed DC or PDC-pulsed DC.

Discussion

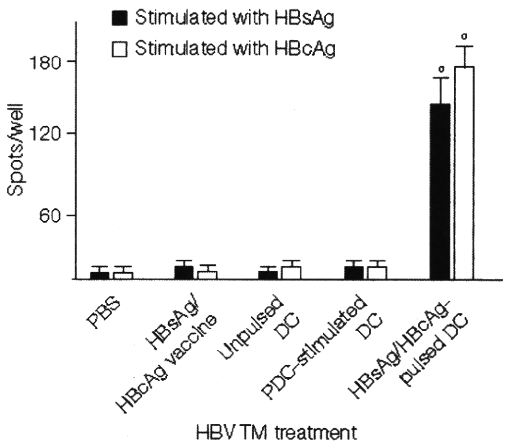
Several RCTs have documented the low therapeutic efficacy and considerable side effects of antiviral drugs used for CHB patients [2]. However, these drugs will remain the most useful and powerful tools for management of CHB patients [21] until more effective therapeutic regimens with fewer side effects can be developed. As an alternative therapeutic approach, polyclonal immune modulators have been used for

Table 2. IFN-γ-secreting T-cells in the spleen of HBV TM resulting from immunization with HBsAg/HBcAg-pulsed DC

HBV TM treatment	HBsAg-specific ELISPOT	HBcAg-specific ELISPOT
PBS	7 ±2	9 ±2
HBsAg/HBcAg vaccine	13 ±3	5 ±1
Unpulsed DC	11 ±4	12 ±3
PDC-pulsed DC	16 ±6	19 ±7
HBsAg/HBcAg-pulsed DC	198 ±23°	365 ±34°

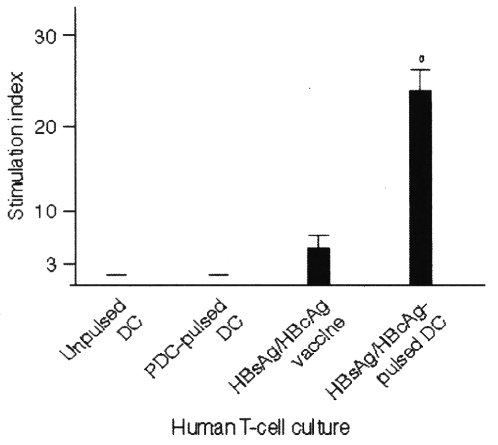
HBV transgenic mice (TM) were injected with phosphate-buffered saline (PBS) or immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-based vaccines, unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBsAg/HBcAg-pulsed DC, twice at an interval of 4 weeks. HBV TM were euthanized 4 weeks after the second immunization, and T-cells were stimulated with HBsAg or HBcAg on an ELISPOT plate to assay the production of inteferon-γ spot. The spots were counted after deducting spots from control plates. Data are mean ±SD of three separate experiments. °$P<0.05$ compared with other groups.

Figure 4. IFN-γ-producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC



Significant numbers of interferon (IFN)-γ producing cytotoxic T-lymphocytes (CTL) were detected among liver non-parenchymal cells from HBV transgenic mice (TM) immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). Data are mean ±SE of four separate experiments. °$P<0.05$ compared to HBV TM immunized with other vaccine or DC formulations. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

Figure 5. Antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulses DC *in vitro*



Antigen-specific proliferation of human peripheral blood T-cells from patients with chronic hepatitis B (CHB) resulting from stimulation with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). The levels of blastogenesis in cultures containing T-cells and unpulsed DC were regarded as a stimulation index of 1.0. Data are mean and ±SD of five separate experiments. *$P<0.05$ compared with HBV transgenic mice (TM) immunized with other vaccine or DC formulations. PDC, pyruvate dehydrogenase complex.

more than three decades in CHB patients; however, the clinical outcome with these agents has not been satisfactory [5]. To develop more effective immune therapy against HBV, vaccine therapy in which vaccines containing HBsAg were administered alone as well as in combination with other antiviral drugs in CHB patients [22,23]. Although some intermediate outcomes of CHB patients has been improved by this vaccine therapy, the present regimen of vaccine therapy still has limitations [6], as described in the *Introduction*.

