厚生労働科学研究費補助金

第3次対がん総合研究事業

ヒトT細胞白血病ウイルス1型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

（H21－3次対がん－一般－004）

平成23年度　総括研究報告書

研究代表者　松岡　雅雄
（京都大学ウイルス研究所・教授）

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厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
総括研究報告書

ヒトＴ細胞白血病ウイルス１型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

研究代表者：京都大学ウイルス研究所　教授　松岡　雅雄

研究要旨

HTLV-1 感染者は全世界で約 2000 万人存在し約 108 万人の感染者が居る日本は最大の蔓延国である。ATL は家族内発生が多いことでも知られており発症への遺伝的要因の関連が推定されている。本課題では全ゲノム関連解析（GWAS）にて、ATL および HAM 感受性 SNP の探索を行った。ATL 415 検体、HAM 429 検体と無症候性 HTLV-1 感染者 308 検体を用いて、アリル頻度、ジェノタイプ分布を統計解析した結果、ATL に関しては、$p<1x10^{-7}$ の SNP を 3 個、HAM に関しては $p<1x10^{-7}$ の SNP を 6 個同定した。さらにプロウイルス量に関連する遺伝子座を 1 番染色体に見出した。これらの SNP の解析は HTLV-1 関連疾患発症の高危険群同定に繋がるのみならず、HTLV-1 病原性発現機序の理解にも貢献すると予想される。

Ａ．研究目的

ヒトＴ細胞白血病ウイルス１型（human T-cell leukemia virus type 1: HTLV-1）感染により成人Ｔ細胞白血病（adult T-cell leukemia: ATL）、HTLV-1 関連脊髄症（HTLV-1 associated myelopathy: HAM）が惹起されるが、その発症には遺伝的背景が深く関連している。本研究では感受性遺伝子多型を解析し発症危険群の同定を目的としている。HTLV-1 感染者が多い唯一の先進国である日本以外では不可能な研究であり、本研究は国際的な責務であるとも言える。本研究の成果は免疫療法を用いた発症予防法の確立などに大きく寄与するものと考えられる。H21-22 年度には、熊本大学および鹿児島大学にて収集された検体を対象として、イルミナ社の Human610k-Quad BeadChip を用いて全ゲノム関連解析(GWAS)を行った。今年度は、同大学での新たな検体の収集を進めると同時に、地域による遺伝子多型の分布を考慮するために、その他の地域（長崎県、東京都、沖縄県）由来の検体収集と再現性の検証を目的とした。加えて今年度は、患者／キャリアの単純な全体比較のみならず、患者を病態で分類した複数のサブグループ間での比較解析を試みた。

Ｂ．研究方法

1) ATL、HAM 感受性 SNP の探索

ATL、HAM 患者、無症候性キャリアの検体（熊本県、鹿児島県、長崎県、沖縄県、東京都由来）を対象とし、イルミナ社の Human610k-Quad BeadChip を用いて全ゲノム関連解析(GWAS)を行う。

2) HTLV-1 プロウイルス量関連 SNP の探索

プロウイルス量による QTL 解析を行う。プロウイルス量情報と GWAS 結果が揃っている検体に絞り、プロウイルス量と関連する遺伝子座の有無を調べる。

（倫理面への配慮）

本研究は国の倫理指針に基づいた京都大学倫理委員会の承認を得て遂行している。検体は全て匿名化する。

Ｃ．研究結果

全ゲノム関連解析 (GWAS)を用いて、ATL 検体、HAM 検体、無症候性 HTLV-1 感染者における遺伝子多型の比較解析を行った。具体的にはゲノムスキャンにより 1 次スクリーニング（熊本県、鹿児島県由来検体）、および 2 次スクリーニング（長崎県、鹿児島県、東京都、沖縄県由来検体）を実施し、最終的に 1 次と 2 次スクリーニングの結果を総合し、ATL 患者 415 検体、HAM 患者 429 検体と無症候性 HTLV-1 感染者 308 検体を用いて、アリル頻度、ジェノタイプ分布を統計解析した。その結果、ATL に関しては、$p<1x10^{-5}$ の SNP が 4 個、HAM に関しては $p<1x10^{-5}$ の SNP が 15 個得られた。

加えて今年度は、患者／キャリアの単純な全体比較のみならず、患者を病態で分類した複数のサブグループ間での比較解析に着手した。プロウイルス量と関連するローカスの有

無を調べた結果、有意差を示すローカスが 1 番染色体に確認された ($p=2.68x10^{-8}$)。

D．考察

ATL の発症に関わる遺伝子群の同定は ATL という予後不良な疾患の早期治療を可能にし、治療成績を向上させると期待される。また、感染の分子基盤における宿主遺伝子の役割を考える上で、従来の分子生物学的、細胞生物学的方法で関与が示唆される宿主遺伝子に加え、遺伝学的見地から新たな関連遺伝子を提示することが可能である。今年度の解析により、HTLV-1 プロウイルス量と相関する SNP の候補が検出された。プロウイルス量は ATL 発症の危険因子であり、当該 SNP の解析は ATL 発症の高危険群同定法に繋がるのみならず、生体内における HTLV-1 感染動態の分子基盤の理解にも貢献すると予想される。

今後の計画として、今回の解析で得られた候補 SNP の再現性検証を行うと同時に、次世代遺伝子解析装置を用いて HTLV-1 キャリア及び HAM 患者における全エクソン配列を決定し、HAM 発症の感受性に関与する遺伝子の特定を試みる。

E．結論

ATL 患者 415 検体、HAM 患者 429 検体と無症候性 HTLV-1 感染者 308 検体を用い、ATL に関しては、$p<1x10^{-5}$ の SNP を 4 個、HAM に関しては $p<1x10^{-5}$ の SNP を 15 個同定した。さらにプロウイルス量と関連するローカスが 1 番染色体に確認された。

F．健康危険情報

該当事項なし

G．研究発表

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H．知的財産権の出願・登録状況

１．特許取得

該当事項なし

２．実用新案登録

該当事項なし

３．その他

該当事項なし

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分担研究報告書

ヒトＴ細胞白血病ウイルス１型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

研究分担者：京都大学医学研究科附属ゲノム医学センター　松田　文彦

研究要旨
ATL 患者 447 検体、無症候性 HTLV-1 感染者 314 検体、HAM 患者 460 検体のゲノムスキャン結果を用いて、患者と対照群をいくつかのサブグループに分け、アリル頻度、ジェノタイプ分布を統計解析した。さらに、患者／キャリアーの単純な全体比較のみならず、患者を病態で分類した比較解析に着手した。

A．研究目的

本研究は、イルミナ社の Human610k アレイを用いてゲノムワイドアプローチによるゲノム多型（SNP）の比較を試み、成人Ｔ細胞白血病(ATL)と HTLV-I 関連脊髄症（HAM）の発症に関わる遺伝子群を特定し、発症機構の解明と発がん危険性の予測を行うことを目的とする。

B．研究方法

SNP を用いた全ゲノム関連解析を行い、疾患関連 SNP を同定する。さらに、疾患関連 SNP の生物学的活性の解析：同定された疾患感受性 SNP が発症を促進する機序を明らかにするため、SNP がその分子に付与する機能について解析する。

（倫理面への配慮）

本研究は全て国の倫理指針に基づいた倫理委員会での審議、許可を得ている。また、検体は全て匿名化する。

C．研究結果

前年度までに申請者らは、イルミナ社の Human610k アレイを用いて、ATL 患者 447 検体、無症候性 HTLV-1 感染者 314 検体、HAM 患者 460 検体のゲノムスキャンを実施した。結果の品質管理をおこなった後、患者と対照群をいくつかのサブグループに分け、アリル頻度、ジェノタイプ分布を統計解析した。まずグループ 1：ATL 患者と HTLV-1 感染者間には $p<1x10^{-7}$ の SNP が 6 個、グループ 2: ATL 患者と HTLV-1 感染者・HAM 患者間には $p<1x10^{-7}$ の SNP が 5 個得られた。どちらものグループで $p<1x10^{-7}$ の SNP が 3 個存在した。次にグループ 3: HAM 患者と HTLV-1 感染者間には $p<1x10^{-7}$ の SNP が 10 個、グループ 4: HAM 患者と HTLV-1 感染者・ATL 患者間には $p<1x10^{-7}$ の SNP が 12 個得られた。グループ 3 と 4 両方で $p<1x10^{-7}$ の SNP は 6 個存在した。

