38) Kondo, E., Akatsuka, Y., Nawa, A., Kuzushima, K., Tsujimura, K., Tanimoto, M., Kodera, Y., Morishima, Y., Kuzuya, K., Takahashi, T.: Retroviral vector backbone immunogenicity: identification of cytotoxic T-cell epitopes in retroviral vector-packaging sequences. ***Gene Ther.*** 12(3):252-258, 2005.

39) Tajima, K., Ito, Y., Demachi, A., Nishida, K., Akatsuka, Y., Tsujimura, K., Hida, T., Morishima, Y., Kuwano, H., Mitsudomi, T., Takahashi, T., Kuzushima, K.: Interferon-γ differentially regulates susceptibility of lung cancer cells to telomerase-specific cytotoxic T lymphocytes. ***Int J Cancer.*** 110(3): 403-412, 2004.

40) Tajima, K., Demachi, A., Ito, Y., Nishida, K., Akatsuka, Y., Tsujimura, K., Kuwano, H., Mitsudomi, T., Takahashi, T., Kuzushima, K. Identification of an epitope from the epithelial cell adhesion molecule eliciting HLA-A*2402-restricted cytotoxic T-lymphocyte responses. ***Tissue Antigens.*** 64(6): 650-659, 2004.

41) Gondo, H., Himeji, D., Kamezaki, K., Numata, A., Tanimoto, T., Takase, K., Aoki, K., Henzan, H., Nagafuji, K., Miyamoto, T., Ishikawa, F., Shimoda, K., Inaba, S., Tsukamoto, H., Horiuchi, T., Nakashima, H., Otsuka, T., Kato, K., Kuroiwa, M., Higuchi, M., Shibuya, T., Kamimura, T., Kuzushima, K., Tsurumi, T., Kanda, Y., Harada, M.: Reconstitution of HLA-A*2402-restricted cytomegalovirus-specific T-cells following stem cell transplantation. ***Int J Hematol.*** 80(5):441-448, 2004.

42) Torikai, H., Akatsuka, Y., Miyazaki, M., Warren, E.H. 3rd., Oba, T., Tsujimura, K., Motoyoshi, K., Morishima, Y., Kodera, Y., Kuzushima, K., Takahashi, T.: A novel HLA-A*3303-restricted minor histocompatibility antigen encoded by an unconventional open reading frame of human TMSB4Y gene. ***J Immunol.*** 173(11): 7046-7054, 2004.

43) Azuma, T., Otsuki, T., Kuzushima, K., Froelich, C.J., Fujita, S., Yasukawa, M.: Myeloma cells are highly sensitive to the granule exocytosis pathway mediated by WT1-specific cytotoxic T lymphocytes. ***Clin Cancer Res.*** 10(21):7402-7412, 2004.

44) Ishida, T., Iida, S., Akatsuka, Y., Ishii, T., Miyazaki, M., Komatsu, H., Inagaki, H., Okada, N., Fujita, T., Shitara, K., Akinaga, S., Takahashi, T., Utsunomiya, A., Ueda, R.: The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-Cell leukemia/ lymphoma. ***Clin Cancer Res.*** 10(22): 7529-7539, 2004.

45) Storek, J., Zhao, Z., Lin, E., Berger, T., McSweeney, PA., Nash, RA., Akatsuka, Y., Metcalf, M.D., Lu, H., Kalina, T., Reindl, M., Storb, R., Hansen, J.A., Sullivan, K.M., Kraft, G.H., Furst, D.E., Maloney, D.G.: Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases). ***Clin Immunol.*** 113: 285-298, 2004.

46) Watanabe, N., Kamachi, Y., Koyama, N., Hama, A., Liang, J., Nakamura, Y., Yamamoto, T., Isomura, M., Kudo, K., Kuzushima, K., Kojima, S.: Expansion of human CMV-specific cytotoxic T lymphocytes to a clinical scale: a simple culture system using tetrameric HLA-peptide complexes. ***Cytotherapy.*** 6(5):514-522, 2004.

47) Akiyama, Y., Kuzushima, K., Tsurumi, T., Yamaguchi, K.: Analysis of HLA-A24-restricted CMVpp65 peptide-specific CTL with HLA-A*2402-CMVpp65 tetramer. ***Immunol Lett.*** 95(2):199-205, 2004.

48) Takami, A., Sugimori, C., Feng, X., Yachie, A., Kondo, Y., Nishimura, R., Kuzushima, K.,

Kotani, T., Asakura, H., Shiobara, S., Nakao, S.: Expansion and activation of minor histocompatibility antigen HY-specific T cells associated with graft-versus-leukemia response. ***Bone Marrow Transplant.*** 34(8):703-709, 2004.