The immune modulator effects of antigen-pulsed DC have been evaluated in HBV TM by other investigators, as well as our group [10,11]. Jiang *et al.* [10] used a murine model of HBV that expressed only HBsAg; the clinical implications of their study for translation research in patients with CHB are limited. We found that HBsAg-pulsed DC induced anti-HBs, but almost no HBsAg-specific cellular immunity, in HBV TM [11]. Also, we did not find therapeutic potentiality of HBsAg pulsed in CHB patients [24].

The present study has some noteworthy features. First, we used a vaccine that contains both HBsAg and HBcAg. We also prepared HBsAg/HBcAg-pulsed DC vaccine using a technique that we have been working with for more than a decade [14–16,19,20]. Next, an *in vitro* study revealed that HBsAg/HBcAg-pulsed DC could induce proliferation of both HBsAg- and HBcAg-specific lymphocytes from normal C57BL/6 mice. Subsequently, a preclinical trial using HBsAg/HBcAg-pulsed DC was accomplished in HBV TM. The data conclusively showed that HBsAg/HBcAg-pulsed DC induced anti-HBs in the sera, HBsAg and HBcAg-specific lymphocytes in the spleen, and HBsAg and HBcAg-specific CTLs in the spleen and the liver. Also, HBsAg/HBcAg-pulsed DC were able to reduce HBV DNA levels in HBV TM. Despite extensive immune modulating capacities of such preparations in HBV TM, we did not find any biochemical or histological evidence of liver injury with this approach, suggesting the viral clearance was mediated by non-cytotoxic effects of DC-based vaccine.

Our main target was to develop an effective immune therapeutic strategy for patients with CHB. Accordingly, we used a vaccine preparation that was a human-grade HBsAg and HBcAg. Indeed, clinical trials with this vaccine have already been performed in normal volunteers [18]. Accordingly, data from this study can be used to support the need for clinical trials using this vaccine. Further support of this concept was accumulated from data that showed that HBsAg/HBcAg-pulsed DC induced proliferation of both HBsAg- and HBcAg-specific T-cells from CHB patients (Figure 5).

The synergistic effect on the resulting immune response of HBsAg/HBcAg stimulation could be explained by the simultaneous stimulation of diverse Toll-like receptor (TLR) on DC. The nuclear content inside the *E.-coli*-derived recombinant HBcAg has been characterized as RNA (TLR3 and TLR7 ligands) [25–27]. Also, there is a recognized interaction between HBsAg and CD14, a component of TLR4 [28]. Finally, the aggregation of HBsAg and HBcAg in the liquid combined HBsAg/HBcAg formulation has been previously reported [25,29]. When these antigens were loaded on DC, antigen-pulsed DC were able to induce or activate HBsAg- and HBcAg-specific immune responses in HBV TM.

In conclusion, we have shown an improved immune therapeutic approach against chronic HBV infection. The antigens that we used are safe for human consumption and have been used in normal volunteers [18]. We prepared immunogenic antigen-pulsed DC with these antigens. HBsAg/HBcAg-pulsed DC induced both humoral and cellular immune responses in HBV TM *in vivo*; in addition, these DC induced proliferation of lymphocytes from CHB patients *in vitro*. We have already used HBsAg-pulsed DC in patients with CHB and confirmed its safety; however, only HBsAg-pulsed DC was not sufficiently effective to contain HBV replication and liver damages [24]. The next challenge will be to assess if HBsAg/HBcAg-pulsed DC can cause sustained control of HBV replication and reduction of liver damage in CHB, a finding that could not be completely assessed in HBV TM. The answer to that question will depend

on results of clinical trials in CHB patients, which are warranted based on the findings of this study.

Disclosure statement

The authors declare no competing interests.