さらに、患者／キャリアーの単純な全体比較のみならず、患者を病態で分類した比較解析に着手した。HAM を発症する HTLV-1 感染者は 1%未満であり、大多数の感染者は無症候性 HTLV-1 感染者として経過する。HAM 患者では無症候性 HTLV-1 感染者に比べプロウイルス量が 10 倍以上に増加し最大の発症危険因子であることが明らかになっており、プロウイルス量の測定が HAM の診断や症状悪化の指標として臨床現場で応用されている。そこで、まずプロウイルス量による QTL 解析を 3 セットで行った。プロウイルス量情報と GWAS 結果が揃っている検体に絞り、Set1 は HAM 患者と無症候性 HTLV-1 感染者計 686 人、Set2 は HAM 患者のみ 400 人、Set3 は無症候性 HTLV-1 感染者のみ 286 人とし、プロウイルス量と関連する遺伝子座の有無を調べた。その結果、Set2 内に有意差を示す遺伝子座が 1 番染色体に確認された ($p=2.13x10^{-8}$)。再現性を確認するため、現在 GWAS 未実施の無症候性 HTLV-1 感染者検体のタイピングを Taqman 法により調べている。

D．考察

これらの SNPs は、疾患感受性遺伝子の候補となるものであるが、独立した集団の検体を用いた再現性検証が必須であるため検体数の増加につとめる。新たに収集された検体を用いて候補 SNP のタイピングを行い、結果の比較を通して、真の感受性遺伝子の同定を目指す。

E．結論

ATL・HAM それぞれの疾患感受性と関連するゲノム上の SNP の候補が複数個同定された。今後の計画として、次世代遺伝子解析装置を用いて無症候性 HTLV-1 感染者及び HAM 患者における全エクソン配列を決定し、ゲノム領域における稀少 SNP、及び遺伝子に直接的な影響を与える SNP や変異を迅速に同定し、HAM 発症の感受性に関与する遺伝子の特定を試みる。また全エクソン塩基配列の決定を介して遺伝子に

直接的な影響を与える SNP や変異に関しても評価し、発症リスク群の特定を可能とすることを目的とする。

F．健康危険情報

特になし

G．研究発表

1．論文発表

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2．学会発表

特になし

H．知的財産権の出願・登録状況

1．特許取得

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2．実用新案登録

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3．その他

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ヒトＴ細胞白血病ウイルス１型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

研究分担者：琉球大学大学院医学研究科 准教授　齊藤峰輝

研究要旨：本年度も、HTLV-1 関連疾患感受性遺伝子多型解析に用いる検体を可能な限り多く確保し、多型同定後の病態における役割の解明に資するため、昨年度に引き続き HAM 患者の臨床情報収集と検体バンクの整備を行った。一方、HTLV-1 マイナス鎖にコードされるウイルス遺伝子 HBZ の HAM 病態形成における意義を明らかにするため、77 症例の HAM 患者末梢血単核球を用いて HBZ, tax, FoxP3 mRNA 発現を定量し、HTLV-1 tax subgroup、HTLV-1 プロウイルス量（PVL）との関連を検討した。沖縄県の症例では tax A が 63%、 tax B が 37%、鹿児島県の症例では tax A が 22%、 tax B が 78%と、沖縄県の症例に tax A が有意に多かった。興味深いことに、tax B HTLV-1 感染 HAM 患者の細胞あたりの HBZ mRNA 発現量は、tax A HTLV-1 感染 HAM 患者より有意に高かった。また、tax B HTLV-1 感染 HAM 患者では、HBZ mRNA 発現量と FoxP3 mRNA 発現量との間に有意な正の相関関係が認められたが、tax A HTLV-1 感染 HAM 患者では認められなかった。tax A HTLV-1 感染者は HAM 発症リスクが高いことが報告されており、HBZ mRNA 発現量との関連に興味が持たれる。

Ａ．研究目的

ヒト T 細胞白血病ウイルス1型（HTLV-1）は、世界ではじめてヒト疾患との関連が見いだされたレトロウイルスであり、成人 T 細胞白血病（ATL）および HTLV-1 関連脊髄症（HAM）の原因ウイルスである。平成 20 年度に国立感染症研究所から報告された約 20 年ぶりの全国調査によると、我が国にはいまだに約 108 万人もの HTLV-1 感染者が存在しており、従来多かった九州・沖縄では減少しているものの、都市部では逆に増加している。ほとんどの HTLV-1 感染者が生涯にわたって未発症の無症候性キャリアー（asymptomatic healthy carrier: HC）として経過し、HAM や ATL を発症するのは感染者全体の 5%前後ではあるものの、最も予後不良の白血病の一つである ATL は死亡者数が年間 1000 人を超え、HAM 患者では約 40%が経過中に歩行不能となり生活の質が著しく障害される。よって、HTLV-1 感染症の制圧は我が国の公衆衛生上・医療上の緊急の課題である。本研究の目的は、HTLV-1 関連疾患（ATL, HAM）患者の疾患感受性遺伝子多型解析に用いる検体を可能な限り多く確保し、多型同定後、その病態における役割の解明に資するための研究資源と病態解析法を整備することである。

Ｂ．研究方法

鹿児島大学病院脳神経センター神経内科、琉球大学病院神経内科の協力のもと、昨年度に引き続き HAM 患者の臨床情報を収集した。同時に、十分な説明と書面による同意を得たのちに末梢血を採取し、血漿、末梢血単核球（PBMC）、ゲノム DNA、cDNA を分離・保存した。PBMC は密度勾配遠心法にて分離し、AllPrep^{TM} DNA/RNA Mini Kit（QIAGEN）を使用してゲノム DNA と total RNA を同時に抽出した。さらに PrimeScript RT reagent Kit（Takara）を用いて逆転写反応を行い鋳型 cDNA を合成した。

HTLV-1 マイナス鎖にコードされるウイルス遺伝子 HBZ の HAM 病態形成における意義を明らかにするため以下の実験を行った。 1) HTLV-1 tax には LTR での cosmopolitan A, B 分類に対応した 2 つの subgroup（taxA, taxB）があり、tax A HTLV-1 感染者の方が HAM 発症リスクが高いことが報告されている（Furukawa et al. J Infect Dis. 182:1343-9, 2000.）。合計 77 例の HAM 患者について、PCR-RFLP 法で tax subgroup を決定した。

2) 各症例の PBMC 1 個あたりの HBZ、Tax および FoxP3 mRNA 発現を定量し、HTLV-1 subgroup、HTLV-1 プロウイルス量（PVL）との関連を検討した。

（倫理面への配慮）

本研究は参加各施設の倫理委員会の承諾を得た後に施行した。十分な説明と同意のも

と、書面による研究協力承諾書が得られた被験者から採取した検体のみを用い、完全に匿名化した後に行った。臨床情報と検体とは非連結匿名化した。

C．研究結果

昨年度に引き続き、HAM 患者から主治医の協力のもと匿名で臨床情報を収集した。同時に各患者から末梢血を採取した後に、血漿・PBMC・ゲノム DNA・cDNA を分離、保存した。PBMC は viable stock とした。病歴等の臨床情報に加え、各種一般臨床検査所見・免疫学的検査所見を含めた臨床情報データベースを作成した。

ゲノム DNA を用いて沖縄県の HAM 症例 27 例、鹿児島県の HAM 症例 50 例、合計 77 例の HAM 患者について PCR-RFLP 法で tax subgroup を解析した。沖縄県の症例では tax A が 17 例(63%)、tax B が 10 例(37%)、鹿児島県の症例では tax A が 11 例（22%)、tax B が 39 例（78%）と、沖縄県の症例に tax A が有意に多かった（χ^2=11.0, p=0.0009, Odds Ratio=6.03, CI 95%: 2.15-16.86.)。tax A HTLV-1 感染 HAM 患者、tax B HTLV-1 感染 HAM 患者いずれの群においても、PBMC 中の HBZ mRNA 発現量と PVL との間に有意な正の相関関係が認められた。tax B HTLV-1 感染 HAM 患者の細胞あたりの HBZ mRNA 発現量は tax A HTLV-1 感染 HAM 患者より有意に高かった(p=0.00012)。また、興味深いことに tax B HTLV-1 感染 HAM 患者では、PBMC 中の HBZ mRNA 発現量と FoxP3 mRNA 発現量との間に有意な正の相関関係が認められたが（p=0.0277)、tax A HTLV-1 感染 HAM 患者ではこの相関関係が認められなかった（p=0.533)。