49) Sugaya, N., Kimura, H., Hara, S., Hoshino, Y., Kojima, S., Morishima, T., Tsurumi, T., Kuzushima, K.: Quantitative analysis of Epstein-Barr virus (EBV)-specific $CD8^+$ T cells in patients with chronic active EBV infection. ***J Infect Dis.*** 190(5):985-988, 2004.

50) Akazawa, T., Masuda, H., Saeki, Y., Matsumoto, M., Takeda, K., Tsujimura, K., Kuzushima, K., Takahashi, T., Azuma, I., Akira, S., Toyoshima, K., Seya, T. : Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice. ***Cancer Res.*** 64(2):757-764, 2004.

51) Tsujimura, K., Obata, Y., Matsudaira, Y., Ozeki, S., Taguchi, O., Nishida, K., Okanami, Y., Akatsuka, Y., Kuzushima, K., Takahashi T.: Immunity against mouse thymus-leukemia antigen (TL) protects against development of lymphomas induced by a chemical carcinogen, N-butyl-N-nitrosourea. ***Cancer Sci.*** 95(11): 914-919, 2004.

52) Nishida, T., Akatsuka, Y., Morishima, Y., Hamajima, N., Tsujimura, K., Kuzushima, K., Kodera, Y., Takahashi T. : Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant. ***Br. J Haematol.*** 124(5):629-635, 2004.

53) Kondo, E., Akatsuka, Y., Kuzushima, K., Tsujimura, K., Asakura, S., Tajima, K., Kagami, Y., Kodera, Y., Tanimoto, M., Morishima, Y., Takahashi, T.: Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. ***Blood,*** 103(2): 630-638, 2004.

54) Li, S., Morishima, Y.: Association of polymorphic MHC microsatellites with GVHD, survival, and leukemia relapse in unrelated hematopoietic stem cell transplant donor/recipient pairs matched at five HLA loci. ***Tissue Antigens***. 63(4):362-8, 2004.

55) Iida, H., Morishima, Y.: Twenty years' experience in allogeneic hematopoietic stem cell transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia in the Nagoya Blood and Marrow Transplantation Group. ***Int J Hematol.*** 79(1):79-84, 2004.

56) Izutsu, K., Morishima, Y.: Japan Marrow Donor Program. Unrelated bone marrow transplantation for non-Hodgkin lymphoma: a study from the Japan Marrow Donor Program. ***Blood.*** 103(5):1955-60, 2004.

57) Morishima, Y.: Efficacy and Safety of Imatinib Mesylate for Patients in the First Chronic Phase of Chronic Myeloid Leukemia: Results of a Japanese Phase II Clinical Study. ***Int J Hematol*** 80:261-266, 2004.

58) Ogura, M., Morishima, Y.: Durable Response but Prolonged Cytopenia after Cladribine Treatment in Relapsed Patients with Indolent non-Hodgkin's Lymphomas: Results of a Japanese Phase II Study. ***Int J Hematol*** 80: 267-277, 2004

59) Karnan, S., Morishima, Y.: Analysis of chromosomal imbalances in de novo CD5-positive diffuse large-B-cell lymphoma detected by comparative genomic hybridization. ***Genes Chromosomes Cancer.***, 39:77-81, 2004.

60) Tagawa, H., Morishima, Y.: Genome-wide

array-based comparative genomic hybridization of diffuse large B-cell lymphoma: comparison between CD5-positive and CD5-negative cases. ***Cancer Res.*** 64(17):5948-55, 2004.

61) Akatsuka, Y., Morishima, Y.: Major and minor histocompatibility antigens in allogeneic hematopoietic stem cell transplantation. ***Cur. Opin. Organ. Transplant.,*** 9: 64-71, 2004.

62) 葛島清隆. 「EBV特異的CTLの臨床応用」最新医学別冊「新しい診断と治療のABC」、印刷中、最新医学社

63) 葛島清隆. 「がんワクチンの免疫モニタリング」特集・がんワクチン、分子細胞治療 6巻2号 p15-21,2007、先端医学社

64) 鳥飼宏基、赤塚美樹. マイナー抗原を標的とした同種免疫反応とその臨床応用. 血液フロンティア **17**: 319-328, 2007.

65) 赤塚美樹. 造血細胞移植におけるマイナー組織適合抗原の役割. 臨床血液 **47**: 1353-1363, 2006.