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Accepted for publication 25 March 2010

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Regulatory natural killer cells in murine liver and their immunosuppressive capacity

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Keywords
hepatic NK cells – immune tolerance – interleukin-10 – liver immunity – regulatory NK cells

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Received 29 September 2009
Accepted 28 March 2010

DOI:10.1111/j.1478-3231.2010.02253.x

Abstract

Background: Abundant amounts of natural killer (NK) cells are present in the liver, most of which are endowed with direct cytotoxic and inflammatory cytokine production capacities. However, the control of compromised immunity in the liver may be accomplished by a population of regulatory NK cells possessing suppressive or tolerogenic functions. *Aims:* To identify and characterize regulatory NK cells in murine liver. *Methods:* NK cells were isolated from the liver of C57BL/6 mice by magnetic-activated cells sorting (MACS). NK cells were stimulated with different agents and those cells that produced interleukin (IL)-10 were detected by flow cytometry and isolated by MACS. IL-10-producing NK cells were regarded as regulatory NK cells and the functional capacities of liver-derived regulatory NK cells were assessed *in vitro*. *Results:* The frequencies of regulatory NK cells in the liver were $4.1 \pm 0.3\%$ of hepatic NK cells and $0.45 \pm 0.02\%$ of liver nonparenchymal cells. Regulatory NK cells produced abundant amounts of IL-10 in culture. These cells also suppressed the proliferative capacities of T cells and B cells *in vitro*. However, another population of NK cells that did not produce IL-10 (immunogenic NK cells) could not suppress lymphocyte proliferation. *Conclusions:* The presence of regulatory NK cells in the liver and their immunosuppressive capacities endowed these cells with the critical function of maintaining homeostasis under normal conditions. Exaggerated or impaired functions of these cells may also contribute to different pathological processes.

Hepatic immunity is a complex and poorly defined area because the exact mechanisms that regulate immunological events of the liver in health and diseases are poorly understood. The liver is composed of hepatic parenchyma cells (nearly 70% of total cellular population), liver nonparenchymal cells (NPCs, nearly 30%) and intracellular matrix (1). The liver remains at the centre of immune tolerance and immune responses. Different food products, inflammatory substances, allergens and drug metabolites constantly enter the liver through the gut or the bloodstream. Under physiological conditions, the liver induces a state of immunological tolerance to these substances to prevent extreme and detrimental immune reactions (2). On the other hand, many hepatotrophic viruses including hepatitis A to E viruses, parasites and bacteria enter the liver and induce antimicrobial immunity, which ultimately leads to the inflammation and destruction of hepatocytes (3, 4). Taken together, hepatic homeostasis is maintained by a highly regulated and well-coordinated balance between the tolerogenic and immunogenic properties of different immunocytes.

Studies have revealed that most of the immunocytes are capable of inducing both tolerance and immunity. Macrophages are well known for their phagocytic activities and production of abundant amounts of inflammatory cytokines, but some specific cells of their lineage have tolerogenic capacities (5, 6). Dendritic cells (DC) are initiators and regulators of immune responses because they can recognize, process, and present antigens to lymphocytes for the induction of immunity (7). However, regulatory DC downregulate the magnitude of immunity and thus play an important role in the maintenance of homeostasis (8). Similarly, immunogenic T cells and regulatory T cells ensure balanced immunity (9).

In this context, natural killer (NK) cells have occupied an interesting and puzzling position in hepatic immunity (10–12). They are regarded as basic pillars of innate immunity by virtue of their potent capacities to contain microbial agents, viral-infected cells and cancer cells. These functions are mediated by their direct cell-killing properties (cytotoxic NK cells) or by the production of inflammatory cytokines (immunogenic NK cells).

However, how compromised innate immunity can be regulated in the presence of only one population of cytotoxic or immunogenic NK cells remains unknown. The issue becomes more important because the frequency of NK cells is usually higher in the liver compared with those in other parenchymal organs (13). In this context, the existence of immunosuppressive NK cells that produce interleukin (IL)-10, an anti-inflammatory cytokine, has been reported in murine decidua and human peripheral blood (14, 15). However, it remains unclear whether immunosuppressive or immunoregulatory NK cells are present in the liver.