D．考察

これまでに、HBZ 遺伝子が全ての ATL 症例で発現していること、HBZ 遺伝子の発現抑制により ATL 細胞の増殖が抑制されること、HBZ 遺伝子を発現するトランスジェニックマウスにおいて T リンパ腫のみならず CD4 陽性 T リンパ球の皮膚、肺への浸潤も認められることが報告されており、HBZ は HTLV-1 関連疾患の病態形成に極めて重要な遺伝子である。我々は、昨年度までに HAM 患者の病態と HBZ 発現との関連を解析し、炎症反応が強い進行期の HAM 患者は慢性期の患者と比較して HBZ mRNA が高発現しており、病勢との有意な相関が認められる一方で、Tax mRNA 発現と病勢との有意な相関は見いだせないことを報告した。過去の研究から、HTLV-1 の転写調節因子をコードする tax 遺伝子には、LTR での cosmopolitan A, B 分類に対応した subgroup が存在し、そのうち tax subgroup A に感染した個体が HAM に罹患しやすいことが明らかになっている（Furukawa Y et al. J Infect Dis. 182:1343-9, 2000)。また、このウイルス型は、HAM の発症率が日本と比較して約 7 倍程度高いジャマイカなど、カリブ海諸国の HAM 患者が持つウイルス型と高い相同性があることも報告されている。今回の検討で、沖縄の HAM 症例は、鹿児島（本土）の症例と比較して tax subgroup A の感染者が有意に高いことが明らかになった。最近、ジャマイカと日本の HTLV-1 感染者の解析から、HTLV-1 に対する宿主免疫応答に差がみられるとの報告もなされている（Birmann BM et al. Int J Cancer. 124: 614-21, 2009)。また、HBZ が Foxp3 遺伝子の転写を誘導することで、感染リンパ球を制御性 T リンパ球へ変換して発がん・炎症に関与する可能性が指摘されている（Satou Y et al. PLoS Pathogens 2011:e1001274.)。本研究で明らかになった、tax B HTLV-1 感染 HAM 患者においてのみ PBMC 中の HBZ mRNA 発現量と FoxP3 mRNA 発現量との間に正の相関関係が認められたことが、どのような生物学的意味を持つのかについて興味が持たれる。今後、HAM 患者のみならず、tax subgroup が異なる HTLV-1 感染者の臨床的・免疫学的特徴を明らかにすることは、HAM の病態解明のためにも有用であると考えられる。

E．結論

昨年度に引き続き、HAM患者から臨床情報およびに末梢血を採取し、血漿、リンパ球、ゲノムDNA、cDNAを分離、保存した。HTLV-1 tax subgroupの違いにより、感染者の生体内におけるウイルス遺伝子発現動態が異なる可能性を指摘した。

F．健康危険情報

特になし

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Ｈ．知的財産権の出願・登録状況
１．特許取得
特になし

２．実用新案登録
特になし

３．その他
特になし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

ヒトＴ細胞白血病ウイルス１型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

研究分担者：熊本大学医学部附属病院　講師　野坂　生郷

研究要旨
ヒトＴ細胞白血病ウイルスＩ型（HTLV-1）は成人Ｔ細胞白血病（ATL）やHTLV-1関連脊髄症(HAM)を惹起する。しかし、ATLの発症は全HTLV-1感染者の数%にすぎず、発症までに長期間を経て発症することより宿主側の因子も関与している可能性がある。本研究では、遺伝子の多型を解析することでその発症危険因子の同定を試みている。昨年度に引き続き、特に成人Ｔ細胞白血病、無症候性キャリアの臨床病態の調査、検体収集を行った。さらに腫瘍化の際に活性化される糖代謝がATL細胞株では、非常に活性化されており、高LDH血症やPETなどの異常高集積という臨床所見と一致しており、病態評価などにも有用な可能性もあると考えられる。

Ａ．研究目的

ATLはHTLV-1感染を契機に発症するＴ細胞リンパ腫瘍である。しかしその発症頻度は、全HTLV-1感染者の５％程度であり、非常に少なく、さらに家族内での発症も報告されていることから、宿主側因子の背景も発症機序に関与している可能性が考えられる。本研究では、宿主側因子の遺伝子的背景を多型の違いやその機能を解析することにより、HTLV-1関連疾患への発症のメカニズムの解明また、病態解明に結びつくと考えられる。成人Ｔ細胞白血病に焦点をあて、臨床病態の解析を含め、免疫機構、さらに糖代謝についても解析した。

Ｂ．研究方法

研究同意を得られた無症候性キャリア、成人Ｔ細胞白血病患者の各病型も含めた臨床病態の調査を行い、末梢血を採取、リンパ球、DNA、RNAを抽出、cDNAを作成した。またHTLV-1感染細胞株、ATL由来の細胞株を用いて、腫瘍解糖系経路に関連する遺伝子の発現をreal time PCR法で測定した。またフローサイトメトリーを使用し、CD4陽性Ｔ細胞におけるFoxP3の発現程度を解析した。

（倫理面への配慮）

本研究を行うに当たり、ヘルシンキ宣言、遺伝子治療臨床研究に関する指針、疫学研究に関する倫理指針および倫理研究における倫理指針を遵守し、当施設の倫理委員会の承認を受け、患者様に説明を十分行い、同意を得て実施した。

Ｃ．研究結果

新たに９例のATL患者、２例の無症候性キャリア患者の同意を得て、臨床情報の収集および検体の収集、抽出を行った。細胞株の解析においては、好気下においても乳酸産生を起こす腫瘍解糖系に関連するPDK1やLDHAの発現の亢進が認められた。さらにCD4細胞におけるFoxP3の発現頻度についても検討した。高頻度での発現は認めたが、発現程度は、症例によって様々であり、HTLV-1感染のみでなく、腫瘍化によって、発現程度に差があることが示唆された。

Ｄ．考察

ATLは、HTLV-1感染により起こされる疾患であり、ウイルス蛋白であるTaxやHBZなどにより、細胞の不死化、腫瘍化がin vitroやin vivoで証明されているが、その発症頻度の低さや発症までの期間を考えると宿主側因子の関与が考えられる。その中で、遺伝子の多型についての検討を行った。さらに腫瘍化した際に起こりうると考えられている腫瘍解糖系の亢進が病態を反映している可能性があると考えている。またターゲットと思われる制御性Ｔ細胞の遺伝子の発現の様々なパターンは、免疫状態の違いを反映している可能性もあり、今後さらに臨床情報とも比較し、検討する必要があると思われる。

E．結論

ATLにおいてFoxP3陽性CD4Tリンパ球の分布にバリエーションを認めた。さらに腫瘍解糖系の亢進もあり、ATL の腫瘍化や病態に関与している可能性があると思われた。

F．健康危険情報

収集検体は、数mLの末梢血であり、さらに、通常検査の際に同時に行うため、採取に関する危険性はないものと考えている。また、量も少しであるため、採取に伴う貧血等の可能性もないと考える。

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H．知的財産権の出願・登録状況

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

研究成果の刊行に関する一覧表

研究代表者　京都大学ウイルス研究所　教授　松岡雅雄

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HTLV-1 bZIP factor impairs cell-mediated immunity by suppressing production of Th1 cytokines

Kenji Sugata,[1] Yorifumi Satou,[1] Jun-ichirou Yasunaga,[1] Hideki Hara,[2] Kouichi Ohshima,[3] Atae Utsunomiya,[4] Masao Mitsuyama,[2] and Masao Matsuoka[1]

[1]Laboratory of Virus Control, Institute for Virus Research, Kyoto University, Kyoto, Japan; [2]Department of Microbiology, Kyoto University Graduate School of Medicine, Kyoto, Japan; [3]Department of Pathology, School of Medicine, Kurume University, 67 Asahimachi, Kurume, Fukuoka, Japan; [4]Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan

Adult T-cell leukemia (ATL) patients and human T-cell leukemia virus-1 (HTLV-1) infected individuals succumb to opportunistic infections. Cell mediated immunity is impaired, yet the mechanism of this impairment has remained elusive. The *HTLV-1 basic leucine zipper factor* (*HBZ*) gene is encoded in the minus strand of the viral DNA and is constitutively expressed in infected cells and ATL cells. To test the hypothesis that HBZ contributes to HTLV-1–associated immunodeficiency, we challenged transgenic mice that express the *HBZ* gene in CD4 T cells (HBZ-Tg mice) with herpes simplex virus type 2 or *Listeria monocytogenes*, and evaluated cellular immunity to these pathogens. HBZ-Tg mice were more vulnerable to both infections than non-Tg mice. The acquired immune response phase was specifically suppressed, indicating that cellular immunity was impaired in HBZ-Tg mice. In particular, production of IFN-γ by CD4 T cells was suppressed in HBZ-Tg mice. HBZ suppressed transcription from the *IFN-γ* gene promoter in a CD4 T cell–intrinsic manner by inhibiting nuclear factor of activated T cells and the activator protein 1 signaling pathway. This study shows that HBZ inhibits CD4 T-cell responses by directly interfering with the host cell-signaling pathway, resulting in impaired cell-mediated immunity in vivo. (*Blood*. 2012;119(2):434-444)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that mainly infects CD4 T cells,[1] a critical cell population for the host defense against foreign pathogens. HTLV-1 is known as the causal agent of adult T-cell leukemia (ATL),[2-4] a leukemia derived from CD4 T cells, and chronic inflammatory diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis,[5,6] alveolitis,[7] and uveitis. It has also been recognized that HTLV-1 infection is complicated by opportunistic infections caused by *Pneumocystis jiroveci*, herpes zoster virus, cytomegalovirus, or *Strongyloides stercoralis*.[8] However, the mechanism by which HTLV-1 causes immune deficiency has remained unknown.

