2. Ogura M, Uchida T, Maruyama D, Uike N, Choi I,Ishizawa K, Itoh K, Ando K, Taniwaki M, Shimada N, Kobayashi K, Tobinai K.Phase I and pharmacokinetic study Everolimus (RAD001) in Patients withrelapsed or refractory Non-Hodgkin Lymphoma (NHL).51th American society of Hematology annual meeting and expositionDecember 5-8, 2009, New Orleans, LA, USA

《国内学会》

1. 鵜池直邦、本多絵美、宮下 要、崔 日承、油布祐二. 再発・難治性低悪性度 B 細胞リンパ腫に対する Ibritumomab tiuxetan (Zevalin) の有効性と安全性，第 49 回日本リンパ系網内学会総会，2009 年 7 月 9ー11 日，兵庫
2. 鵜池直邦、本多絵美、宮下 要、崔 日承、油布祐二. 治療関連急性前骨髄性白血病 4 例の臨床的特徴と予後，第 71 回日本血液学会学術総会（一般口演），2009 年 10 月 23ー25 日，京都
3. 油布祐二、本多絵美、宮下 要、崔 日承、鵜池直邦. び漫性大細胞型 B 細胞性リンパ腫の表面免疫グロブリンクラス別の特徴，第 71 回日本血液学会学術総会，2009 年 10 月 23ー25 日，京都
4. 崔 日承、久家知子、本多絵美、宮下 要、油布祐二、織田信弥、鵜池直邦. 本態性血小板血症における *JAK2 cMPL* 遺伝子変異−単一施設における解析−，第 71 回日本血液学会学術総会，2009 年 10 月 23ー25 日，京都
5. 宮下 要、本多絵美、崔 日承、油布祐二、阿南憲一、麻生範雄、鵜池直邦. カップ様(cup-like)の核形態を呈する HLA-DR 陰性急性骨髄性白血病の臨床病理学的解析，第 71 回日本血液学会学術総会（一般口演），2009 年 10 月 23ー25 日，京都
6. 本多絵美、宮下 要、崔 日承、油布祐二、鵜池直邦、衛藤徹也、高瀬 謙、加藤光次. MLL/AF6キメラ遺伝子を伴う急性骨髄性白血病の 3 例，第 71 回日本血液学会学術総会（一般口演），2009 年 10 月 23ー25 日，京都
7. 阿部光一郎、鵜池直邦. ゼヴァリン療法の実際ーゼヴァリン治療の施行経験「核医学治療の現在と未来」(シンポジウム)，第 49 回日本核医学会学術総会， 2009 年 10 月 1〜3 日，旭川

厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

ATLに対する同種造血幹細胞移植の治療成績とウイルス遺伝子型に関する研究

研究分担者：今村　雅寛　北海道大学大学院医学研究科　教授

研究要旨

ATL に対する同種造血細胞移植の治療成績にかかわる要因として、HTLV-I ウイルスの遺伝子型の関与があるか否かを検討する目的で、北海道における 9 例の ATL 症例の骨髄血あるいは末梢血を用いて解析した。一定の DNA 量が抽出でき、解析し得た 5 症例の検討からは、北海道と九州地区に多い遺伝子型には偏りのある可能性も示唆されたが、症例数が少ないため、確固とした結論を得るためには、さらなる症例の蓄積が必要である。

A. 研究目的

北海道の ATL 症例に対する同種造血幹細胞移植の治療成績と HTLV-I ウイルスの遺伝子型に関連性があるのか否かを検討することを目的とした。

B. 研究方法

北海道の ATL 症例（急性型 6 例、リンパ腫型 2 例、くすぶり型 1 例）の 9 例から倫理委員会の承認を得た後、各症例の同意を得て骨髄血あるいは末梢血を採取し、 DNA を抽出した。それを PCR 法で増幅し、HTLV-I ウイルスの全て領域（約 9kb）の塩基配列の比較を行った。このうち、十分な DNA 量を抽出できたのは、急性型 3 例とくすぶり型 1 例の 4 例であった。急性型で不十分ながら DNA の抽出が出来たのが 1 例であった。また、同種造血幹細胞移植を受けたのは 4 例であり、いずれも現在生存中であるが、この中で遺伝子解析が出来たのは、3 例であった。

（倫理面への配慮）

本研究は、ATL 症例の骨髄血あるいは末梢血から DNA を抽出し、HTLV-I ウイルスの遺伝子の塩基配列を解析することで、北海道地区とその他の地区に差があるか否かを検討し、同種造血幹細胞移植の治療成績との関連性を明らかにするもので、各施設での倫理委員会の承認を得て行った後、各症例の同意のもとに行った。採取血液量も必要最低限に抑えて、個人情報の保護に留意して行った。

C. 研究結果

三陸に多い ATK に由来する HTLV-I ウイルスの塩基配列 J02029 と九州に多い Box3-44 を対照として、北海道の HTLV-I ウイルスの遺伝子型を解析すると、北海道の ATL 症例の HTLV-I ウイルスの遺伝子型は九州地区に多いものと比較的似ていたが、HD-1 と HD-4 は同じサブグループに属し、HD-2、HD-8 と HD-9 は同じグループに属していることが明らかとなった。しかし、九州地方のサンプルでは HD-1 と HD-4 に近いサブグループの頻度が 8/54（15%）であった。このことは、北海道では HD-1 と HD-4 に近いサブグループの頻度が高い可能性がある。

D. 考察

世界的には HTLV-I ウイルスの遺伝子型を Cosmopolitan 型、Central Africa 型、Melanesia 型に分類後、Cosmopolitan 型を、さらに West African subgroup、North African subgroup 型、Japanese subgroup 型、Transcontinental subgroup 型に分類する。日本における HTLV-I ウイルスの遺伝子型に関しては、大きく分けて和人型、琉球型、アイヌ型に分類する方法がある。和人型は Japanese subgroup 型とほぼ同一で、

アイヌ型に関しては殆ど解析が進んでいないが、アイヌ型と琉球型似ており Cosmopolitan Transcontinental subgroup に属することが示唆されている。今回解析し得た北海道の ATL5 症例の HTLV-I 遺伝子型は、世界的な分類法に照らしてどれに属するかは、今後の検討課題であるが、大きく分けて HD-1 と HD-4 を含むサブグループと、HD-2、HD-8、HD-9 を含むサブグループの 2 つからなり、いずれのサブグループも九州のものに類似していた。しかし、HD-1 と HD-4 のサブグループは九州では 15%にしか認められないにもかかわらず、北海道では 40%に認められていた。まだ、少数例の解析であり、しかも感染経路が不明瞭な時点で、明確な結論は出し得ないが、北海道における同種造血幹細胞移植が、他地区のそれに比して比較的良好な成績を示していることと、何らかの関連性があることも否定できない。より明確な結論を導くためには、今後のさらなる症例の蓄積が期待される。

E. 結論

北海道における ATL 症例の HTLV-I ウイルス遺伝子型は、九州地区のものに類似していたが、大きく分けて 2 つのサブグループに分類された。HD-1 と HD-4 からなるサブグループは、九州地区より北海道で多く認められる傾向にあった。同種造血幹細胞移植の治療成績との関連で、HTLV-I ウイルスの遺伝子型がいかなる役割を果たしているのかは、まだ明らかではないが、その答えを得るためにはさらなる症例の蓄積が望まれる。

F．健康危険情報

該当なし

G. 研究発表

1．論文発表

なし

2．学会発表

なし

H. 知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

末梢性 T 細胞リンパ腫と ATL の細胞起源に関する免疫組織化学的研究

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研究要旨
末梢性 T 細胞リンパ腫（PTCL）と成人 T 細胞白血病（ATL）の細胞起源を免疫組織化学的に検討した．マスター制御因子の発現の検討からは，3 例が T-Bet 陽性で Th1 由来，3 例が GATA3 陽性で Th2 由来と考えられた．FOXP3，RORγt 陽性 (Treg，Th17 由来) の症例は認めなかった。マスター制御因子陰性の 4 例のうち，2 例は CCR7，CD62L の両方が陽性で T_{CM} 由来，1 例は BCL6，CXCL13，PD-1 のいずれもが陽性で T_{FH} 由来が示唆された。ATL では，現在のところ一定の傾向は見出されていない。

A．研究目的

ヘルパーT 細胞 (Th)サブセットへの分化を制御するマスター因子が明らかにされている．Th，Th1，Th2，Th17，Treg への分化に関与するマスター制御因子は各々Th-POK，T-Bet，GATA3，ROPγt，FOXP3 である．今回の研究では，PTCL-NOS と ATL の細胞起源を免疫組織化学的に検討し，病型診断や治療効果判定に応用する。

B．研究方法

PTCL-NOS 10 例，ATL 10 例から採取された骨髄血，末梢血，リンパ節などを，患者の同意を得たのち検索の対象とした。免疫組織化学は型のとおり行い，ケモカイン受容体を中心とした細胞表面抗原とマスター遺伝子産物について特異抗体を用いて検討した。検討したケモカイン受容体と細胞表面抗原は，CXCR3，CXCL13，CCR3，4，5，6，7，CD62L，PD-1，CD4，CD25 であり，マスター制御因子は，Th-POK，T-Bet，GATA3，RORγt，Foxp3，BCL6 である。25%以上の細胞で発現が見られる場合に陽性とした。陽性，陰性コントロールとして，扁桃腺炎，リンパ節炎，血管免疫芽球性 T 細胞リンパ腫，B 細胞リンパ腫 (濾胞性リンパ腫，びまん性大細胞型 B 細胞性リンパ腫) の検体を用いた。

C．研究結果

1) PTCL-NOS

① 3 例がヘルパーT 細胞のプロフィール (CD4 陽性，CD8 陰性，細胞傷害性分子陰性) を示していなかった。

②エフェクターTh 関連抗原の発現
9 例で，CD45RA 陰性，CD45RO 陽性．全例で Th-POK が陽性。

③ Th1 関連抗原の発現
3 例で T-Bet 陽性で，うち 2 例は Th1 関連ケモカイン受容体である CXCR3 が陽性。

④ Th2 関連抗原の発現
3 例で GATA3 陽性であった。うち 1 例は Th2 関連ケモカイン受容体である CCR3，CCR4 が陽性。

⑤ Treg，Th17 のマスター制御因子の発現
FOXP3，ROPγt は全例で陰性。

⑥ TCM ，TFH 関連抗原の発現
2 例で TCM に関連する CCR7，CD62L の両方が陽性。他の 1 例で，TFH 関連抗原である BCL6，CXCL13，PD-1 のいずれもが陽性であり，本例では t(5；9)(q33；q22) の染色体転座を認めた。1 例で検索したいずれの Th サブセットの抗原も陰性。

2) ATL

現在のところ，一定の傾向は認められていない。

D. 考察

PTCL-NOS の 10 例では，全てが CD45RO, Th-POK 陽性であり，イフェクターTh 由来と考えられた。マスター制御因子の発現の検討からは，3 例が T-Bet 陽性で Th1 由来，3 例が GATA3 陽性で Th2 由来と考えられた。FOXP3, RORγt 陽性 (Treg, Th17 由来) の症例は認めなかった。これらの制御因子陰性の 4 例のうち，2 例は CCR7, CD62L の両方が陽性でセントラルメモリーT 細胞 (TCM), 1 例は BCL6, CXCL13, PD-1 のいずれも陽性で濾胞ヘルパーT 細胞 (TFH) 由来が示唆された。