This study was performed to detect immunoregulatory NK cells in the liver by looking for a population of IL-10-producing NK cells in murine liver. A trace population of hepatic NK cells produced IL-10 in culture. We isolated IL-10-producing regulatory NK cells and assessed their functional capacities *in vitro*.

Materials and methods

Mice

Adult male C57BL/6J (H-2K^b) mice were purchased from Nihon Clea (Tokyo, Japan). The mice were housed in polycarbonate cages in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle in the pathogen-free animal housing facility at Ehime University Graduate School of Medicine. All animals received humane care and study protocols were in compliance with the institution's guidelines.

Isolation of T lymphocytes and B lymphocytes from the spleen and the liver

We have previously described in detail the methodologies for isolating spleen cells and liver NPCs (16, 17). To produce a single cell suspension from the spleen, the spleens were cut into pieces and passed through a 40-μm pore-size nylon filter (BD Falcon, Durham, NC, USA); the resulting cells were collected and suspended in a culture medium [RPMI 1640 (Iwaki, Osaka, Japan) plus 10% fetal calf serum (Filtron PTY Ltd., Brooklyn, Australia)].

To retrieve liver NPCs, liver tissues were cut into pieces, homogenized, passed through 70-μm pore-size steel meshes (Morimoto Yakuhin Co., Matsuyama, Japan) and suspended in 35% percoll (Sigma Chemical, St Louis, MO, USA). After centrifugation for 15 min at 450*g* at room temperature, a high-density cell pellet was collected and suspended in a culture medium.

T lymphocytes and B lymphocytes were isolated from a spleen single-cell suspension and liver NPCs by a negative selection column method using a mouse pan T isolation kit and a mouse pan B isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany) according to the directions of the manufacturer (18).

Isolation of natural killer cells

Spleen NK cells and liver NK cells were isolated from single-cell suspensions of the spleen and liver NPCs, respectively, by a negative cell selection method using an NK isolation kit (Miltenyi Biotec) with a magnetic-activated cell sorting (MACS) system (AutoMACS, Miltenyi Biotec).

Estimation of interleukin-10- and interferon-γ-producing natural killer cells by the cytometric bead array method

Freshly isolated NK cells were stimulated with CpG oligodeoxynucleotide (CpG ODN; 1 μg/ml, Invitrogen, San Diego, CA, USA), concanavalin A (Con A, 1 μg/ml, Sigma), lipopolysaccharides (LPS, 1 μg/ml, Sigma), CL-097 (1 μg/ml, Invitrogen) and herpes simplex virus-1 (HSV-1, 1×10^5 plaque-forming units, kindly provided by Prof. Masaki Yasukawa, Department of Bioregulatory Medicine, Ehime University Graduate School of Medicine, Ehime, Japan) for 72 h. The levels of IL-10 and interferon (IFN)-γ in culture supernatants were estimated using a commercial kit for the cytometric bead array method, as described previously (18). The levels of IL-10 and IFN-γ in the culture supernatants were calibrated from the mean fluorescence intensities of the standard negative control, standard positive control and samples by cytometric bead array software (BD Biosciences Pharmingen, San Jose, CA, USA) using a Macintosh computer (SAS Institute, Cary, NC, USA). The amounts of IL-10 and IFN-γ were expressed as pg/ml.

Flow cytometric detection of regulatory natural killer cells

Because we found that CpG ODN was the most potent stimulator of IL-10 in NK cells, CpG ODN was used in subsequent experiments to detect regulatory NK cells. The detection of IL-10-producing NK cells was performed according to a method described previously (19). We stimulated freshly isolated NK cells with CpG ODN (1 μg/ml) overnight. After stimulation, cells were labelled with anti-IL-10/CD45 antibody–antibody conjugates (Miltenyi Biotec) for 10 min. After washing, cells were resuspended in 37 °C medium to secrete IL-10 for 45 min. Subsequently, cells were washed and stained with phycoerythrin (PE)-conjugated anti-IL-10 for 10 min. After washing with a washing buffer, cells were incubated with FITC-conjugated anti-NK1.1 (BD Biosciences Pharmingen) and PE-conjugated anti-IL-10. Next, IL-10-secreting NK cells were detected by two-colour flow cytometry.