Another human pathogenic retrovirus, HIV, replicates vigorously in vivo and produces a large number of virions. As a result of abundant viral production, HIV-infected CD4 T cells proceed to apoptosis, a phenomenon that eventually results in AIDS. In contrast, HTLV-1 increases its copy number primarily in the form of a provirus, by promoting the clonal proliferation of infected host CD4 T cells.[9,10] Despite this opposite effect on CD4 T-cell homeostasis compared with HIV, HTLV-1 infection and ATL are frequently accompanied by a deficiency of cellular immunity resembling that seen with AIDS.

HTLV-1 encodes several regulatory and accessory genes in the viral genome.[1,11] The viral proteins expressed by the integrated provirus control viral gene transcription and induce host cell proliferation, enabling HTLV-1 to achieve persistent infection. Among the viral genes of HTLV-1, *HTLV-1 bZIP factor* (*HBZ*), which is encoded in the minus strand,[12] is a constitutively expressed viral gene.[13] It has been reported that there are 2 major transcripts of the *HBZ* gene: spliced HBZ (sHBZ) and unspliced HBZ (usHBZ).[14] Based on the findings that sHBZ is more abundantly expressed than usHBZ[15] and that sHBZ has a functionally stronger effect than usHBZ,[16] we focused on sHBZ in this study.

Recently, we have reported that sHBZ expression increases the number of regulatory T cells (Tregs) by inducing transcription of the *Foxp3* gene in transgenic mice that express the *HBZ* gene in CD4 T cells (HBZ-Tg mice).[17] An increase in Tregs might be implicated in the immunodeficiency observed in ATL patients. Furthermore, previous studies have reported that HBZ suppresses host cell-signaling pathways that are critical for T-cell receptor signaling in the immune response, such as the NF-κB[18] and AP-1 pathways.[19] These findings led us to hypothesize that HBZ might have important roles in the dysregulation of cellular immunity associated with HTLV-1 infection.

To verify this hypothesis, we used HBZ-Tg mice that express sHBZ in CD4 T cells and studied well-established infection models of 2 pathogens. The first model involves intravaginal viral infection with herpes simplex virus type-2 (HSV-2). IFN-γ production by CD4 T cells is critical for the exclusion of HSV-2 from the host.[20,21] The other model involves infection with the Gram-positive intracellular bacterium, *Listeria monocytogenes* (LM), which is known as an opportunistic pathogen. In LM infection, CD4 T cells play pivotal roles in the acquired immune response by producing IFN-γ and inducing the activation of macrophages, which eliminate LM

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by phagocytosis and subsequent bactericidal activity.[22,23] Indeed, previous reports have shown that some ATL patients are infected with these 2 pathogens.[24,25] Using these 2 infection models, we demonstrated that sHBZ suppresses cell-mediated immunity. Furthermore, we determined the molecular mechanism of this HBZ-mediated immune suppression.

Methods

Mice

Wild-type C57BL/6J mice were purchased from CREA Japan. Transgenic mice expressing the *sHBZ* gene under control of the CD4 promoter/enhancer/silencer have been described previously.[13] All HBZ-Tg mice were heterozygotes for the transgene. All mice used in this study were maintained in a specific pathogen-free facility and handled according to protocols approved by Kyoto University.

Herpes simplex virus type 2 infection

The HSV-2 wild-type strain UW268 and thymidine kinase (TK)-negative strain UWTK (a gift from T. Suzutani, Fukushima Medical University) used in this study were propagated and titrated on Vero cells.[26] Acyclovir was used for propagation of UWTK to block emergence of TK^+ revertant. To increase their susceptibility to HSV-2, we injected mice subcutaneously with medroxyprogesterone acetate, Depo-provera (Sigma-Aldrich), (2 mg/mouse). Five days after this hormone injection, mice were anesthetized using Avertin (Sigma-Aldrich), preswabbed with a type 2 Calgiswab (Puritan), and inoculated intravaginally with 10^3 or 10^4 plaque-forming units (PFU) of UW268. For studies of secondary infection, mice were first immunized intravaginally with 10^6 PFU of UWTK, and 4 weeks later, they were inoculated intravaginally with 10^5 PFU of UW268. Vaginal secretions were collected by 3 pipettings with 15 μL of PBS, swabbed with a Calgiswab, and added to 955 μL of 5% FCS-DMEM and stored at −80°C. HSV-2 titers were determined by plaque assay on Vero cells. Five days after primary infection, lavage fluid from the vaginal tract was harvested similarly by 3 pipettings with 20 μL of PBS.

At 6 days after infection, the vaginal tissues of infected mice were fixed in 10% formalin in phosphate buffer and embedded in paraffin. H&E staining was performed according to standard procedures. The presence of HSV-2 antigen in tissues was detected using rabbit polyclonal anti–herpes simplex virus type 2 (Dako North America). Images were captured using a Provis AX80 microscope (Olympus) equipped with OLYMPUS DP70 digital camera, and detected using a DP manager system (Olympus; original total magnification ×200).

Splenic CD4 T cells from HSV-2 primary-infected mice were stimulated in a 96-well plate coated with CD3 mAb (1 μg/mL) and CD28 mAb (1 μg/mL) for 24 hours. For antigen specific stimulation, CD4 T cells were cocultured for 48 hours in the presence of irradiated T cell–depleted splenocytes as antigen-presenting cell (APC) and heat-inactivated HSV-2 (heat inactivated at 56°C for 2 hours) at a multiplicity of infection of 1. Supernatant was collected and stored at −20°C until assay.

Evaluation of resistance and immune response to LM in mice

Wild-type LM strain EGD was used in this study. The bacterial suspension was prepared as described previously.[27] For primary infection, mice were inoculated intravenously with 10^3 colony-forming units (CFUs) of LM and the bacterial burden in the spleen was determined on day 2 or 5 after infection.

For studies of secondary infection, mice were immunized intravenously with 10^3 CFUs of LM. From day 3 through day 6.5 after immunization, the drinking water supplemented with ampicillin (2 mg/mL) was given to clear any remaining LM. On day 7, mice were challenged with 10^6 CFUs of LM, and the spleens and sera were harvested after 3 or 12 hours. Spleens were homogenized in PBS, and the number of viable bacteria was determined by plating 10-fold serial dilutions on tryptic soy agar plates and counting the CFUs.

For cytometric assays, immunized mice were re-inoculated with 10^7 CFUs of LM. Splenocytes were harvested after 12 hours, cultured in the presence of protein transport inhibitor for 6 hours, and evaluated by the FACSCanto II (BD Biosciences) for cell surface and intracellular markers.

To determine the functional development of CD4 T cells in immunized mice, we purified splenic CD4 T cells and then stimulated them in a 96-well plate coated with CD3 mAb and CD28 mAb. For LM specific stimulation, CD4 T cells were cocultured with mouse bone marrow–derived macrophages (BMDMs) differentiated in the presence of 100 ng/mL of M-CSF and pulsed with viable LM at a multiplicity of infection of 10. Supernatant after stimulation for 24 hours was collected and stored at −20°C until assay.