しかし，3 例がヘルパーT 細胞のプロフィール (CD4 陽性，CD8 陰性，細胞傷害性分子陰性) を示しておらず，また，ATL では一定の傾向が見出されなかった。今後，多数例の解析や，遺伝子発現プロファイルを用いた細胞起源の同定の検討が必要である。

E. 結論

Th-POK, T-Bet, GATA3, ROPγt, FOXP3 などのマスター制御因子と，ケモカイン受容体を含む表面抗原の発現を免疫組織化学的に検索し，PTCL-NOS の正常対応細胞の候補を同定できた。

F. 健康危険情報

特記すべきものなし

G. 研究発表

1. 論文発表

1. Nakahata S, Saito Y, Hamasaki M, Hidaka T, Arai Y, Taki T, Taniwaki M, Morishita K. Alteration of enhancer of polycomb 1 at 10p11.2 is one of the genetic events leading to development of adult T-cell leukemia/lymphoma. Genes Chromosomes Cancer. 48:768-776, 2009
2. Matsumoto Y, Taki T, Fujimoto Y, Taniguchi K, Shimizu D, Shimura K, Uchiyama H, Kuroda J, Nomura K, Inaba T, Shimazaki C, Horiike S, Taniwaki M. Monosomies 7p and 12p and FLT3 internal tandem duplication: possible markers for diagnosis of T/myeloid biphenotypic acute leukemia and its clonal evolution. Int J Hematol. 89:352-358, 2009
3. Kuroda J, Taniwaki M. Involvement of BH3-only proteins in hematologic malignancies. Crit Rev Oncol Hematol. 71:89-101, 2009
4. Zen K, Yasui K, Gen Y, Dohi O, Wakabayashi N, Mitsufuji S, Itoh Y, Zen Y, Nakanuma Y, Taniwaki M, Okanoue T, Yoshikawa T. Defective expression of polarity protein PAR-3 gene (PARD3) in esophageal squamous cell carcinoma. Oncogene 28:2910-2918, 2009
5. Zen K, Yasui K, Nakajima T, Zen Y, Zen K, Gen Y, Mitsuyoshi H, Minami M, Mitsufuji S, Tanaka S, Itoh Y, Nakanuma Y, Taniwaki M, Arii S, Okanoue T, Yoshikawa T. ERK5 is a target for gene amplification at 17p11 and promotes cell growth in hepatocellular carcinoma by regulating mitotic entry. Genes Chromosomes Cancer 48:109-120, 2009
6. Murakami H, Shimizu K, Sawamura M, Suzuki K, Sugiura I, Kosugi H, Shimazaki C, Taniwaki M, Abe M, Takagi T. Phase II and pharmacokinetic study of thalidomide in Japanese patients with relapsed/refractory multiple myeloma. Int J Hematol 89:636-641, 2009
7. Tobinai K, Watanabe T, Ogura M, Morishima Y, Hotta T, Ishizawa K, Itoh K, Okamoto S, Taniwaki M, Tsukamoto N, Okumura H, Terauchi T, Nawano S, Matsusako M, Matsuno Y, Nakamura S, Mori S, Ohashi Y, Hayashi M, Endo K. Japanese phase II study of 90Y-ibritumomab tiuxetan in patients with relapsed or refractory indolent B-cell lymphoma. Cancer Sci 100:158-164, 2009

2．学会発表

1. 松本洋典, 野村憲一, 大城宗生, 山本未央, 堤 康彦, 小林 勉, 清水大介, 内山人二, 黒田純也, 兼子裕人, 堀池重夫, 島崎千尋, 谷脇雅史. 末梢性T細胞リンパ腫の正常対応細胞の同定. 第13回京都分子血液フォーラム. 2009年7月4日. 京都
2. 小林 覚, 滝 智彦, 西田一弘, 大城宗生, 栗田佳代子, 坂元奈津美, 知念良顕, 名越久朗, 堤 康彦, 松本洋典, 黒田純也, 堀池重夫, 谷脇雅史. びまん性リンパ腫にみられた6q-の切断点におけるIGHD-BACH2再構成の同定. 第68回日本癌学会学術総会. 2009年10月1日. 横浜
3. Nagoshi H, Taki T, Nisida K, Goto M, Kobayashi S, Tsutsumi Y, Matsumoto Y, Kuroda J,Horiike S, Otuki T,Taniwaki M. Alteration of DCC gene in multiple myeloma cell lines. 第71回血液学会学術総会. 2009年10月23日. 京都
4. 堤 康彦, 中山理祐子, 水谷信介, 志村勇司, 大城宗生, 山本未央, 古林 勉, 内山人二, 松本洋典, 黒田純也, 堀池重夫, 中村栄男, 谷脇雅史. 病理組織診断のGrade2とGrade3aで付加的染色体異常に差が認められた濃胞性リンパ腫. 第71回血液学会学術総会 2009年10月23日京都
5. 片山雄太, 松本洋典, 中山理祐子, 水谷信介, 大城宗生, 山本未央, 堤 康彦, 古林 勉, 内山人二, 黒田純也, 堀池重夫, 谷脇雅史. 再発･難治性リンパ腫CNSリンパ腫, 二次性CNSリンパ腫に対しAuto-PBSCTを施行した4症例. 第71回血液学会学術総会. 2009年10月23日. 京都

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

臨床試験の計画と解析，ATL レトロスペクティブデータ

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研究要旨
本研究班でこれまでに実施したATLの血縁者間骨髄移植に関する2つの臨床試験データの統合解析をおこなった。

A. 研究目的

本研究班でこれまでに実施してきた ATL に対する RIST を用いた血縁者間骨髄移植の 2 つの臨床試験データは世界で初めて実施された ATL に対する移植治療の前向き試験データである。これらを統合したレトロスペクティブ解析をおこなった。

B. 研究方法

血縁間骨髄移植の臨床試験データの解析：
本研究班でこれまでに実施した血縁者間骨髄移植に関する RIST の臨床試験データ（第 1 期，第 2 期計 29 例）に関するレトロスペクティブ解析をおこない，生存時間、ウイルス量の経時的変化などを探索した。

C. 研究結果

血縁間骨髄移植の臨床試験データの解析：
登録された 29 例に対する検討では，11 人が原病死、8 人が治療関連死であった。対象症例の中でもっとも遅く死亡が観測されたのは登録から 26 ヶ月後であった。29 人の 5 年生存率は 34%（95%CI：18–51%）、追跡期間中央値は 82 ヶ月であった。その他、生存症例の臨床背景について詳細な集計をおこなった。

生存している10例におけるHTLV-1のウイルス量の経時的変化についても要約した。ATL は HTLV-1 感染が原因で発症する疾患であるため、HTLV-1 ウイルス量の経時的変化については多くの研究者が興味を寄せるところであるが、前向き研究として、その変化を検討したデータはない。今回、移植後の長期生存 10 例に対する検討をおこなった結果、移植後、長期生存例でもウイルス量の高値の状態が続く症例、逆にウイルス量が減少して検出限界下のレベルに至る症例が存在することが判明した。さらに、いったん検出限界以下のレベルになるものの、再びウイルス量の増加が見られる症例もいた。今回の検討ではこの 3 通りであるが、多様なパターンを示していることが判明したので今後のさらなる研究が必要である。（Choi et al, BMT, E-publish, 2010）

D. 考察

血縁者間の骨髄移植によって、一部の症例に疾患治癒と思われる状態が観察されたのは有望な結果であるといえる。しかしながら、移植関連死亡率は高いことは今後の重大な検討課題である。また、移植が実施可能な症例は限られており，また ATL という疾患の特徴から前処置を RIST とした非血縁者間での移植の実施可能性を検討することは重要である。ウイルス量の経時的変化データと併せて現在本研究班で実施中の臨床試験の結果を待ちたい。

E. 結論

29 人の 5 年生存率 34%（95%CI：18–51%）は治癒が困難と認識されていた ATL にとっては有望な結果と考えられる。今後、ATL に対する治療戦略へのさらなる展望が得られることが期待される。

G. 研究発表

1. 論文発表

1. Choi I , Tanosaki R, Uike N, Utsunomiya A, Tomonaga M, Harada M, Yamanaka T, Kannagi M and Okamura J. Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials. Bone Marrow Transplantation, E-publish, 2010

2. 学会発表

なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出 版 地	出版年	ページ
油布祐二、鵜池直邦	成人T細胞白血病/リンパ腫の適切な初期治療は？	神田善伸	臨床に直結する血液疾患診療のエビデンス	文光堂	東京	2009	295-298

雑誌

発　表　者　氏　名	論　文　タ　イ　ト　ル　名	発　表　誌　名	巻　　号	ペ　ー　ジ	出版年
Choi I , Tanosaki R, Uike N, Utsunomiya A,Tomonaga M, Harada M, Yamanaka T, Kannagi M , Okamura J.	Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials.	Bone Marrow Transplantation		E-publish	2010
Shimizu Y, Takamori A, Utsunomiya A, Kurimura M, Yamano Y, Hishizawa M, Hasegawa A, Kondo F, Kurihara K, Harashima N, Watanabe T, Okamura J, Masuda T, Kannagi M.	Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages.	Cancer Sci	100	481-489	2009
Takatsuka N, Hasegawa A, Takamori A, Shimizu Y, Kato H, Ohashi T, Amagasa T, Masuda T, Kannagi M.	Induction of IL-10 and IFN-g-producing T-cell responses by autoreactive T-cells expressing human T-cell leukemia virus type I Tax.	Int Immunol	21	1089-1100	2009
Kinpara S, Hasegawa A, Utsunomiya A, Nishitsuji H, Furukawa H, Masuda T, Kannagi M.	Stromal cell-mediated suppression of human T-cell leukemia virus type-1 expression in vitro and in vivo through type-I interferon.	J Virol	83	5101-5108	2009
Zhao T, Yasunaga J-I, Satou Y, Nakao M, Takahashi M,Fujii M, Matsuoka M.	Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-κB.	Blood	113	2755-2764	2009
Matsuoka M, Green PL.	HBZ gene, a key player in HTLV-1 pathogenesis.	Retrovirology	6	71	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Koyama M, Hashimoto D, Aoyama K, Matsuoka KI, Karube K, Niiro H, Harada M, Tanimoto M, Akashi K, Teshima T.	Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells.	Blood	113(9)	2088-2095	2009
Yamamoto H, Uchida N, Ishiwata K, Araoka H, Takagi S, Tsuji M, Kato D, Matsuhashi Y, Seo S, Matsuno N, Masuoka K, Wake A, Yoneyama A, Makino S, Taniguchi S.	Possible graft-versus-host disease involving the central nervous system soon after cord blood transplantation.	Am J Hematol	Nov;84(11)	764-6	2009
Matsuno N, Wake A, Uchida N, Ishiwata K, Araoka H, Takagi S, Tsuji M, Yamamoto H, Kato D, Matsuhashi Y, Seo S, Masuoka K, Miyakoshi S, Makino S, Yoneyama A, Kanda Y, Taniguchi S.	Impact of HLA disparity in the graft-versus-host direction on engraftment in adult patients receiving reduced-intensity cord blood transplantation.	Blood	114(8)	1689-95	2009
Kim SW, Mori SI, Tanosaki R, Fukuda T, Kami M, Sakamaki H, Yamashita T, Kodera Y, Terakura S, Taniguchi S, Miyakoshi S, Usui N, Yano S, Awano Y, Nagatoshi Y, Harada M, Morishima Y, Okamoto S, Saito AM, Ohashi Y, Ueda R, Takaue Y.	Busulfex (i.v. BU) and CY regimen before SCT: Japanese-targeted phase IIpharmacokinetics combined study.	BoneMarrow Transplant	43	611-617	2009
Nakahata S, Saito Y, Hamasaki M, Hidaka T, Arai Y, Taki T, Taniwaki M, Morishita K.	Alteration of enhancer of polycomb 1 at 10p11.2 is one of the genetic events leading to development of adult T-cell leukemia/lymphoma.	Genes Chromosomes Cancer	48	768-776	2009
宇都宮與	成人T細胞白血病/リンパ腫へのアプローチ	血液・腫瘍科	58(4)	434-440	2009

Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells

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Dendritic cells (DCs) can be classified into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can prime antigen-specific T-cell immunity, whereas in vivo function of pDCs as antigen-presenting cells remains controversial. We evaluated the contribution of pDCs to allogeneic T-cell responses in vivo in mouse models of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation by an add-back study of MHC-expressing pDCs into major histocompatibility complex-deficient mice that were resistant to GVHD. Alloantigen expression on pDCs alone was sufficient to prime alloreactive T cells and cause GVHD. An inflammatory environment created by host irradiation has the decisive role in maturing pDCs for T-cell priming but this process does not require Toll-like receptor signaling. Thus, functional outcomes of pDC–T-cell interactions depend on the immunologic context of encounter. To our knowledge, these results are the first to directly demonstrate an in vivo pathogenic role of pDCs as antigen-presenting cells in an antigen-specific T cell–mediated disease in the absence of other DC subsets and to provide important insight into developing strategies for tolerance induction in transplantation. (Blood. 2009;113:2088-2095)

Introduction

The interaction of naive T cells and dendritic cells (DCs) is essential for initiating primary immune responses. DCs can be divided into 2 distinct subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) according to their immunophenotype and functional properties.[1-3] pDCs represent a $CD11c^{int}$ $B220^{+}$ DC subset that differs from the $CD11c^{high}$ $B220^{-}$ major histocompatibility complex (MHC) class II^{high} cDCs, commonly viewed as the classic stimulators of naive T cells. One distinctive feature of pDCs is their capacity to rapidly produce high levels of type I interferon (IFN) in response to viral and bacterial stimuli, highlighting the importance of pDCs in innate immune responses.[2-8] pDCs express low levels of surface MHC and classical costimulatory molecules; therefore, they are poor T-cell stimulators.[5-11] In contrast, pDCs matured with CD40 ligands or Toll-like receptor (TLR) ligands are potent antigen-presenting cells (APCs), capable of stimulating naive T-cell proliferation and differentiation to helper, killer, memory, and regulatory T cells in vitro.[7,12,13] In vivo, injection of pDCs activated by synthetic oligodeoxynucleotides containing unmethylated cytosine-guanine motifs (CpG), but not immature pDCs, is capable of eliciting antigen-specific $CD8^{+}$ T-cell responses.[10,14] On the other hand, OVA-pulsed pDCs protected mice against OVA-induced asthma development.[15] pDCs in the tumor-draining lymph nodes express indole 2, 3-dioxygenase, and suppress antitumor T-cell responses.[16] In patients with ovarian cancer, large numbers of pDCs, which induced interleukin 10 (IL10)–producing regulatory T cells, were found in ascites.[17] pDCs mediate tolerance and prolong survival of cardiac allografts.[11,18,19] Several recent clinical observations also suggest that pDCs play important regulatory roles in transplant outcome. An increased ratio of pDCs/cDCs is associated with the successful withdrawal of immunosuppressants after liver transplants.[20] In allogeneic hematopoietic stem cell transplantation (HSCT), low pDC count in the peripheral blood is a risk for graft-versus-host disease (GVHD),[21] while larger numbers of pDCs in donor bone marrow (BM) are associated with increased relapse.[22] Collectively, accumulating data suggest that pDCs are mostly tolerogenic in vivo. However, it remains unclear whether pDCs as APCs have a causative role in antigen-specific T cell–mediated diseases in vivo, although pDCs are involved in the pathogenesis of systemic lupus erythematosus (SLE) and psoriasis through IFN-α production.[23,24]

GVHD, the major obstacle to successful outcome after allogeneic HSCT, is mediated by donor T cells stimulated by recipient DCs.[25-27] MHC class I– or II–deficient ($H2\text{-}Ab1^{-/-}$) mice are resistant to CD8- and CD4-dependent GVHD, respectively.[26-28] When $H2\text{-}Ab1^{-/-}$ mice are repopulated with syngeneic MHC class II–expressing DCs, these mice succumb to acute GVHD.[26,28] Thus, recognition of MHC class II alloantigens on host-derived DCs, alone, is sufficient to prime donor $CD4^{+}$ T cells and cause lethal acute GVHD. CD4-mediated GVHD can develop even in the absence of MHC class II alloantigen expression on GVHD target cells, such as epithelium, endothelium, and parenchyma.[26,28] Thus, this GVHD model system using $H2\text{-}Ab1^{-/-}$ mice presents a stringent test of the allostimulatory capacity of a DC subset when the donor and recipient differ at only MHC class II loci. Using this

Submitted July 14, 2008; accepted December 30, 2008. Prepublished online as *Blood* First Edition paper, January 14, 2009; DOI 10.1182/blood-2008-07-168609.

The online version of the article contains a data supplement.

Presented in abstract form at the 49th Annual Meeting of the American Society of Hematology, Atlanta, GA, December 10, 2007.

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model system with modification, we addressed whether pDCs as APCs have a causative role in antigen-specific T cell–mediated diseases, such as GVHD, or induce tolerance.

Methods

Mice

Female C57BL/6 (B6: H-2^b, CD45.2$^+$), B6D2F1 (H-2$^{b/d}$), and BALB/c (H-2^d) mice were purchased from Charles River Japan (Yokohama, Japan). C3H/HeJ (C3H: H-2^k) and AKR/J (AKR: H-2^k) mice were purchased from Japan SLC (Shizuoka, Japan). B6-Ly5a (H-2^b, CD45.1$^+$), and C3H-background β_2m-deficient (*β2m*$^{-/-}$: C3.129P2(B6)-*B2m*^{tm1Unc}/Dcr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II$^{-/-}$ mice (*H2-Ab1*$^{-/-}$: B6.129-*Abb*^{tm1} N12) were from Taconic (Germantown, NY). B6-background Toll–IL-1 receptor domain-containing adaptor inducing IFN-β/myeloid differentiation factor 88 double-deficient (TRIF/MyD88 DKO) mice[29] were kindly provided by Dr Kiyoshi Takeda at Kyushu University (Fukuoka, Japan). The age of the mice ranged between 9 and 16 weeks. Mice were maintained in a specific pathogen-free condition and received normal chow and hyperchlorinated drinking water for the first 3 weeks after transplantation. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Kyushu University.

Cell isolation

To expand DCs, we injected mice subcutaneously once daily with 10 μg recombinant human fms-like tyrosine kinase 3 ligand (FL; Amgen, Seattle, WA) for 10 consecutive days, and cDCs, pDCs, and B cells were isolated, as previously described, with a modification.[28,30] Briefly, cDCs were enriched from splenocytes using CD11c microbeads and the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by cell sorting of CD11c^{high} B220$^-$ cells using a BD FACSAria (BD Biosciences, San Jose, CA). B cells were enriched from splenocytes with B220 microbeads and the AutoMACS, and sorted as CD11c$^-$ B220$^+$ cells. pDCs were enriched from BM by depleting CD3$^+$, CD19$^+$, CD11b$^+$, CD49b$^+$, and Ly-76$^+$ cells using a cocktail of biotin-conjugated mAbs, streptavidin-microbeads, and the AutoMACS system, followed by a FACS sorting of CD11c^{int} B220$^+$ cells. CD4$^+$ T cells were negatively selected from splenocytes by depleting CD8$^+$, CD49b$^+$, CD11b$^+$, Ly-76$^+$, and B220$^+$ cells. CD8$^+$ T cells were negatively selected from splenocytes by depleting CD4$^+$, CD49b$^+$, CD11b$^+$, Ly-76$^+$, and B220$^+$ cells, using the AutoMACS system, followed by a FACS sorting of CD4$^-$CD8$^+$ cells. T-cell depletion (TCD) of donor BM cells was performed using CD90 microbeads and the AutoMACS system.

Induction and assessment of GVHD

GVHD was induced as previously described.[28] In brief, mice received 11 Gy total body irradiation (TBI), split into 2 doses separated by 4 hours to minimize gastrointestinal toxicity, and injected intravenously with 2 × 10^6 each APC subset on day −1. On day 0, mice were injected intravenously with 2 × 10^6 CD4$^+$ or CD8$^+$ T cells with or without 5 × 10^6 TCD-BM. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly using a scoring system that evaluated changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10).[31] Acute GVHD was also assessed by detailed histopathologic analysis of the liver and intestine. Slides stained with hematoxylin and eosin were examined systematically using a semiquantitative scoring system.[32] Pictures from tissue sections were taken at room temperature using an ProgRes 3012 mF digital camera (Jenoptik Laser Optik Systeme, Jena, Germany) mounted on an Olympus BX51 microscope (Olympus, Tokyo, Japan) and analyzed using a ProgRes PlugIn for PCI software version 5.0 (Jenoptik Laser Optik Systeme). Images were acquired using an UPlan Apochromat 10×/0.40 numeric aperture (NA) or a Plan Apochromat 40×/0.90 NA WLSM objective, depending on the desired magnification.

Cell culture and enzyme-linked immunosorbent assay

All culture media and incubation conditions have been described previously.[33] CD4$^+$ T cells were cultured at a concentration of 2 × 10^5 cells/well with 10^4 irradiated (20 Gy) APCs. After culturing for 3 days, supernatants were harvested for cytokine measurements, and cells were pulsed with ^{3}H-thymidine (1 μCi per well) for further 16 hours. Proliferation was determined using a Topcount NXT (Packard Instruments, Meriden, CT). In some experiments, 1 μM CpG 1668 (Sigma-Aldrich Japan, Ishikari, Japan) or 100 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich Japan) was added to the culture. To determine secretion of IFN-α and IL-12 p70 from APCs, 10^5 APCs were incubated with 1 μM CpG 2216 (Sigma-Aldrich Japan) for 16 hours. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ (BD Biosciences), IFN-α (PBL Biomedical Laboratories, Piscataway, NJ), and IL-12 p70 (R&D Systems, Minneapolis, MN) was performed according to the manufacturer's protocols with the sensitivity of 31.3 pg/mL, 12.5 pg/mL, and 2.5 pg/mL, respectively.