Isolation of interleukin-10-producing regulatory and immunogenic natural killer cells by magnetic-activated cell sorting

We stimulated NK cells with CpG ODN for their detection by flow cytometry as well as for the isolation of

regulatory NK cells. After detecting IL-10-producing NK cells with PE-conjugated anti-IL-10, we additionally labelled the cells with anti-PE microbeads, as described previously (19). The IL-10-producing NK cells (regulatory NK cells) were isolated by a positive selection method using MACS (15). The cells of negative fraction, non-IL-10 producing cells, were regarded as immunogenic NK cells. These cells were suspended in RPMI 1640 medium plus 10% fetal calf serum.

Enzyme-linked immunospot assay

Freshly isolated 1×10^5 NK cells were stimulated with CpG ODN in an IL-10-coated enzyme-linked immunospot (ELISPOT) plate (Mabtech, Nacka Strand, Sweden) for 24 h. After the cells were removed, a detection antibody (2A5-biotin) was added to the wells. After 2 h of incubation, the plates were incubated with streptavidin–alkaline phosphatase for 1 h. After the plate was washed, the substrate solution, BCIP/NBT, was put into each well. The reaction was stopped by washing the plates extensively with tap water. The numbers of spot-forming units (SFU) were counted using an ELISPOT reader (KS ELISPOT, Carl Zeiss, Thornwood, NY, USA) and were deducted from number of background SFU of control wells. The data were finally shown as numbers of SFU/well.

Lymphoproliferative assays

T cells and B cells (1×10^5 cells/200 µl well) were stimulated with Con A and LPS in a U-bottom 96-well plate (Corning, Tokyo, Japan) with or without regulatory NK cells and immunogenic NK cells for 120 h. [^{3}H]-thymidine (1.0 µCi/ml, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) was diluted in sterile RPMI 1640 medium and added to the cultures for the last 16 h. The cells were harvested automatically by a multiple cell harvester (LABO MASH, Futaba Medical, Tokyo, Japan) onto filter paper (LM 101-10, Futaba Medical). The levels of incorporation of [^{3}H]-thymidine (1.0 mCi/L, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) were determined in a liquid scintillation counter (Beckman-LS 5000, Beckman Instruments Inc., Fullerton, CA, USA) from the level of blastogenesis. The levels of blastogenesis in cultures without regulatory NK cells or immunogenic NK cells were regarded as the control. The data were expressed as counts per minute (CPM).

Statistical analysis

Data were analysed by unpaired *t*-tests if the data were normally distributed and by the Mann–Whitney rank-sum test if they were skewed. Data were expressed as means ± standard error of mean (mean ± SEM). Differences were considered significant if $P<0.05$.

Results

Interleukin-10 production by natural killer cells in response to stimulation by CpG oligodeoxynucleotide

We checked the capacity of IL-10 production of freshly isolated NK cells from the liver because of stimulation with various stimulants, as described in the 'Methods' section. The production of IL-10 by liver NK cells was stimulated with all stimulants; however, stimulation with CpG ODN induced the maximum amounts of IL-10 (Fig. 1a). Conversely, CpG ODN induced relatively lower amounts of IFN-γ compared with other stimulants (Fig. 1). Accordingly, CpG ODN was used to induce IL-10 in successive experiments to detect regulatory NK cells.