Analysis of virus vector-transduced CD4 T cells

Retroviral transduction was performed as described previously.[17] The spliced HBZ gene was cloned into a retroviral vector, pMXs-Ig (a gift from T. Kitamura, The University of Tokyo), to generate pMXs-Ig-HBZ. This plasmid DNA was transfected into the packaging cell line, Plat-E. For retroviral transduction, $CD25^-CD4^+$ cells were enriched by a CD4 enrichment kit (BD Biosciences PharMingen) and were activated by anti-CD3 Ab (0.5 μg/mL) and rIL-2 (50 U/mL) in the presence of T cell–depleted and x-irradiated (20 Gy) C57BL/6J splenocytes as APCs in 12-well plates. After 16 hours, activated T cells were transduced with viral supernatant in the presence of 4 μg/mL polybrene and centrifuged at 1700*g* for 60 minutes. Then, transduced CD4 T cells were stimulated by phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (1 μg/mL) or plate-coated CD3 mAb (1 μg/mL) and CD28 mAb (1 μg/mL) in the presence of protein transport inhibitor and analyzed by a flow cytometry as shown in Figure 3. Dead cells were excluded using forward and side scatter and LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) by flow cytometry. Thereafter, intracellular cytokines were measured.

For generation of the lentivirus vector, sHBZ cDNA was cloned into pCS2-EF-GFP (a gift from H. Miyoshi, RIKEN BioResource Center) as previously described.[13] In brief, 293FT cells were cotransfected with the lentivirus vector, pCMV-Δ8/9 and pVSVG and supernatant containing virus was used for transduction. The lentivirus titer was determined on 293FT cells.

Empty vectors that express only GFP were used as controls for retroviral and lentiviral transductions.

IFN-γ promoter assay

Nucleotides −670 to +64 of the IFN-γ promoter region were amplified by PCR using human genomic DNA as a template, and cloned into pGL4.22 (Promega). The PathDetect pAP-1-Luc and pNFAT-Luc Cis-Reporter Plasmids were purchased from Promega. Transfection and luciferase assay were performed according to supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

ChIP assay

sHBZ-expressing Jurkat cells were stimulated with PMA and ionomycin. ChIP assay was performed as reported previously.[28] ChIP DNA samples were subjected to the StepOnePlus real-time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences of the primers for the human IFN-γ promoter were: 5′-TACCAGGGC-GAAGTGGGGAG-3′ (sense) and 5′-GGTTTTGTGGCATTTGGGTG-3′ (anti-sense).

Statistical analysis

For in vitro and in vivo experiments, multiple data comparisons were performed using the Student unpaired *t* test.

Results

High susceptibility of HBZ-Tg mice to HSV-2 infection

We first evaluated the susceptibility of HBZ-Tg mice to HSV-2 infection. Recently, we reported that HBZ-Tg mice frequently develop T-cell lymphoma and dermatitis after 10 weeks.[17] Therefore, HBZ-Tg mice without skin symptoms at 7 to 10 weeks of age were used in this study. It has been reported that the host immune response against primary HSV-2 infection can be divided into 2 stages: the innate immune response plays a dominant role by day 2 after infection, whereas cellular immunity plays an important role later, after day 5 after infection.[29] IFN-γ production by CD4 T cells is known as a critical factor in the cellular immune response against pathogens.[29] To determine whether cellular immunity is impaired in HBZ-Tg mice, we pretreated HBZ-Tg and non-Tg mice with Depo-provera for efficient infection and inoculated them with HSV-2 through the vaginal route.[30] The viral titer of HSV-2 in the lesion was measured. In this primary infection assay, there was no significant difference in the viral titers between non-Tg and HBZ-Tg mice at day 2 after inoculation (Figure 1A), when innate immunity is responsible for the host defense. In contrast, at day 6 after infection, when acquired immunity becomes important, HBZ-Tg mice showed significantly higher viral titers of HSV-2 than non-Tg mice (Figure 1A). Immunohistochemical analysis revealed that abundant viral antigens were detected in the vaginal epithelial cells and ganglia of HSV-2 challenged HBZ-Tg mice but not in non-Tg mice (Figure 1B).

To explore the mechanism of this immune deficiency, we examined cytokine production by CD4 T cells stimulated with antibodies to CD3 and CD28 or with heat-inactivated HSV-2 and APC. On day 6 after infection, the production of Th1 effector cytokines, including IFN-γ, IL-2, and TNF-α, was significantly reduced in CD4 T cells from HBZ-Tg mice compared with non-Tg mice (Figure 1C). Furthermore, IFN-γ concentration in vaginal wash fluids at day 5 after infection was significantly suppressed in HBZ-Tg compared with non-Tg mice (Figure 1D). When we challenged mice with a 50% lethal dose of HSV-2, the survival rate of non-Tg mice at day 20 after infection was 53%. In contrast, HBZ-Tg mice could not survive a viral challenge at the same dose (Figure 1E).

To study acquired immunity against HSV-2, we immunized and challenged mice as shown in Figure 1F. First, mice were immunized by TK-negative HSV-2 strain, the attenuated mutant of HSV-2, and then they were challenged with wild-type HSV-2. The vaginal virus titer in HBZ-Tg mice at day 3 after challenge was similar to that in nonimmune non-Tg mice (Figure 1F), whereas HSV-2 was not detected in immune non-Tg mice. The difference in viral titer between non-Tg and HBZ-Tg mice was much more remarkable in these secondary infection experiments than in the previous primary infection experiments, implicating impaired acquired immunity in HBZ-Tg mice. These results demonstrate that expression of sHBZ in CD4 T cells induces a deficiency in the immune response against HSV-2 and impairs the production of IFN-γ, IL-2, and TNF-α.

HBZ-Tg mice have an impaired T cell–dependent immune response to LM

We next evaluated the susceptibility of HBZ-Tg mice to infection with LM via an intravenous route. As with HSV-2 infection, production of IFN-γ by CD4 T cells plays a crucial role in the growth inhibition and elimination of LM in vivo.[31,32] On day 2 or 5 after primary infection with LM, we removed spleens and evaluated the bacterial burdens in the organs. The number of LM recovered from HBZ-Tg spleen on day 2 was comparable to that from non-Tg mice, yet the bacterial burden in HBZ-Tg mice at day 5 was higher than that in non-Tg mice (Figure 2A), suggesting a reduced protection in HBZ-Tg mice against LM, especially when acquired immunity is being established. We next a performed secondary infection experiment to evaluate the T cell–dependent immunity that developed after primary infection. Non-Tg mice immunized with a small dose of LM and later challenged with a high dose exhibited a significant level of bacterial elimination 12 hours after challenge compared with nonimmunized mice (Figure 2B). By contrast, such a significant level of bacterial elimination was not observed in immunized HBZ-Tg mice (Figure 2B), indicating that acquired LM-specific immunity is impaired in HBZ-Tg mice.

Characterization of cytokine production in the LM-infected mice

We next measured the concentration of several cytokines in the sera and homogenized spleen supernatant of HBZ-Tg and non-Tg mice during secondary infection with LM. IFN-γ, TNF-α, IL-2, IL-6, and IL-10 were decreased in HBZ-Tg mice (Figure 2C) compared with non-Tg mice. On the other hand, IL-12, which is mainly secreted by APCs, was increased in HBZ-Tg at 12 hours. To explore whether impaired production of Th1 cytokines by CD4 T cells is responsible for the decrease in levels of IFN-γ, TNF-α, and IL-2 in the serum, we enriched CD4 T cells from the spleens of immunized mice and then stimulated the cells ex vivo nonspecifically (with mAbs to CD3 and CD28) or specifically (with BMDMs pulsed with viable LM). The ability of CD4 T cells from HBZ-Tg mice to produce IFN-γ and IL-2 in response to either kind of stimulation was markedly impaired compared with that of cells from non-Tg mice (Figure 2D). In contrast, a considerable amount of TNF-α production was detected in tests of both HBZ-Tg and non-Tg CD4 T cells after stimulation with LM-pulsed BMDMs. However, this level of TNF-α was almost comparable with that observed in the culture of LM-pulsed BMDMs alone (Figure 2D). Therefore, the TNF-α detected in this experiment was probably produced by the macrophages, not by the CD4 T cells. These results strongly suggest that the ability of CD4 T cells to produce Th1 cytokines is impaired in HBZ-Tg mice.

Because IFN-γ is reported to play a pivotal role in the acquired protection of mice against LM,[22,23] we focused on IFN-γ production by LM-specific CD4 T cells. Splenic cell suspensions were prepared from 2 groups of mice immunized and challenged according to the protocol shown in Figure 2B. Cells were cultured for 6 hours in the presence of protein transport inhibitor and then subjected to flow cytometric analysis for IFN-γ production by intracellular cytokine staining. The number of IFN-γ–producing CD4 T cells in HBZ-Tg mice was remarkably reduced compared with that in non-Tg mice (Figure 3A). In contrast, IFN-γ production by CD8 T cells showed no significant difference between non-Tg and HBZ-Tg mice (Figure 3A). In addition, there were no differences between HBZ-Tg mice and control littermates in both total and $CD4^+$ splenocytes (supplemental Figure 1).