Flow cytometric analysis

The mAbs used were fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, or allophycocyanin-conjugated anti–mouse TCRβ, CD4, CD8α, CD11c, CD11b, CD19, CD49b, CD45.1, CD45.2, CD90.2, CD86, B220 (CD45R), H-2K^b, H-2K^d, H-2K^k, I-A^b, Ly6C (BD Biosciences), mPDCA-1 (Miltenyi Biotec), and Foxp3 (eBioscience, San Diego, CA). Cells were stained as previously described.[33] Irrelevant IgG$_{2a/b}$ mAbs were used as a negative control. For intracellular IFN-γ staining, splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti–IFN-γ mAbs. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, CD4$^+$ T cells were stained in phosphate-buffered saline (PBS) with 1 μM CFSE (Molecular Probes, Invitrogen, Eugene, OR), as described earlier.[34] At least 5000 live samples were acquired for analysis. Dead cells were identified as 7-amino-actinomycin D (BD Biosciences) or 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) positive cells. The cells were analyzed using a FACSCalibur or a FACSAria flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Statistical analysis

Mann-Whitney U tests were used to analyze cell counts and clinical scores. We used the Kaplan-Meier product limit method to obtain the survival probability and the log-rank test was applied to compare the survival curves. We defined *P* values of less than .05 as statistically significant.

Results

Isolation and characterization of pDCs

Low frequency of pDCs in vivo has hampered the study of this cell population. To expand DCs in vivo, mice were injected with 10 μg FL for 10 days.[6,28,30] As previously reported,[35,36] FL treatment resulted in an increase in frequency of CD11c$^+$B220$^+$ pDCs to 6.7% in spleen and 18.6% in BM (Figure 1A). pDCs, cDCs, and B cells were then isolated as described in the methods section. The purity of each population was greater than 98% with less than 0.1% contamination by the others (Figure 1A). pDCs appeared plasmacytoid round shaped with excentered nuclei and basophilic cytoplasm (Figure 1B). They expressed low levels of CD86 and MHC class II but high levels of Ly6C and mPDCA-1 (Figure 1C). These cells did not express CD19, CD49b, or CD11b, thus ruling out contamination of B cells, natural killer (NK) cells, and IFN-producing killer

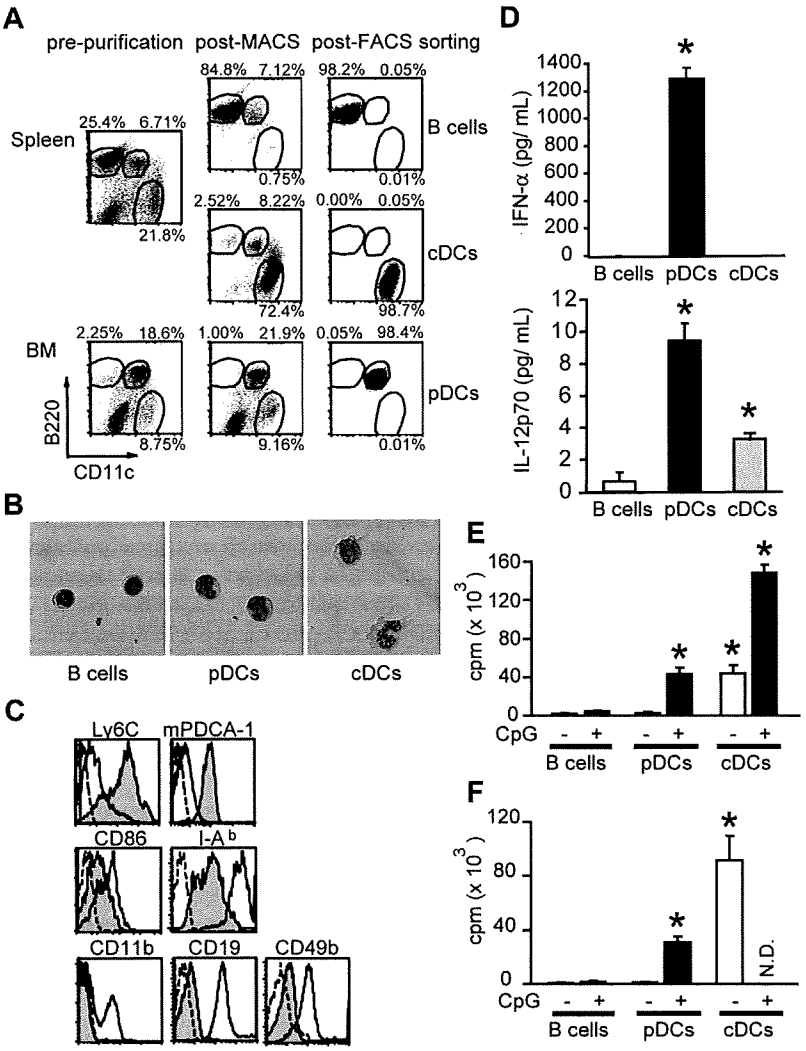

Figure 1. Isolation of pDCs and cDCs. B cells (CD11c$^-$ B220$^+$), pDCs (CD11c^{int} B220$^+$), and cDCs (CD11c^{high} B220$^-$) were isolated from the spleen or BM of FL-treated mice. (A) Two-dimensional counter plots of B220 and CD11c staining. Percentages of cells are shown enclosed in circles. (B) Cell morphology stained with May-Giemsa (magnification ×400). (C) Immunophenotyping. Filled histograms; pDCs, broken-lined open histograms; pDCs stained with isotype controls, solid-lined open histograms for Ly6C, mPDCA-1, CD86, I-A^b, and CD11b; cDCs, those for CD19 and CD49b; B cells and enriched CD49b$^+$ cells as positive controls, respectively. (D) Production of IFN-α (top) and IL-12p70 (bottom) after incubation of cells with CpG 2216 for 16 hours (mean ± SD). (E,F) Aliquots of 2 × 10^5 BALB/c CD4$^+$ T cells were cultured with 10^4 cells from each APC subset isolated from FL-treated (E) and FL-untreated mice (F), with or without 1 μM CpG 1668, and their proliferation 3 days later was shown as mean plus or minus SD. N.D. indicates not done. Data are representative of at least 2 similar experiments. *$P < .05$ compared with B cells.

DCs[37,38] in the pDC fraction. After stimulation with CpG 2216, pDCs secreted high levels of IFN-α and IL-12 p70, whereas cDCs secreted moderate levels of IL-12 p70, but not IFN-α, as has been described[39](Figure 1D).

Next, we examined the allostimulatory capacity of pDCs in mixed lymphocyte reaction. Freshly isolated pDCs were poor inducers of allogeneic CD4$^+$ T-cell proliferation as previously reported[3] (Figure 1E). However, pDCs matured with CpG 1668 were capable of priming T cells nearly as effectively as cDCs (Figure 1E). Similar results were obtained when pDCs were isolated from mice without treatment with FL (Figure 1F).

MHC class II–expressing pDCs alone sufficiently stimulate donor CD4$^+$ T cells to cause GVHD in MHC class II–deficient mice

H2-Ab1$^{-/-}$ B6 mice are resistant to CD4-dependent GVHD.[26,28] We studied whether the add-back of MHC class II$^{+/+}$ pDCs could prime alloreactive T cells using this system with modification. In our previous studies, we used a bm12 → B6 model across MHC class II disparity alone. In contrast, the B6 and BALB/c strain combination used in the current study differs at both MHC class I and class II loci. To avoid the confounding effects of MHC class I and CD8$^+$ T cells, therefore, purified CD4$^+$ T cells were used to induce GVHD, while whole T cells were used in the previous studies. Contamination of CD8$^+$ T cells in the CD4$^+$ cell fraction was < 0.1%. H2-Ab1$^{-/-}$ B6 (H-2^b) mice were irradiated with 11 Gy TBI and injected with 2 × 10^6 pDCs, cDCs, or B cells isolated from wild-type (WT) B6 mice on day −1. Mice were then injected with 2 × 10^6 CD4$^+$ T cells from BALB/c (H-2^d) donors that differ at MHC and multiple minor histocompatibility antigens (miHAs) from B6 mice on day 0. On day 0, we confirmed homing of the injected cDCs and pDCs to spleen and lymph nodes (LNs), where mature DCs form long-lived contacts with T cells (data not shown).[40] Flow cytometric analysis of the mesenteric LNs (mLNs) and spleen on day +6 demonstrated the significant expansion of donor CD4$^+$ T cells (H-2K^{d+}CD4$^+$) in animals that had been repopulated with cDCs or pDCs compared with those with B cells (Figure 2A). No CD8$^+$ T-cell expansion was observed (< 0.1%), confirming the stimulation of only CD4$^+$ donor T cells in this system. Similarly, CFSE-labeled donor CD4$^+$ T cells showed robust cell division in animals preinjected with cDCs or pDCs, while those underwent some homeostatic divisions in animals preinjected with B cells (Figure 2B). Donor CD4 expansion in these recipients was associated with increased expression of IFN-γ (data not shown). Evaluation of Foxp3 expression on CD4$^+$ T cells ruled out the possibility of expansion of Foxp3$^+$ regulatory T cells in response to pDCs (data not shown). We then investigated the effects of delayed add-back of pDCs on GVHD. H2-Ab1$^{-/-}$ mice were irradiated on day −4 and injected with pDCs on day −1, followed by the injection of CFSE-labeled BALB/c CD4$^+$ T cells