Detection of interleukin-10-producing natural killer cells by enzyme-linked immunospot

We obtained evidence that NK cells produced considerable amounts of IL-10 because of stimulation with CpG ODN. To obtain further evidence of IL-10 production by NK cells, we performed an IL-10-specific ELISPOT assay. As shown in Fig. 2, NK cells stimulated with CpG ODN produced IL-10-specific spots in the ELISPOT assay; however, almost no spot was detected in unstimulated NK cells. The SFU value in the control culture without stimulation was $3.0 \pm 1.0/1 \times 10^5$cells. On the other hand, the SFU value was $163.7 \pm 7.1/1 \times 10^5$ cells ($P < 0.01$) in cultures containing CpG ODN-stimulated NK cells.

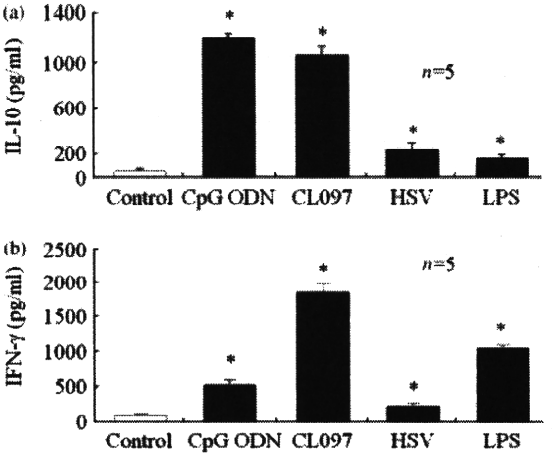

Fig. 1. Induction of interleukin (IL)-10 from liver natural killer (NK) cells. Liver-derived NK cells were stimulated with CpG ODN (CpG oligodeoxynucleotide, a double-stranded DNA), CL097 (an agonist of toll-like receptor 8), herpes simplex virus-1 (HSV-1), and lipopolysaccharide (LPS) for 24 h. The amounts of IL-10 (a) and interferon (IFN)-γ (b) in culture supernatant were measured by the cytometric bead array method and expressed as pg/ml. The mean and SEM of five separate experiments are shown. *$P < 0.05$, compared with control (not stimulated with any stimulant).

Increased frequency of regulatory natural killer cells in the liver

A representative staining pattern of NK cells and IL-10-producing regulatory NK cells in the spleen and the liver is shown in Fig. 3. First, we estimated the frequency of NK cells in the spleen and the liver [cells in the upper left quadrant ($NK1.1^+$ and $CD3^-$)] (Fig. 3a). The amounts of NK cells in the liver were about three times of those in the spleen. Next, we stimulated freshly isolated NK cells with CpG ODN to induce IL-10 production. Subsequently, regulatory NK cells that produced IL-10 were detected by flow cytometry and their frequencies were assessed. The frequency of IL-10-producing NK cells was almost similar in the liver and the spleen (Fig. 3b). However, the frequencies of total NK cells were significantly higher in the liver (Fig. 3a). Accordingly, the ultimate proportion of regulatory NK cells was significantly higher in the liver ($0.45 \pm 0.02\%$, $n = 5$) than in the spleen ($0.15 \pm 0.03\%$, $n = 5$) ($P < 0.05$) (Table 1).

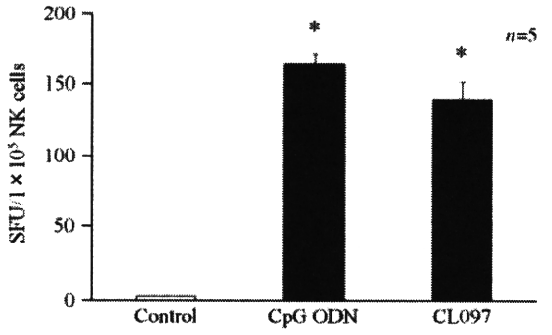

Fig. 2. Interleukin (IL)-10 production by liver natural killer (NK) cells in an enzyme-linked immunospot (ELISPOT) assay. Liver-derived NK cells isolated by a magnetic cell sorter were checked for IL-10 production in an ELISPOT assay after stimulation with CpG oligodeoxynucleotide (CpG ODN) and CL-097. Almost no IL-10 was produced by NK cells without activation by stimulators (Control). Abundant numbers of IL-10-specific spots because of stimulation with CpG ODN and CL-097. Data of five separate experiments have been shown. $^*P < 0.05$, compared with controls.