We recently reported that the proportion of $Foxp3^+$ $CD4^+$ T cells is increased in HBZ-Tg mice.[17] A previous study reported that Foxp3 expression inhibits the production of IFN-γ,[33] suggesting that a decreased proportion of effector T cells in HBZ-Tg mice might be responsible for the low number of IFN-γ–producing CD4

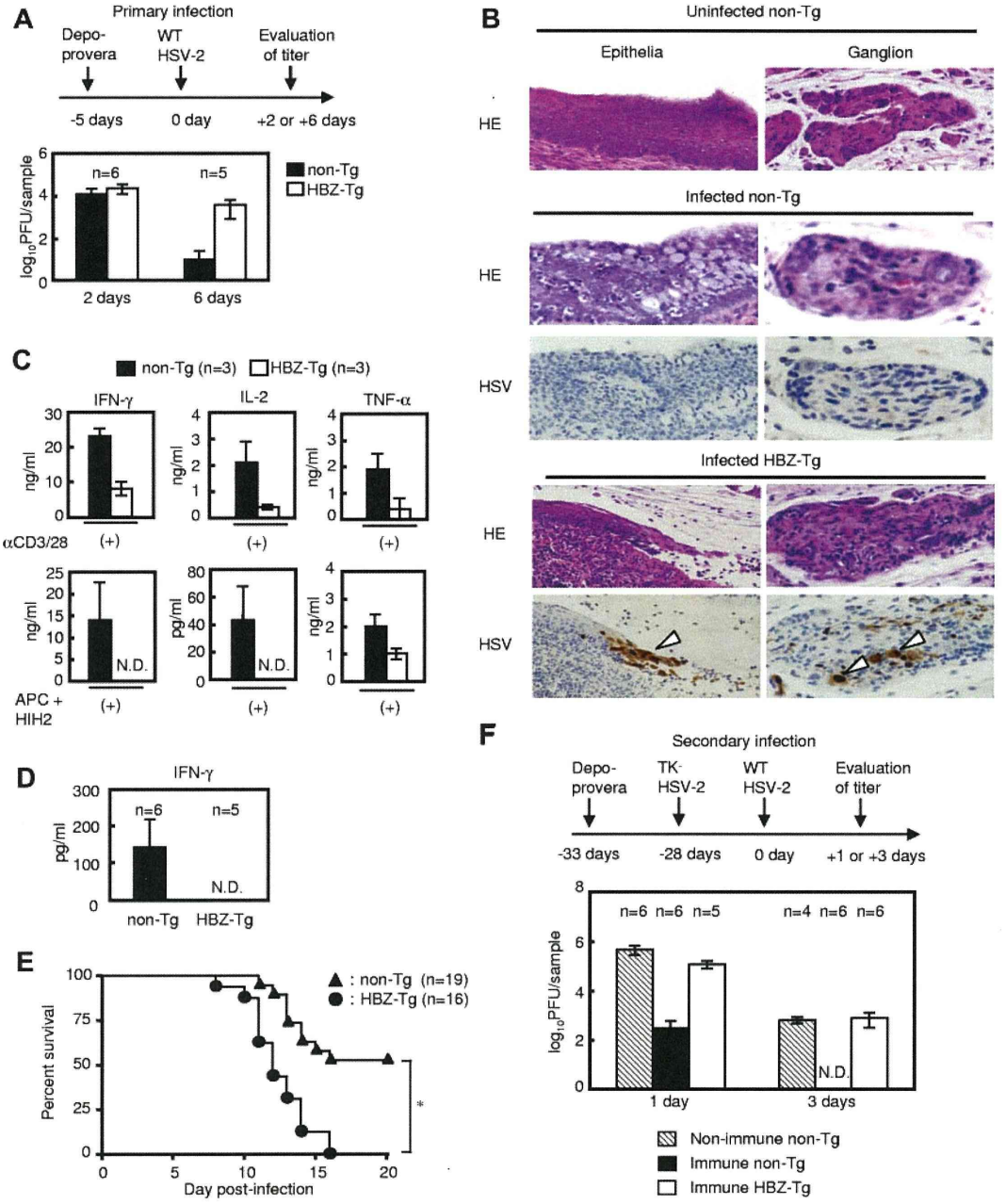

Figure 1. Transgenic mice expressing sHBZ in CD4 T cells are highly susceptible to intravaginal infection with HSV-2. (A) Virus titer in vaginal washes in primary infection. (B) Histologic analysis of epithelia and ganglion in vaginal tissue from mice infected with HSV-2. Uninfected vaginal tissues are presented as controls. HE indicates H&E stain; and HSV, immunohistochemical analysis for the viral antigen. Arrowheads indicate HSV-2–positive cells. (C) Cytokine production by splenic CD4 T cells from mice infected with 10^4 plaque-forming units (PFU) of HSV-2. Cells were stimulated with mAbs to CD3 and CD28 or APC plus heat-inactivated HSV-2 (HIH2) in ex vivo culture. (D) IFN-γ concentration in vaginal wash fluid harvested at day 5 after infection. (E) Survival curve of non-Tg or HBZ-Tg mice infected with 10^3 PFU of HSV-2. *$P < .05$ (log-rank test). (F) Viral titer in vaginal washes during HSV-2 secondary infection. To evaluate adaptive immunity against HSV-2 infection, mice were immunized and infected with the virus as shown in the upper panel. Bars represent the mean ± SD of all mice per genotype. Two or 3 independent experiments have been performed. N.D. indicates not detected.

T cells. However, the impairment of IFN-γ production was still observed in the Foxp3-negative effector CD4 T-cell population (Figure 3B), indicating that the reduction in IFN-γ production is independent of Foxp3 expression. These results collectively indicate that transgenic expression of sHBZ in CD4 T cells results in a reduction in effector cytokine production by CD4 T cells.

sHBZ directly inhibits IFN-γ production in a CD4 T cell–intrinsic manner

To determine whether sHBZ-mediated IFN-γ suppression was induced by a cell-intrinsic effect of sHBZ in CD4 T cells or by a dysregulated immunologic status in vivo indirectly caused by sHBZ expression, we used a retrovirus vector to express sHBZ in naive CD4 T cells. Wild-type CD4 T cells transduced with sHBZ showed lower IFN-γ production than empty vector-transduced cells (Figure 4A-B), demonstrating that sHBZ directly suppresses IFN-γ production in CD4 T cells. It is noteworthy that sHBZ suppressed IFN-γ production in human CD4 T cells as well as mouse T cells. This suppression was not limited to IFN-γ but was also observed for TNF-α (Figure 4C) and IL-2 (Figure 4D). Expression level of the *HBZ* gene transcript was much higher than that of HBZ-Tg mice (supplemental Figure 2). IL-4 production was