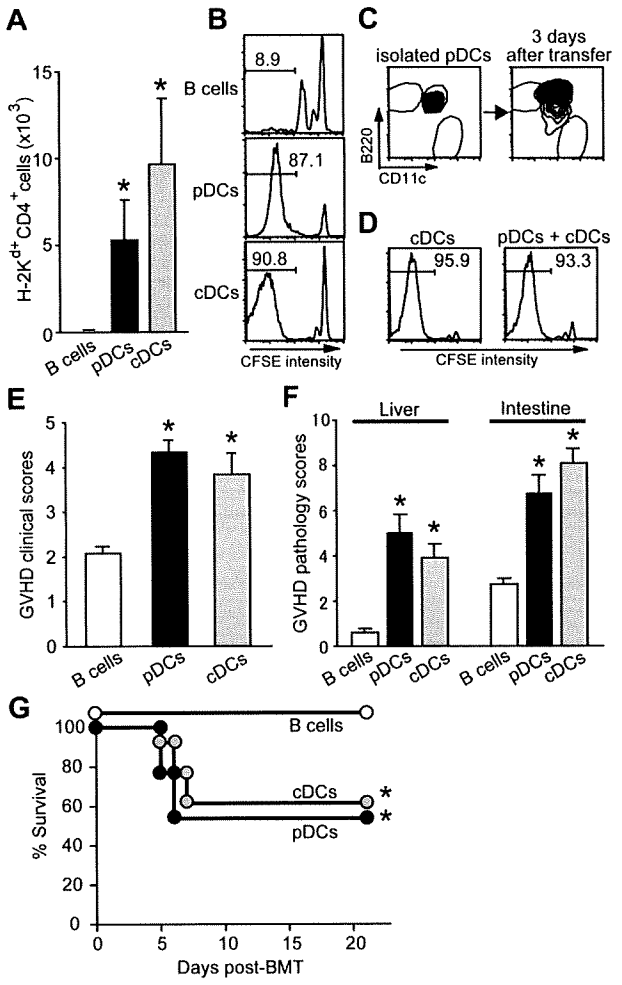

Figure 2. Host pDCs or cDCs alone are sufficient to stimulate alloreactive CD4$^+$ T cells and induce GVHD. (A,B) Totals of 2×10^6 BALB/c (H-2K^{d+}) CD4$^+$ T cells were transferred to irradiated H2-Ab1$^{-/-}$ mice preinjected with 2×10^6 WT B cells, pDCs, or cDCs. Expansion of H-2K^{d+} donor CD4$^+$ T cells in the mLNs (mean ± SD; A) and cell division of CFSE-labeled donor CD4$^+$ T cells in the spleens (B). (C) pDCs were injected to irradiated H2-Ab1$^{-/-}$ mice. Expression levels of B220 and CD11c on I-A^{b+} cells were analyzed 3 days after transfer and compared with those before transfer. (D) Aliquots of 2×10^6 CFSE-labeled BALB/c CD4$^+$ T cells were transferred to irradiated H2-Ab1$^{-/-}$ mice preinjected with 10^6 pDCs plus 10^6 cDCs or 2×10^6 cDCs from WT B6 mice. Cell divisions of donor CD4$^+$ T cells in the spleens on day 7 are shown. (E,F) BALB/c CD4$^+$ T cells were transferred as above and GVHD clinical scores (E), and pathology scores in the liver and intestine (F) on day 6 are shown as mean plus or minus SEM. (G) Lethally irradiated H2-Ab1$^{-/-}$ mice preinjected with pDCs, cDCs, or B cells were injected with 2×10^6 CD4$^+$ T cells and 5×10^6 TCD-BM from BALB/c mice. Survival after BMT is shown. Results from 2 similar experiments were combined. *$P < .05$ compared with B cells.

on day 0. Flow cytometric analysis of the spleen showed robust division of donor CD4$^+$ T cells, thus suggesting that TBI-mediated inflammation persist at least for 3-4 days after TBI (data not shown). In these experiments, we did not have syngeneic controls, since it was apparent in a previous study of DC add-back[28] that there was no expansion of donor T cells in syngeneic controls, where H2-Ab1$^{-/-}$ B6 recipients that had been repopulated with WT B6 DCs were injected with CD4$^+$ T cells from congeneic B6-Ly5a mice.

To rule out the possibility that allostimulatory capacity of pDCs is due to FL treatment, pDCs were isolated from mice without treatment with FL, and their allostimulatory capacity was evaluated similarly. Again, CD4$^+$ T cells primed by pDCs underwent robust cell divisions (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) and differentiated into effectors secreting high levels of IFN-γ (Figure S1B) in H2-Ab1$^{-/-}$ mice 7 days after transfer, indicating that the ability of pDCs to prime T cells is not due to FL treatment. Therefore, we restricted our subsequent analysis to FL-expanded pDCs.

There was still a possibility that pDCs differentiate into cDCs fully endowed with APC function after transfer to H2-Ab1$^{-/-}$ mice.[41] To examine this possibility, we isolated and injected pDCs into irradiated H2-Ab1$^{-/-}$ mice. Flow cytometric analysis of the spleens, 3 days later, showed that pDCs still maintained the phenotype of pDCs (Figure 2C), thus ruling out this possibility. Next, we evaluated whether pDCs were capable of down-regulating T-cell activation induced by cDCs. To investigate this, CFSE-labeled donor CD4$^+$ T cells were transferred to H2-Ab1$^{-/-}$ mice preinjected with cDCs alone or with cDCs and pDCs. However, flow cytometric analysis of the spleen 7 days after transfer showed that the addition of pDCs did not suppress donor CD4 expansion mediated by cDCs (Figure 2D).

We then tested the hypothesis that alloantigen expression on pDCs alone is sufficient for the induction of GVHD target organ damage. H2-Ab1$^{-/-}$ mice were irradiated and injected with 2×10^6 WT pDCs, cDCs, or B cells on day −1. Mice were then injected with 2×10^6 BALB/c CD4$^+$ T cells. Mice preinjected with pDCs or cDCs developed significant GVHD, as assessed by the clinical scores[31] (Figure 2E) and pathology scores on day +6[32] (Figure 2F), compared with those with B cells. Liver histology of mice preinjected with pDCs showed standard histologic features of acute GVHD, including mononuclear cell infiltration in bile ducts and portal triads, and hepatocellular damage with acidophilic bodies (Figure 3B). Histopathology of the small and large intestine also showed significant changes in these mice, including villous atrophy with epithelial apoptosis (Figure 3E), as well as lymphocytic infiltration and granulation tissue formation (Figure 3H). To note, these pathologic features were similar to those observed in recipients preinjected with cDCs (Figure 3C, F, and I), whereas animals preinjected with B cells showed no significant pathologic signs of GVHD, as previously described[28] (Figure 3A,D,G). These results demonstrated that pDCs alone are sufficient to activate donor CD4$^+$ T cells to trigger GVHD as effectively as cDCs. Finally, we evaluated whether pDC-mediated T-cell activation induces GVHD mortality. Lethally irradiated H2-Ab1$^{-/-}$ mice preinjected with 2×10^6 WT pDCs, cDCs, or B cells on day −1 were injected with 2×10^6 CD4$^+$ T cells and 5×10^6 TCD-BM from BALB/c donors on day 0. Mice preinjected with pDCs or cDCs developed lethal GVHD (Figure 2G).

pDCs are solely sufficient to activate alloreactive CD8$^+$ T cells in β_2m-deficient mice

We examined whether presence of allogeneic pDCs could also stimulate donor CD8$^+$ T cells in β_2m$^{-/-}$ mice with impaired cellular expression of functional MHC class I. Irradiated β_2m$^{-/-}$ C3H mice (CD90.2$^+$) were injected with WT pDCs, cDCs, or B cells on day −1, followed by the injection with 2×10^6 CD8$^+$ T cells from MHC-matched, miHA-mismatched AKR mice (CD90.1$^+$) on day 0. Flow cytometric analysis of the mLNs and spleen on day +6 showed significantly greater expansion (Figure 4A) and IFN-γ production (Figure 4B) of donor CD8$^+$ T cells (CD90.2$^-$ CD8$^+$) in mice preinjected with pDCs or cDCs than in those with B cells. Thus, alloantigen expression on pDCs can solely prime alloreactive CD8$^+$ T cells in vivo as potently as cDCs, although we were unable to examine whether these CD8$^+$ T cells could cause GVHD target organ injury because miHA expression

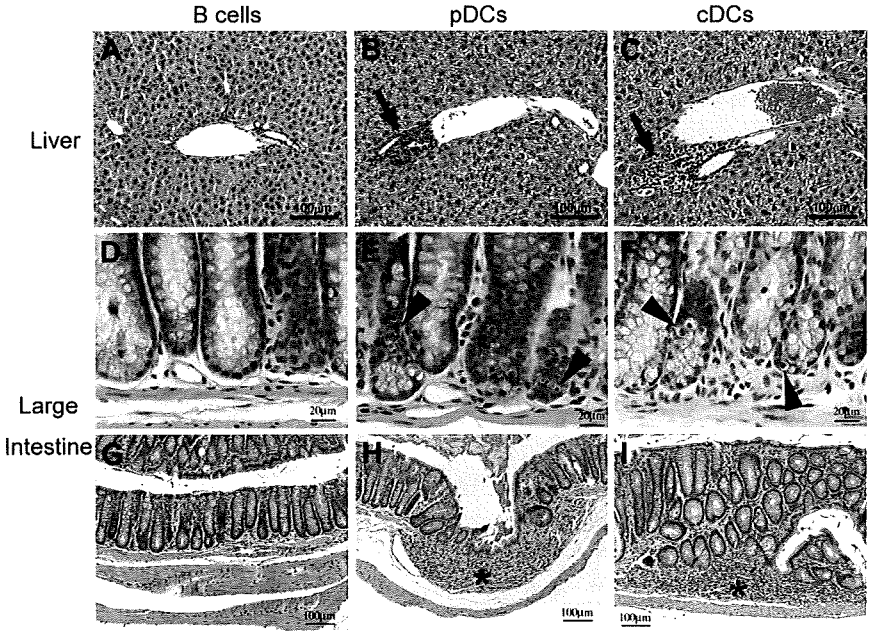

Figure 3. pDCs or cDCs alone mediate standard acute GVHD. Histologic findings of the liver (A-C) and large intestine (D-I). Periportal mononuclear infiltrates in the liver (panels B,C), and crypt cell apoptosis (panels E,F) and granulation tissue (* in panels H,I) in the large intestine are shown.

on target epithelium was required to induce GVHD in MHC-matched, miHA mismatched HSCT.[27,42]

Cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells

pDCs have the ability to produce large amounts of cytokines; therefore, they can be pathogenic through cytokine production as in SLE.[23] To confirm the pathogenic role of pDCs functioning as APCs in GVHD, CFSE-labeled BALB/c $CD4^+$ T cells were adoptively transferred to irradiated H2-$Ab1^{-/-}$ mice preinjected with H2-$Ab1^{-/-}$ pDCs. Flow cytometric analysis of the spleens 6 days after transfer showed that almost 90% of CFSE-labeled T cells had progressed through at least 3 cell divisions in animals preinjected with WT pDCs or cDCs, whereas only a small population did so with some homeostatic divisions in mice with H2-$Ab1^{-/-}$ pDCs or cDCs (Figure 5). These results demonstrate that cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells.

Irradiation is critical for pDCs to prime alloreactive T cells

Activation of pDCs is critical to prime alloreactive T cells as shown in Figure 1. Since it has been shown that irradiation induces maturation of cDCs in vivo,[27] we hypothesized that pretransplantation TBI also plays an important role in activating pDCs. To test this hypothesis, we irradiated mice with 11Gy TBI and performed a flow cytometric analysis of the spleens and mLNs 10 hours after TBI. Expression of CD86 and MHC class II was up-regulated on both pDCs and cDCs isolated from irradiated mice, compared with those from unirradiated mice (Figure 6A). Next, we investigated whether maturation of pDCs are mediated by direct effects of irradiation on pDCs or by effects of irradiation on host tissues. To examine this, 80×10^6 BM cells and splenocytes collected from B6 ($CD45.2^+$) mice were transferred to congeneic B6-Ly5a ($CD45.1^+$) mice that had been irradiated 1 hour before transfer. Flow cytometric analysis of the mLNs isolated 10 hours later showed up-regulated MHC class II expression on $CD45.2^+$ pDCs in irradiated mice compared with those isolated from unirradiated mice (Figure 6B). Collectively, irradiation, likely from the inflammation, is responsible for maturation of pDCs.