Suppressive effects of regulatory natural killer cells on T cells proliferation

To assess the functional capacities of regulatory NK cells, the effect of these cells on mitogen-induced lymphocyte proliferation was evaluated. T cells and B cells were stimulated with Con A and LPS, respectively, without or in the presence of regulatory NK cells. The levels of blastogenesis of T cells (1×10^5 cells) owing to stimulation with Con A were $77\,502 \pm 13\,407$ CPM ($n = 5$).

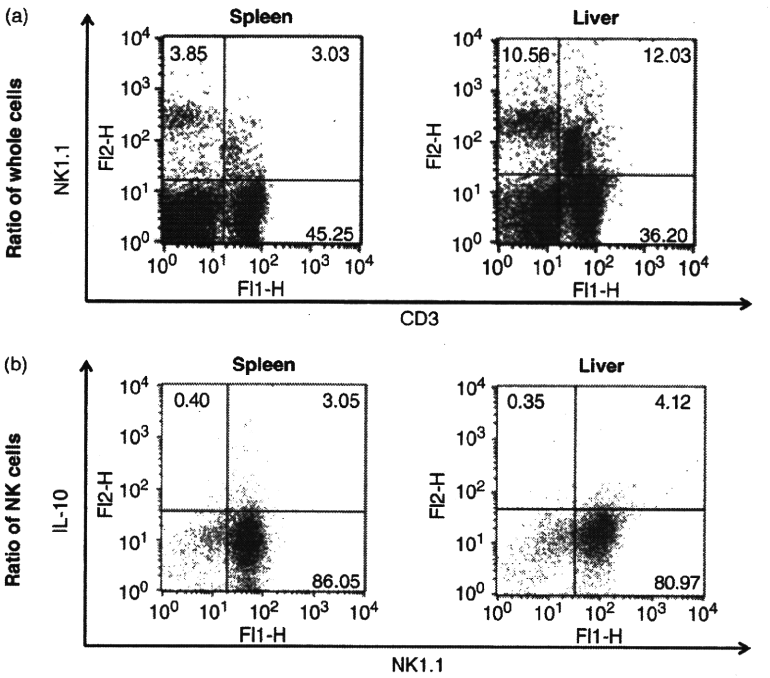

Fig. 3. Flow cytometric detection of natural killer (NK) cells and regulatory NK cells in the spleen and the liver. A representative staining pattern of NK cells and interleukin (IL-10)-producing regulatory NK cells have been shown. (a) Natural killer cells were defined by $NK1.1^+$ and $CD3^-$ fraction by dual-colour flow cytometry. The cells in the upper left quadrant indicate NK cells. (b) NK cells were isolated from the spleen and the liver. NK cells were stimulated with CpG oligodeoxynucleotide and regulatory NK cells were confirmed by expressions of IL-10, as described in the Methods section. Cells in the right upper quadrant are IL-10-producing regulatory NK cells.

However, the addition of 1×10^4 IL-10-producing regulatory NK cells caused a significant reduction of levels of blastogenesis (56 326 ± 3286 CPM ($n = 5$, $P < 0.05$) (Fig. 4). Similarly, the levels of LPS-induced proliferation of B cells (1×10^5 cells) were 34 950 ± 599 CPM ($n = 5$). The addition of regulatory NK cells (1×10^4 cells) caused a significant suppression of levels of B cells proliferation (25 073 ± 971 CPM) ($n = 5$) ($P < 0.05$) (Fig. 4).

Immunogenic effect of nonregulatory natural killer cells

To develop more insights on the role of NK cells and regulatory NK cells in T and B cells proliferation, we isolated a population of NK cells that did not produce IL-10. These cells were regarded as immunogenic NK cells. As expected, the immunogenic NK cells induced an increased proliferation of T cells in the presence of Con A (levels of blastogenesis; without immunogenic NK cells vs with immunogenic NK cells, 60 179 ± 3642 CPM vs 85 475 ± 4753 CPM, $n = 5$, $P < 0.05$) (Fig. 5). Similarly, immunogenic NK cells induced a significantly higher proliferation of B cells in the presence of LPS ($P < 0.05$) (Fig. 5).