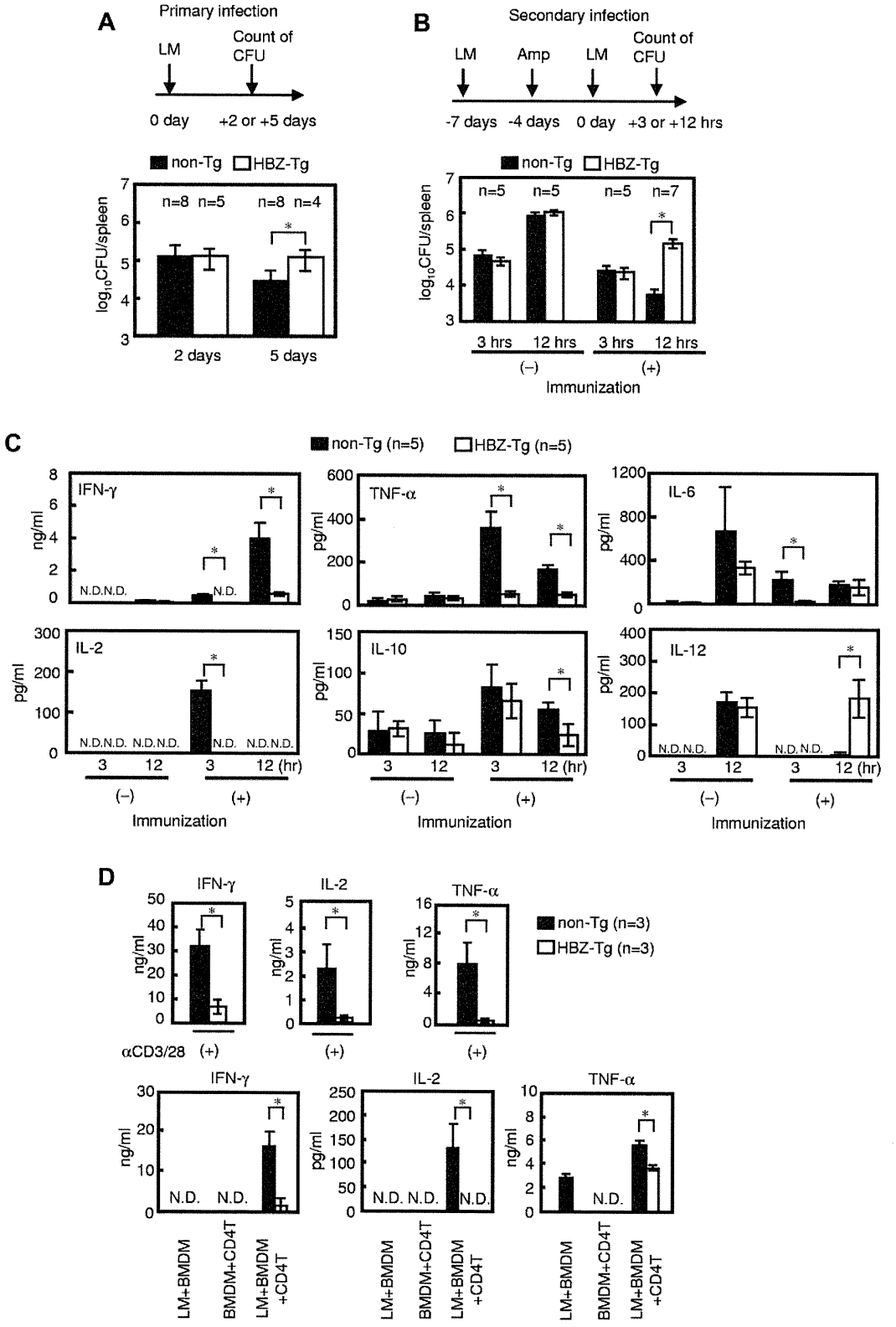

Figure 2. HBZ-Tg mice show decreased immune response to primary and secondary infection with LM. Bacterial loads of spleens from mice challenged with LM in primary (A) and secondary (B) infection are shown. (C) Concentrations of IFN-γ, TNF-α, IL-2, IL-6, and IL-12 in serum and IL-10 in homogenized spleen supernatant from the secondarily infected mice. (D) Cytokine production by CD4 T cells from secondarily infected mice. Mice were immunized as shown in panel B. CD4 T cells were stimulated ex vivo with mAbs to CD3 and CD28 or with LM-infected WT-BMDMs. Bars represent the mean ± SD of all mice per genotype. Two independent experiments have been performed; representative results are shown. *$P < .05$ by Student t test. N.D. indicates not detected.

not detected in CD4 T cells (supplemental Figure 3A). Although production of Th1 cytokines was reduced in sHBZ-expressing CD4 T cells, IL-6 and IL-10 production was not altered by sHBZ expression (supplemental Figure 3B-C). These results collectively suggest that sHBZ expression in HTLV-1–infected CD4 T cells inhibits transcription of the *IFN-γ*, *TNF-α*, and *IL-2* genes, which

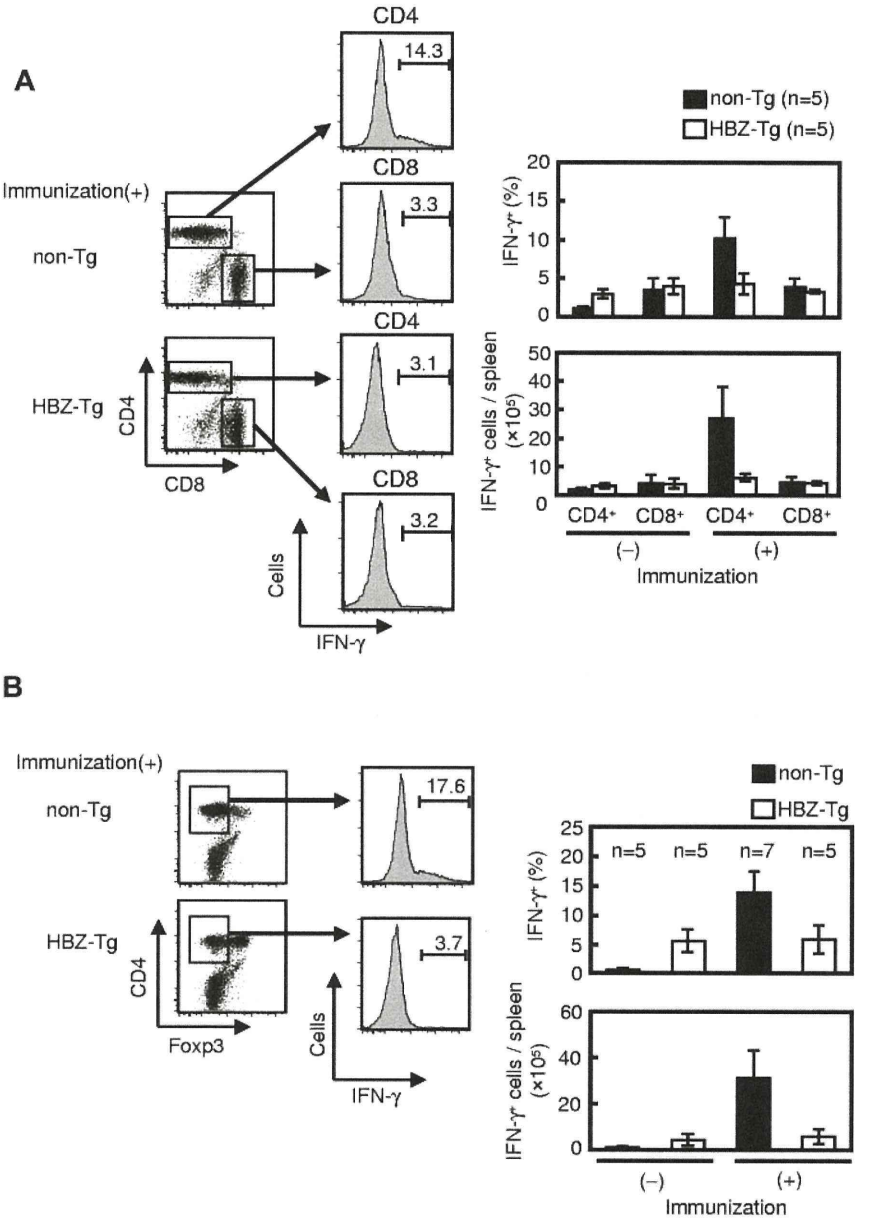

Figure 3. IFN-γ production by CD4 splenocytes from LM secondarily infected HBZ-Tg mice decreases in CD4⁺ Foxp3⁻ T cells. Mice were immunized and challenged as shown at the top of Figure 2B, and their splenocytes were harvested at 12 hours after challenge and analyzed for intracellular IFN-γ production. (A) Splenocytes were gated by CD3 expression, and IFN-γ production was measured in living CD4 or CD8 T cells using FACS. (B) IFN-γ production in CD3⁺ CD4⁺ Foxp3⁻ cells was determined. Bars represent the mean ± SD of all mice per genotype. Two independent experiments have been performed.

play important roles in the immune response against foreign pathogens.

sHBZ suppresses the activity of the IFN-γ promoter by inhibiting the NFAT and AP-1 signaling pathways

To further elucidate the mechanism of sHBZ-mediated IFN-γ inhibition, we performed a promoter assay using a human −670 to +64 IFN-γ promoter construct in the human T-cell line Jurkat. Previous reports have demonstrated that NFAT, AP-1, and NF-κB signaling pathways are involved in the regulation of *IFN-γ* transcription.[34] We found that PMA and ionomycin treatment enhanced IFN-γ promoter activity, and sHBZ suppressed this enhancement in a dose-dependent manner (Figure 5A). In contrast, another viral protein, Tax, enhanced the promoter activity as reported previously (Figure 5B),[35] an observation that is in line with previous findings that Tax is capable of activating the NF-κB and AP-1 signaling pathways.[36] Previous studies have demonstrated that the level of *sHBZ* transcripts in ATL patients and HTLV-1 carriers is approximately 4-fold higher than the level of *tax* transcripts.[15] The activation of the IFN-γ promoter by Tax was inhibited by sHBZ when sHBZ was expressed at levels similar to those in HTLV-1 carriers (Figure 5C), suggesting that sHBZ can have an inhibitory effect on Tax-mediated IFN-γ induction in HTLV-1 infected cells.