These results suggest that pDCs are incapable of priming alloreactive T cells in unirradiated mice as effectively as in irradiated mice. To test this hypothesis, we transferred CFSE-labeled BALB/c $CD4^+$ T cells to unirradiated H2-$Ab1^{-/-}$ mice

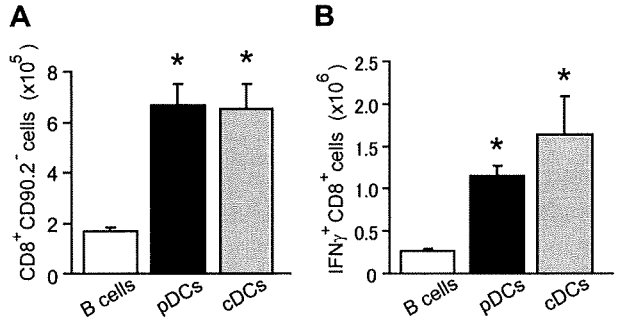

Figure 4. Alloantigen expression on pDCs alone is sufficient to stimulate alloreactive $CD8^+$ T cells. $CD8^+$ T cells from AKR ($CD90.2^-$) mice were transferred to irradiated $\beta 2m^{-/-}$ C3H ($CD90.2^+$) mice preinjected with WT B cells, pDCs, or cDCs. Expansion of $CD90.2^-$ donor $CD8^+$ T cells in mLNs (A) and IFN-γ expression on donor $CD8^+$ T cells in spleens (B) 6 days after transfer are shown as means plus or minus SEM. Data are representative of 2 similar experiments. *$P < .05$.

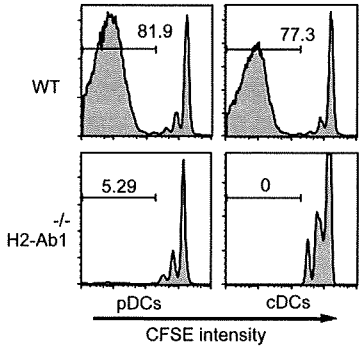

Figure 5. Cognate interaction between pDCs and T cells is required for T-cell activation. CFSE-labeled BALB/c $CD4^+$ T cells were transferred to irradiated H2-$Ab1^{-/-}$ mice preinjected with 2×10^6 pDCs and cDCs isolated from WT or H2-$Ab1^{-/-}$ B6 mice. Cell divisions of H-$2K^{d+}$ donor $CD4^+$ T cells in the spleens 6 days after transfer are shown. Data are representative of 2 similar experiments.

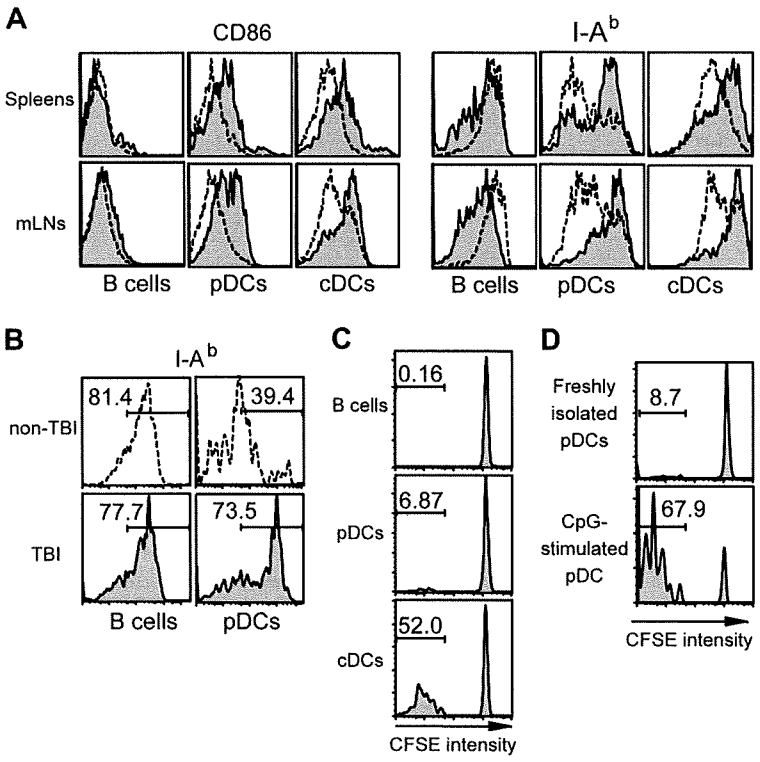

Figure 6. Host irradiation is prerequisite for pDC maturation to prime T cells. (A) Expression of CD86 and I-A^b expression on B cells, pDCs, and cDCs in the spleen and mLNs isolated from irradiated mice 10 hours after irradiation (filled histograms) and from unirradiated control mice (broken-lined open histograms). (B) Unirradiated (top) or irradiated (bottom) B6-Ly5a (CD45.1$^+$) mice were injected with 80 × 10^6 BM cells and splenocytes isolated from FL-treated B6 (CD45.2$^+$) mice. Expression of I-A^b on CD45.2$^+$ B cells and pDCs in mLNs 10 hours after injection is shown. (C) Aliquots of 20 × 10^6 CFSE-labeled BALB/c (H-2K^{d+}) CD4$^+$ T cells were transferred to unirradiated H2-Ab1$^{-/-}$ mice preinjected with 6 × 10^6 WT pDCs or cDCs. Cell division of donor CD4$^+$ T cells in the spleens on day +6 is shown. (D) Similarly, CFSE-labeled BALB/c CD4$^+$ T cells were transferred to unirradiated H2-Ab1$^{-/-}$ mice preinjected with freshly isolated pDCs or pDCs stimulated with CpG 1668 1 μM for 24 hours in vitro. Cell division of donor CD4$^+$ T cells in the spleens on day +6 is shown. Data are representative of 2 similar experiments.

preinjected with WT B cells, pDCs, or cDCs. Donor CD4$^+$ T cells were significantly proliferated in mice preinjected with cDCs 6 days after transfer (Figure 6C), although this cell division appeared to be less potent compared with that in irradiated animals. In contrast, few cell divisions were observed in mice preinjected with B cells or pDCs. We next evaluated whether maturation of pDCs was critical for T-cell activation. Isolated pDCs were cultured with CpG 1668 for 24 hours and injected into unirradiated H2-Ab1$^{-/-}$ mice, followed by the transfer of CFSE-labeled BALB/c CD4$^+$ T cells. CpG-stimulated pDCs stimulate proliferation of donor T cells even in unirradiated mice (Figure 6D).

TLR signaling is not required for pDCs to prime alloreactive T cells

Stimulation with TLR ligands is crucial for the maturation and activation of pDCs.[7,14] We, therefore, hypothesized that maturation of pDCs after TBI is mediated by TLR engagement. To test this hypothesis, we used TRIF/MyD88 DKO mice, where the TLR-dependent signaling pathway was critically abolished.[29] pDCs isolated from TRIF/MyD88 DKO mice were phenotypically identical to WT pDCs (data not shown) but did not respond to CpG or LPS stimulation (Figure 7A), as has been shown.[29] However, TRIF/MyD88 DKO cDCs and pDCs were capable of stimulating donor CD4$^+$ T-cell division in irradiated H2-Ab1$^{-/-}$ mice 6 days after transfer (Figure 7B) and induced significant pathologic GVHD (Figure 7C) as effectively as WT cDCs and pDCs. Thus, TLR signaling is not required for pDCs and cDCs to prime alloreactive T cells.

Discussion

GVHD is initiated by the interaction of alloreactive donor T cells and host DCs.[25,28] Our GVHD model system using MHC-deficient mice presents a stringent test on the allostimulatory functions of a subpopulation of APCs.[28] Using this model system with modification, we addressed whether pDCs are pathogenic in GVHD. We found that alloantigen expression on pDCs alone sufficiently stimulates donor T cells to trigger GVHD in the absence of other APC subsets. Induction of GVHD required cognate interaction of pDCs and T cells, since MHC class II expression on pDCs was absolutely required for stimulating donor CD4$^+$ T cells. It has been suggested that pDCs are involved in the pathogenesis of SLE and psoriasis through IFN-α production[23,24]; however, to our knowledge, our study is the first to directly demonstrate in vivo pathogenic role of pDCs as APCs in an antigen-specific T cell–mediated disease in the absence of other DC subsets.

Our results are in sharp contrast to previous reports suggesting that pDCs mediate tolerance in vivo. In cancer patients, pDCs are incapable of inducing antitumor immune responses but, instead, may induce regulatory T cells that inhibit immunity.[16,17] In animal model of asthma, pDCs in the lung prevent asthmatic reactions to harmless inhaled antigens.[15] In experimental cardiac transplantation, pDCs mediate tolerance and prolong survival of allografts.[11,18,19] It has been suggested that pDCs functioning as APCs generate suppressive or regulatory T cells that mediate tolerance.[12,13,16-18] In contrast, our study showed that alloantigen-presenting pDCs were incapable of suppressing activation of alloreactive T cells but, instead, induced immunity even in the absence of other APC subsets. Differences between previous studies and our study may be attributed to pretransplantation irradiation essential for performing HSCT, which was used in our study.

We have shown that TBI is critical for pDC maturation to prime T cells; TBI up-regulates expression of MHC and costimulatory molecules on pDCs. It has been shown that TBI induces phenotypic and functional maturation of cDCs.[27] Our results

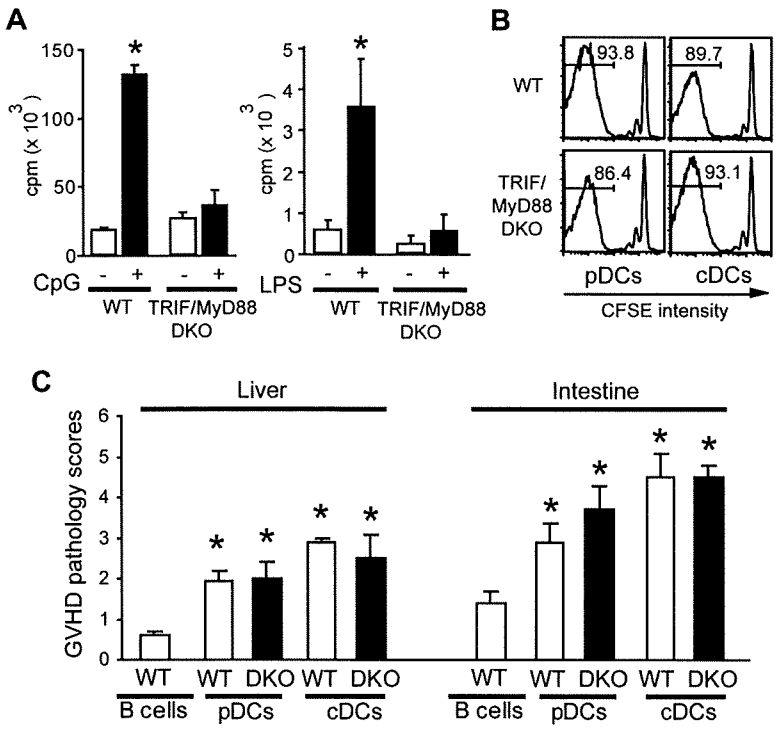

Figure 7. TLR signaling is not required for pDCs to prime alloreactive T cells. (A) A total of 2×10^5 BALB/c $CD4^+$ T cells were cultured with 10^4 WT or TRIF/MyD88 DKO (DKO) pDCs with or without CpG 1668 1 μM (left) or LPS 10 μg/mL (right) to determine cell proliferation. Data are shown as mean (± SD). (B,C) CFSE-labeled BALB/c $CD4^+$ T cells ($H\text{-}2K^d$) were transferred to irradiated $H2\text{-}Ab1^{-/-}$ mice preinjected with pDCs or cDCs isolated from WT or DKO B6 mice. Cell divisions of $H\text{-}2K^{d+}$ donor $CD4^+$ cells in the spleens (B) and GVHD pathology scores in the liver and intestine (C) are shown. Data from 3 similar experiments are combined and shown as means plus or minus SEM (n = 7). $^*P < .05$.