Table 1. Frequencies of different immunocytes in spleen cells and liver nonparenchymal cells

	Liver	Spleen
T cells	34.6 ± 1.6	40.5 ± 3.3
B cells	32.2 ± 1.5	47.1 ± 1.2
Natural killer (NK) cells	11.0 ± 0.5*	4.6 ± 0.4
Regulatory NK cells	0.45 ± 0.02*	0.15 ± 0.03
Natural killer T cells	14.6 ± 1.2	3.9 ± 0.5
Monocytes	12.6 ± 2.3	8.6 ± 1.9

Different populations of immunocytes among single cell suspensions from the spleen and nonparanchymal cells of the liver were assessed by flow cytometry. Data of five separate experiments are shown.
*$P < 0.05$, compared with that of spleen.

Discussion

Natural killer cells represent a primary pillar of innate immunity and are endowed with excellent capacities to destroy different types of cells. They also produce various proinflammatory cytokines. It is natural to assume that NK cells ensure an inflammatory mucosal milieu *in vivo*. However, an exception to this rule is observed in the context of hepatic immunity. The liver harbours increased frequencies of NK cells compared with other parenchymal organs (13). However, the liver is recognized as a tolerogenic organ because most of the dangerous and nonself elements entering the liver do not induce immune responses (20). Moreover, the liver is comparatively resistant to transplant rejection even in the context of HLA mismatch transplantation (21). These realities suggest that some immunocytes of the liver may have a potent tolerogenic activity; however, the nature of these cells has not been properly explored until now.

We targeted NK cells to determine the role of these cells in hepatic tolerance because tolerogenic NK cells have been detected in placental decidua and peripheral blood and they have been referred to as regulatory NK cells (14, 15).

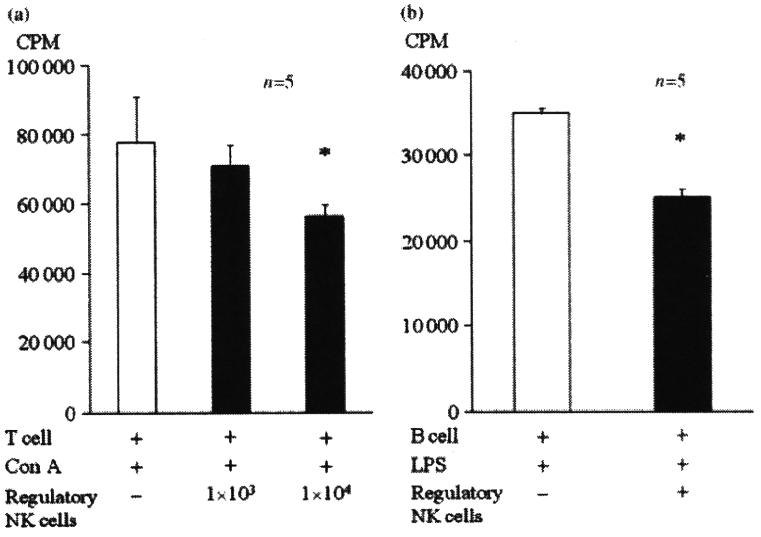

Fig. 4. Suppression of the proliferation of lymphocytes by liver regulatory natural killer (NK) cells. T cells and B cells were stimulated by concanavalin A (Con A) (a) and lipopolysaccharides (LPS) (b), and with or without regulatory NK cells. The levels of proliferation of T cells and B cells were shown as counts per minute (CPM). Data are shown as mean and standard error of mean of five separate experiments. The addition of hepatic regulatory NK cells suppressed the proliferation of T and B cells. Data of five separate experiments have been shown. *$P < 0.05$, compared with proliferation of T and B cells in the absence of regulatory NK cells.

Liver International (2010)