To identify the region of the IFN-γ promoter responsible for sHBZ-mediated suppression, we conducted further analyses using serially deleted promoter constructs. The human IFN-γ promoter (−670 to +64) contains NFAT, AP-1, STAT, ATF, and T-bet binding regions, and these transcription factors are reported to be involved in IFN-γ expression. The suppressive effect of sHBZ on the IFN-γ promoter was reduced by the deletion between dM2 and dM3 ($P < .001$; Figure 5D: a deletion, which removes 2 NFAT sites, an AP-1 site, and a STAT binding site). Because HBZ has a suppressive effect on the NFAT and AP-1 signaling pathways,[17,19] these binding sites might be associated with the suppressive effect of sHBZ. To further explore this possibility, we generated the promoter constructs with point mutation for each NFAT or AP-1 sites, and performed the promoter assay. The point mutation for

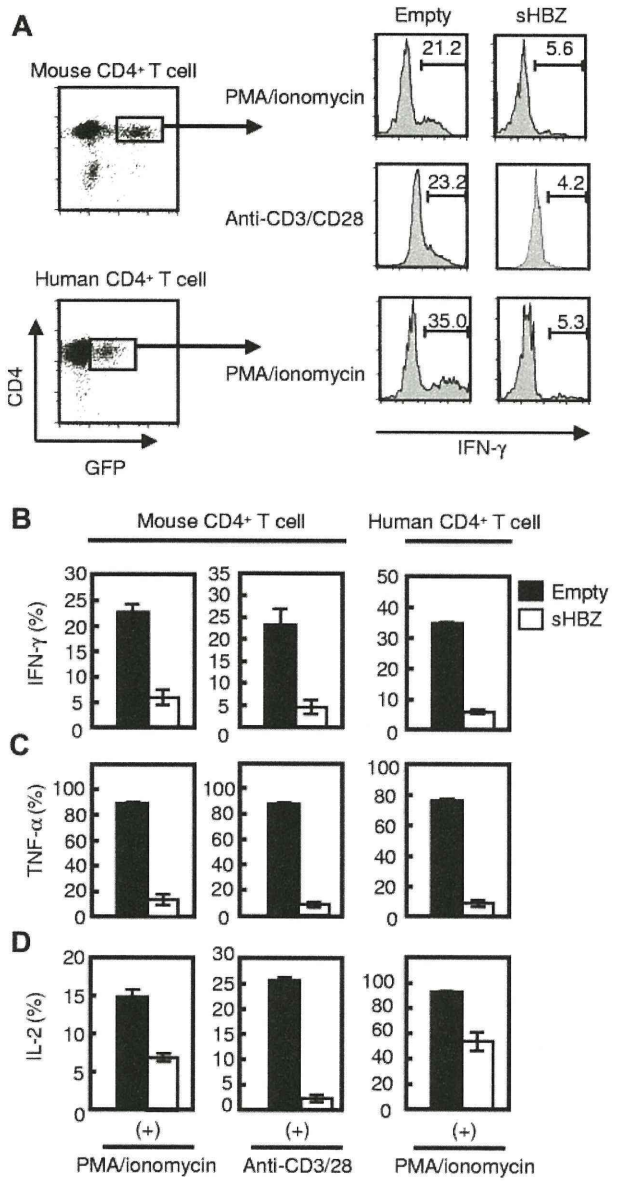

Figure 4. sHBZ directly inhibits IFN-γ production in both human and mouse CD4 T cells. Mouse and human CD4 T cells were transduced with recombinant retroviruses or lentiviruses, respectively, expressing sHBZ, and stimulated with PMA and ionomycin or antibodies to CD3 and CD28. Then, intracellular cytokines in living HBZ-expressing CD4 T cells were measured using FACS. (A) GFP$^+$ and CD4$^+$ cells were gated as shown in the left panel and evaluated for intracellular production of IFN-γ, TNF-α, or IL-2 by flow cytometry. Representative histograms of IFN-γ are shown. (B-D) Percentages of IFN-γ$^+$ (B), TNF-α$^+$ (C), or IL-2$^+$ (D) cells in mouse and human CD4 T cells. Representative data from 2 independent experiments in triplicate (mean ± SD) are shown.

−163 to −153 (P = .025) but not −279 to −269 (P = .057) NFAT binding site remarkably reduced suppressive effect of promoter activity by HBZ (Figure 5E). We next characterized effect of sHBZ on AP-1 binding sites in the IFN-γ promoter. The point mutation for −193 to −183 AP-1 binding site partially impaired the inhibitory effect (P = .042; Figure 5F). Three point mutations of all AP-1 binding sites much more reduced the HBZ-mediated suppressive effect on the promoter (P = .001; Figure 5F). These results indicate that NFAT and AP-1 binding sites are involved in the suppressive effect of HBZ on this promoter.

To further elucidate the involvement of the AP-1 or NFAT signaling pathway in the sHBZ-induced impairment of IFN-γ production, we used sHBZ mutants, which are unable to exert an inhibitory effect on NFAT or AP-1 signaling. We have reported that activation and central domains of HBZ interacted with NFAT.[17] We constructed deletion mutants and 7 amino-acid substitution mutants of sHBZ central domain and assessed their abilities to function in the NFAT or AP-1 signaling pathway (Figure 6A-B; supplemental Figure 4A-C). We found 2 mutants of interest: sHBZ-CDm7 and sHBZ-ΔAD. sHBZ-CDm7 contained amino acid substitutions in the central domain of sHBZ, and these mutations abrogated the inhibitory effect of sHBZ on the activity of an NFAT reporter plasmid (Figure 6A). In contrast, sHBZ-ΔAD, which contains a deletion of the activation domain of sHBZ, did not have suppressive activity on the AP-1 signaling pathway (Figure 6B). We confirmed that expression levels of the sHBZ mutants were comparable with that of WT-sHBZ (supplemental Figure 4D). Consistent with the findings of the reporter assay with the deleted promoters, sHBZ-CDm7 and sHBZ-ΔAD showed remarkable reduction in the inhibitory effect on the IFN-γ promoter (Figure 6C). Furthermore, we generated retrovirus vectors that express these sHBZ mutants, transduced them to mouse CD4 T cells, and evaluated their effect on IFN-γ production. We found that these 2 sHBZ mutants lost their inhibitory effect on IFN-γ production compared with WT-sHBZ (Figure 6D). Previous reports have shown that bZIP domain of HBZ plays a role in suppression for transcriptional activity of AP-1 family, including c-Jun and JunB.[19,37] In this study, deletion mutant of bZIP domain in sHBZ did not influence NFAT and AP-1 pathway in Jurkat cell (Figure 6A-B) and IFN-γ production in mouse CD4$^+$ T cell (supplemental Figure 5A), indicating that not bZIP domain but activation domain of HBZ is essential for suppression of AP-1 pathway in this study.

In addition, we performed a ChIP assay to explore recruitment of the transcription factors NFAT and AP-1 to the IFN-γ promoter in the presence of sHBZ. This experiment showed that sHBZ inhibited recruitment of NFATc2 and c-Jun to the IFN-γ promoter containing 2 NFAT sites and one AP-1 binding site (Figure 6E). These results suggest that sHBZ physically inhibits DNA binding of c-Jun and NFATc2 and suppresses the NFAT and/or AP-1 signaling pathways, which are critical for IFN-γ production in CD4 T cells.

Impaired production of IFN-γ in primary ATL cells

Jurkat T cells express *IFN-γ* gene transcripts after stimulation with PMA and ionomycin. sHBZ expression in Jurkat cells remarkably reduced the level of *IFN-γ* mRNA (Figure 7A). It is critical to study IFN-γ expression in naturally HTLV-1–infected T cells. Therefore, we examined IFN-γ production in PBMCs from ATL patients (supplemental Table 1). PBMCs were stimulated by PMA and ionomycin for 5 hours, and intracellular IFN-γ was stained. We found that IFN-γ production by CD4 T cells was remarkably decreased in ATL patients compared with healthy donors (Figure 7B). In addition, TNF-α and IL-2 production also was suppressed in CD4 T cells from ATL patients. These data suggest that impaired production of IFN-γ is observed not only in HBZ-Tg or ectopically transfected cells but also in primary CD4 T cells from ATL patients.

Discussion

Viruses that cause chronic infections, including hepatitis C virus, HIV, Epstein-Barr virus, and HTLV-1, have strategies to evade the host immune system and to replicate in vivo despite detectable immune responses.[38] For HTLV-1, it has been reported that p12 binds to free human major histocompatibility complex class