confirm and extend these findings. TBI also maturates pDCs, and this process is dependent on a TBI-mediated inflammatory environment. Although pDCs need to be in a certain state of maturation to prime naive T cells, cDCs can stimulate donor T cells even in unirradiated mice. Thus, the capacity of pDCs to prime naive T cells is far less efficient than cDCs in an uninflamed environment. Host pDCs may not be responsible for the induction of transfusion-associated GVHD in humans.[43,44]

It has been shown that TBI plays an important role in the pathogenesis of GVHD; GVHD is less severe in recipients that have had gentle rather than intensive preconditioning treatments.[45,46] TBI activates and damages host tissue to secrete endogenous factors, such as heat-shock proteins and proinflammatory cytokines like tumor necrosis factor-α and IL-1. TBI-mediated gut injury allows the translocation of exogenous microbial products, such as LPS.[27,45,47,48] DC maturation can be triggered by such endogenous and exogenous "danger signals" as well as by activated T cells, NK cells, and NKT cells.[1,3,4,49] Since many of these stimuli bind to TLRs, we hypothesized that TLR signaling is required for pDC maturation. However, pDCs from TRIF/MyD88 DKO mice with defective TLR signaling[29] were activated by TBI and activate donor T cells to cause GVHD as potently as WT pDCs. Thus, pDCs can be matured by pathways other than TLR signaling, such as nucleotide-binding oligomerization domain-like receptors, retinoic acid–inducible gene I, melanoma differentiation-associated gene 5, DNA-dependent activator of interferon-regulatory factors, C-type lectin receptors, CD40 ligands, and cytokine and chemokine receptors.[49,50]

Immature cDCs also play a role in maintaining tolerance, and thus, irrespective of the subset of DCs, DC maturation may be a control checkpoint in the initiation of immunity.[3,51] Although this simple concept has been revised by several reports showing that mature cDCs and pDCs can induce regulatory T-cell responses,[12,13] our results indicate that pDCs mature and acquire APC function to induce antigen-specific immunity in the inflamed tissue. Thus, functional outcomes of pDC–T-cell interactions depend on the immunologic context of encounter.

It is important to note that pDCs mediate clinically and histologically standard GVHD that was not indistinguishable from cDC-mediated GVHD. Thus, our results further extend the current paradigm that host DCs play a critical role in initiating GVHD.[25,28] Inactivation of host DCs can be a novel strategy to prevent GVHD.[25] Our results suggest that immunologic elimination of cDCs may not be sufficient for complete prevention of GVHD, thus providing important information for developing strategies aimed at inactivating host DCs to prevent GVHD.

Acknowledgment

This study was supported by research funds from the Ministry of Education, Culture, Sports, Science, and Technology grant 2659153 (T.T.), Health and Labor Science Research grants (T.T.), and the Mitsubishi Pharma Research Foundation (Tokyo, Japan; T.T.).

Authorship

Contribution: M.K. conducted research and wrote the paper; D.H., K.A., K.M., K.K., and H.N. conducted research; M.H., M.T., and K.A. designed the study; and T.T. designed the study and organized the data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol. 2002;2:151-161.
2. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature. 2007;449:419-426.
3. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nat Immunol. 2004;5: 1219-1226.
4. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.
5. Asselin-Paturel C, Boonstra A, Dalod M, et al. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol. 2001;2:1144-1150.
6. Bjorck P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. Blood. 2001;98:3520-3526.
7. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat Immunol. 2000;1:305-310.
8. Nakano H, Yanagita M, Gunn MD. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. J Exp Med. 2001;194: 1171-1178.
9. Krug A, Veeraswamy R, Pekosz A, et al. Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T helper 1 differentiation of antigen-experienced unpolarized T cells. J Exp Med. 2003;197:899-906.
10. Schlecht G, Garcia S, Escriou N, Freitas AA, Leclerc C, Dadaglio G. Murine plasmacytoid dendritic cells induce effector/memory CD8+ T-cell responses in vivo after viral stimulation. Blood. 2004;104:1808-1815.
11. Abe M, Wang Z, de Creus A, Thomson AW. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. Am J Transplant. 2005;5:1808-1819.
12. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med. 2002;195: 695-704.
13. Moseman EA, Liang X, Dawson AJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. J Immunol. 2004;173:4433-4442.
14. Salio M, Palmowski MJ, Atzberger A, Hermans IF, Cerundolo V. CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T-cell responses to endogenous but not exogenous antigens. J Exp Med. 2004; 199:567-579.
15. de Heer HJ, Hammad H, Soullie T, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. J Exp Med. 2004;200:89-98.
16. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest. 2004;114:280-290.
17. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. Nat Med. 2001;7:1339-1346.
18. Ochando JC, Homma C, Yang Y, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nat Immunol. 2006;7:652-662.
19. Bjorck P, Coates PT, Wang Z, Duncan FJ, Thomson AW. Promotion of long-term heart allograft survival by combination of mobilized donor plasmacytoid dendritic cells and anti-CD154 monoclonal antibody. J Heart Lung Transplant. 2005;24:1118-1120.
20. Mazariegos GV, Zahorchak AF, Reyes J, et al. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. Am J Transplant. 2003;3:689-696.
21. Rajasekar R, Mathews V, Lakshmi KM, et al. Plasmacytoid dendritic cell count on day 28 in HLA-matched related allogeneic peripheral blood stem cell transplant predicts the incidence of acute and chronic GVHD. Biol Blood Marrow Transplant. 2008;14:344-350.
22. Waller EK, Rosenthal H, Jones TW, et al. Larger numbers of CD4(bright) dendritic cells in donor bone marrow are associated with increased relapse after allogeneic bone marrow transplantation. Blood. 2001;97:2948-2956.
23. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science. 2001;294:1540-1543.
24. Nestle FO, Conrad C, Tun-Kyi A, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med. 2005; 202:135-143.
25. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science. 1999;285:412-415.
26. Teshima T, Ordemann R, Reddy P, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med. 2002;8:575-581.
27. Zhang Y, Louboutin JP, Zhu J, Rivera AJ, Emerson SG. Preterminal host dendritic cells in irradiated mice prime CD8+ T-cell–mediated acute graft-versus-host disease. J Clin Invest. 2002;109:1335-1344.
28. Duffner UA, Maeda Y, Cooke KR, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol. 2004;172: 7393-7398.
29. Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003;301: 640-643.
30. Teshima T, Reddy P, Lowler KP, et al. Flt3 ligand therapy for recipients of allogeneic bone marrow transplants expands host CD8 alpha(+) dendritic cells and reduces experimental acute graft-versus-host disease. Blood. 2002;99:1825-1832.
31. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood. 1996;88: 3230-3239.
32. Hill GR, Cooke KR, Teshima T, et al. Interleukin-11 promotes T-cell polarization and prevents acute graft-versus-host disease after allogeneic bone marrow transplantation. J Clin Invest. 1998; 102:115-123.
33. Hashimoto D, Asakura S, Miyake S, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. J Immunol. 2005;174:551-556.
34. Hashimoto D, Asakura S, Matsuoka K, et al. FTY720 enhances the activation-induced apoptosis of donor T cells and modulates graft-versus-host disease. Eur J Immunol. 2007;37:271-281.
35. Lou Y, Liu C, Kim GJ, Liu YJ, Hwu P, Wang G. Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. J Immunol. 2007;178:1534-1541.
36. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: Multiple dendritic cell subpopulations identified. J Exp Med. 1996;184:1953-1962.
37. Chan CW, Crafton E, Fan HN, et al. Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. Nat Med. 2006;12:207-213.
38. Pelayo R, Hirose J, Huang J, et al. Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow. Blood. 2005;105:4407-4415.
39. Gilliet M, Boonstra A, Paturel C, et al. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med. 2002;195:953-958.
40. Mittelbrunn M, Martinez Del Hoyo G, Lopez-Bravo M, et al. Imaging of plasmacytoid dendritic cell interactions with T cells. Blood. 2009;113:75-84.
41. Zuniga EI, McGavern DB, Pruneda-Paz JL, Teng C, Oldstone MB. Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. Nat Immunol. 2004;5: 1227-1234.
42. Jones SC, Murphy GF, Friedman TM, Korngold R. Importance of minor histocompatibility antigen expression by nonhematopoietic tissues in a CD4+ T-cell–mediated graft-versus-host disease model. J Clin Invest. 2003;112:1880-1886.
43. Via CS, Sharrow SO, Shearer GM. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-vs-host disease. J Immunol. 1987;139:1840-1849.
44. Hathaway WE, Githens JH, Blackburn WR, Fulginiti V, Kempe CH. Aplastic anemia, histiocytosis and erythrodermia in immunologically deficient children. Probable human runt disease. N Engl J Med. 1965;273:953-958.
45. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood. 1997;90:3204-3213.
46. Clift RA, Buckner CD, Appelbaum FR, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: A randomized trial of two irradiation regimens. Blood. 1990;76:1867-1871.
47. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. Int J Hematol. 2003;78:181-187.
48. Paulos CM, Wrzesinski C, Kaiser A, et al. Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling. J Clin Invest. 2007; 117:2197-2204.
49. Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol. 2007;7:543-555.
50. Sansonetti PJ. The innate signaling of dangers and the dangers of innate signaling. Nat Immunol. 2006;7:1237-1242.
51. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med. 2000;191: 411-416.

Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages

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(Received August 14, 2008/Revised November 4, 2008/Accepted November 9, 2008/Online publication December 18, 2008)

Human T-cell leukemia virus type-1 (HTLV-1)-specific T-cell immunity, a potential antitumor surveillance system *in vivo*, is impaired in adult T-cell leukemia (ATL). In this study, we aimed to clarify whether the T-cell insufficiency in ATL is present before the disease onset or occurs as a consequence of the disease. We investigated T-cell responses against Tax protein in peripheral blood mononuclear cells (PBMCs) from individuals at earlier stages of HTLV-1-infection, including 21 asymptomatic HTLV-1 carriers (ACs) and four patients with smoldering-type ATL (sATL), whose peripheral lymphocyte count was in normal range. About 30% of samples tested showed clear Tax-specific interferon (IFN)-γ producing responses. Proviral loads in this group were significantly lower than those in the other less-specific response group. The latter group was further divided to two subgroups with or without emergence of Tax-specific responses following depletion of CC chemokine receptor 4 (CCR4)$^+$ cells that contained HTLV-1-infected cells. In the PBMCs with Tax-specific responses, CD8$^+$ cells efficiently suppressed HTLV-1 p19 production in culture. The remaining group without the emergence of Tax-specific response after CCR4$^+$ cell-depletion included at least two sATL and one AC samples, which spontaneously produced HTLV-1 p19 in culture, where tetramer-binding, Tax-specific cytotoxic T-lymphocytes were either undetectable or unresponsive. Our results indicated that HTLV-1-specific T-cell responsiveness widely differed among HTLV-1 carriers, and that impairment of HTLV-1-specific T-cell responses was observed not only in advanced ATL patients but also in a subpopulation at earlier stages, which was associated with insufficient control of HTLV-1. (*Cancer Sci* 2009; 100: 481–489)

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL).[1,2] Although the majority of HTLV-1-infected individuals remain asymptomatic throughout their lives, about 5% develop ATL during or after middle age and another small population develops HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and a variety of chronic inflammatory diseases.[3–7] Several epidemiological risk factors have been suggested to be associated with ATL development, including vertical transmission, gender (greater incidence in males than in females),[4,8] and increased numbers of abnormal lymphocytes associated with elevated HTLV-1 proviral loads.[9,10] However, elevation in HTLV-1 proviral loads is also a feature of HAM/TSP patients.[7,11]

ATL is known to be an immunosuppressive condition.[3] Recent reports have shown that ATL cells frequently express Foxp3 and the chemokine receptor CCR4, in addition to CD4 and CD25.[12–16] These molecules are also expressed in regulatory T-cells (Tregs).[17–20] Although isolated ATL cells do not always exhibit suppressive functions *in vitro*, the common phenotypes shared by ATL cells and Tregs suggest that ATL cells may share a common origin with Tregs, or possess immunoregulatory properties.[21] General immunosuppression may be present not only in ATL patients, but also in asymptomatic HTLV-1 carriers (ACs) to some extent.[22,23]

There is a clear difference between ATL and HAM/TSP patients in the host T-cell responses against HTLV-1. Outgrowth of CD8$^+$ HTLV-1-specific cytotoxic T-lymphocytes (CTLs) in response to *in vitro* stimulation is frequently found in peripheral blood mononuclear cell (PBMC) cultures from HAM/TSP patients, but rarely observed in those from ATL patients.[24–26] These CTLs have anti-HTLV-1 effects, as elimination of CD8$^+$ cells among PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture.[27,28] HTLV-1 Tax-specific CTL responses are strongly activated in some ATL patients who obtained complete remission after hematopoietic stem cell transplantation (HSCT), but are not observed in the same patients before transplantation.[29] These findings suggest that Tax-specific CTLs may play a role in immunosurveillance for HTLV-1 leukemogenesis.

Studies on a rat model have indicated that the otherwise-elevated proviral loads in orally HTLV-1-infected rats could be reduced by restoration of HTLV-1-specific T-cell responses.[30,31] Furthermore, DNA vaccines or peptide vaccines targeting Tax, the major target antigen recognized by HTLV-1-specific T-cells, can induce antitumor immunity and eradicate HTLV-1-infected lymphomas.[32,33] These observations imply that antitumor therapeutic vaccines targeting Tax might be promising.

It is important to clarify the immunological status of ACs, since insufficiency in host T-cell responses against HTLV-1 could be an immunological risk factor for ATL. HTLV-1-specific CTL responses are also detectable in ACs.[34,35] However, because a wide survey for HTLV-1-specific T-cell immunity has never been carried out, the questions of the proportion of ACs with proper levels of immune responses and the possible existence of a population of ACs with insufficient anti-HTLV-1 responses before ATL onset, remain unresolved. One reason for the poor status of such immunological surveys among ACs is the absence of simple methods for measuring HTLV-1-specific T-cell responses, as they are restricted by individual human leukocyte antigens (HLAs).

We recently established a detection system for HTLV-1-specific T-cell responses using recombinant Tax proteins fused to

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doi: 10.1111/j.1349-7006.2008.01054.x

glutathione-*S*-transferase (GST), in which Tax antigens are processed by antigen-presenting cells and capable of stimulating both $CD4^+$ and $CD8^+$ T-cells among self PBMCs.[36] In this study, by using this assay, we analyzed HTLV-1-specific T-cell responses in unselected ACs and smoldering-type ATL (sATL) patients. We examined sATL samples together because their peripheral lymphocyte numbers are in the normal range (< 4000/μL) and the prognoses vary among individuals.[37,38] Here, we demonstrated wide diversity in T-cell response patterns against HTLV-1 in ACs and sATL patients. Among them, we found some individuals exhibiting impaired Tax-specific T-cell responses associated with poor control of HTLV-1 both in ACs and sATL patients.

Materials and Methods

Subjects. A total of 21 ACs, five HAM/TSP patients, four sATL patients, two chronic-type ATL (cATL) patients, and two acute ATL patients in long-term remission (>2 and >5 years) after allogeneic HSCT donated peripheral blood samples after providing written informed consent. PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare UK, Buckinghamshire, UK) density gradient centrifugation, and either used immediately or stored frozen in liquid nitrogen in Bambanker stock solution (NIPPON Genetics Co., Tokyo, Japan).

Separation of PBMC fractions. $CD4^+$ or $CD8^+$ cells were depleted from PBMCs by negative selection using 10-fold numbers of Dynabeads M-450 CD4 or CD8 (Dynal Biotec, Oslo, Norway), respectively, according to the manufacturer's instructions. $CCR4^+$ cells were depleted from PBMCs using Dynabeads goat antimouse IgG (Dynal Biotec) following incubation with carboxyfluorescein-conjugated anti-CCR4 monoclonal antibody (mAb) for 45 min at 4°C. The resulting contamination by $CD4^+$, $CD8^+$, or $CCR4^+$ cells was between 0.02% and 3.90% of the total lymphocytes, as analyzed by flow cytometry. The PBMC concentrations were adjusted to 1×10^6 cells/mL before depletion, and the resulting $CD4^+$, $CD8^+$, or $CCR4^+$ cell-depleted fractions were resuspended in medium with the same initial volume, irrespective of the remaining cell numbers.

Recombinant proteins and peptides. GST-fusion proteins of HTLV-1 Tax-A, Tax-B, and Tax-C (corresponding to the N-terminal, central, and C-terminal regions of HTLV-1 Tax, respectively) were prepared as described previously.[36] Briefly, partially overlapping DNA fragments designated Tax-A, Tax-B, and Tax-C were inserted into pGEX-2T (GE Healthcare UK) to express the corresponding proteins fused to GST. DH5α competent cells were transformed with these plasmids, and cultured in 2xYT medium supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) for protein expression. Individual GST–Tax proteins in inclusion bodies were extracted by sonication and purified using Glutathione Sepharose 4B affinity columns (GE Healthcare UK), followed by size exclusion gel chromatography. The purified proteins were stored at –80°C. The concentrations used were 12.5 μg/mL for GST and 18.75 μg/mL for a mixture of GST–Tax A, B, and C proteins (6.25 μg/mL for each protein). In some experiments, a synthetic peptide corresponding to Tax 301-309 (SFHSLHLLF) and Tax 88-96 (KVLTPPITH), representing the major CTL epitopes restricted by HLA-A24 and A11, respectively, was used as an antigen at 10 μM in PBMC cultures.[29,39]

Assay for T-cell responses. Whole PBMCs (2×10^5 cells/well) or various cell-depleted PBMC fractions starting from the same number of whole PBMCs were incubated with various antigens in 96-well round-bottom culture plates in duplicate wells. The culture medium was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mg/mL of sodium bicarbonate. To avoid the potential influence of endotoxin contamination of the recombinant proteins, 10 μg/mL of polymyxin B was added to all assays. After 4 days of culture, the supernatants were harvested and stored at –20°C until analysis. The concentrations of interferon (IFN)-γ in the supernatants were measured using a Human IFN-γ ELISA Kit (BioSource, Camarillo, CA, USA) or OptEIA Human IFN-γ ELISA Set (BD Biosciences). The absorbances at 450 nm were measured using a microplate reader and analyzed with the Microplate Manager III software (Bio-Rad Laboratories). In some experiments, a Human Th1/Th2 Cytokine Kit for a Cytokine Beads Assay (CBA) (BD Biosciences) was used to measure various cytokines, including IFN-γ.

Flow cytometry. For cell surface phenotyping, phycoerythrin (PE)-Cy5-conjugated anti-CD4 and PE-Cy5-conjugated anti-CD8 mAbs, carboxyfluorescein-conjugated anti-CCR4 mAb, and appropriate isotype controls were used. Uncultured PBMCs were incubated with these mAbs individually or in combination for 30 min at 4°C, before being washed in phosphate-buffered saline (PBS) containing 1% FBS and fixed with 1% formaldehyde in PBS. For tetramer staining, PBMCs were stained with PE-Cy5-conjugated anti-CD8 mAb for 30 min at 4°C, and then with PE-conjugated HLA-A*1101/Tax88-96, HLA-A*1101/Tax272–280, or HLA-A*2402/Tax301-309 tetramers (National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University Vaccine Center, Atlanta, GA, USA) for 45 min at 4°C.[29,39] The samples were analyzed using a FACSCalibur and the CellQuest software (BD Biosciences).

HTLV-1 antibody titer. The titers of HTLV-1-specific antibodies in the plasma samples were determined by the particle agglutination method by using Serodia HTLV-1 (FUJIREBIO, Tokyo, Japan) according to the manufacturer's instructions.

HTLV-1 proviral loads. HTLV-1 proviral loads in PBMCs were measured by quantitative real-time polymerase chain reaction (PCR) with HTLV-1 Tax-specific primers through the clinical diagnostic services of SRL Inc. (Tokyo, Japan) or the Group of Joint Study on Predisposing Factors of ATL Development (JSPFAD, Japan) as described previously.[40,41] Proviral DNA copy numbers in various fractions of PBMC samples were measured by SYBR Green quantitative real-time PCR methods using Tax-specific primers (forward: 5′-cggatacccagtctacgtgtttggagactgt-3′, reverse: 5′-gagccgataacgcgtccatcgatggggtcc-3′) and control beta-globin primers (forward: 5′-acacaactgtgttcactagc-3′, reverse: 5′-caacttcatccacgttcacc-3′).

Statistical analysis. The Mann–Whitney *U*-test was used to examine the statistical difference in HTLV-1 proviral loads between two groups by using the Graphpad Prism 4 (Graphpad Software). *P*-values <0.05 were considered significant.

Results

Detection of different patterns of Tax-specific T-cell responses in various diseases associated with HTLV-1 infection. In order to obtain typical patterns of T-cell responses detected by the Tax protein-based assay, we examined PBMCs from HTLV-1-infected patients with various clinical conditions (Fig. 1a). Two ATL patients (#37, #253) who had been in long-term complete remission after HSCT showed clear Tax-specific IFN-γ production, only against GST–Tax protein but not against control GST protein. PBMCs from HAM/TSP patients (#254, #259) produced high levels of IFN-γ in response to GST–Tax, but also in the presence of medium alone or the control GST. In contrast, PBMCs from two cATL patients (#227, #249) showed very weak responses to any stimulation. Although the PBMCs from uninfected individuals showed low levels of background responses that might involve macrophages or natural killer cells, the levels of IFN-γ production in cATL samples were even lower than the background responses.

HTLV-1-infected cells have been reported to express the chemokine receptor CCR4 frequently.[13] As shown in Fig. 1(b),

doi: 10.1111/j.1349-7006.2008.01054.x