66) 葛島清隆. 「EBV感染症に対する細胞療法」日本臨床 64巻増刊号3、ヘルペスウイルス学、p678-681.2006、日本臨床社

67) 葛島清隆. 「テトラマーアッセイ」分子細胞治療 5巻2号 p51-56,2006、先端医学社

2. 学会発表

1) 赤塚美樹. GVLの標的としての造血細胞特異的マイナー適合抗原：第29回日本造血細胞移植学会総会、シンポジウム. 福岡 2007年2月

2) 赤塚美樹. Minor組織適合抗原を標的とした免疫療法：日本輸血学会東海支部会学術集会特別講演、名古屋 2007年1月

3) Kawase T, Akatsuka Y, Torikai H, et al. Alternative Splicing due to an Intronic SNP defines a Novel HLA-B44-Restricted-Hematopoiesis-Restricted Minor Histocompatibility Antigen：第48回米国血液学会総会、Orland、フロリダ 2006年12月

4) Morishima Y. et al. Identification of non-permissive HLA allele mismatch combinations and amino acid substitution responsible for acute graft-versus-host disease in unrelated allogeneic bone marrow transplantation. 48th annual meeting of American Society of Hematology. Dec. 2006. Orland. USA

5) Akatsuka Y, Torikai H, Kawase T, et al. Immunotherapy of hematological malignancies targeting hematopoietic cell-specific minor histocompatibility antigens：第11回アジアパシフィック血液骨髄移植学会、名古屋 2006年11月（ワークショップ、招請）

6) 岡村文子、伊藤嘉規、葛島清隆. EBNA1特異的$CD4^+$T細胞の誘導とその機能解析：ワークショップ「ウイルス宿主防御の攻防」 第54回日本ウイルス学会学術集会、名古屋 2006年11月

7) 鳥飼宏基、赤塚美樹、鬼塚真仁ら. HA-1マイナー組織適合性抗原はHLA-A*0206によっても提示される：第68回日本血液学会総会・第48回日本臨床血液学会総会、福岡 2006年10月

8) 川瀬孝和、赤塚美樹、鳥飼宏基ら. HLA-B*4403拘束性の新規マイナー組織適合性抗原の同定と抗原性発現のメカニズム：第68回日本血液学会総会・第48回日本臨床血液学会総会、福岡 2006年10月

9) 赤塚美樹. マイナー抗原を標的とした移植後再発白血病に対する免疫療法の可能性：第68回日本血液学会総会・第48回日本臨床血液学会総会、シンポジウム. 福岡 2006年10月

10) 川瀬孝和、赤塚美樹、鳥飼宏基他. HLA-B*4403拘束性の新規マイナー組織適合性抗原の同定と抗原性発現の機序：第65回日本癌学会総会、横浜 2006年9月

11) 渡辺一絵、鈴木進、田路真悟、葛島清隆、高橋利忠、赤塚美樹. 抗原特異的CTLの新たな単離方法：第65回日本癌学会総会、横浜 2006年9月

12) 岡村文子、伊藤嘉規、葛島清隆. EBV陽性癌に対するT細胞免疫療法の基盤的研究、第3回EBウイルス研究会　名古屋　2006年6月

13) 森島聡子、赤塚美樹 、中西透ら. 子宮頚癌患者の末梢血におけるHLA-A24拘束性HPV-16 $E6_{49-57}$特異的CTLの誘導：第10回基盤的癌免疫研究会総会、札幌 2006年7月

14) 葛島清隆. ウイルス特異的細胞傷害性T細胞を用いた免疫療法の基盤的研究、分野別シンポジウム２「小児血液・腫瘍難病に対する遺伝子治療・再生治療」：第109回日本小児科学会学術集会、金沢　2006年4月

15) 鳥飼宏基, 赤塚美樹, 宮崎幹則, 辻村朱音, 伊藤嘉規, 辻村邦夫, 元吉和夫, 森島泰雄, 小寺良尚, 葛島清隆, 高橋利忠 HLA-A*3101及び-A*3303拘束性のCTSH遺伝子上の同一多型部位にコードされる2つの新規マイナー組織適合抗原の同定：基盤的癌免疫研究会第９回総会　東京 2005年6月

16) 森島聡子, 赤塚美樹, 那波明弘, 清野透, 鳥飼宏基, 伊藤嘉規, 辻村邦夫, 葛島清隆, 高橋利忠：HPV16型E6上のHLA-A24拘束性CTLエピトープの同定とbortezomib, INF-γの併用効果：基盤的癌免疫研究会第９回総会　東京 2005年6月

H. 知的財産権の出願・登録状況

1) 赤塚美樹、高橋利忠、葛島清隆、森島泰雄.「LOC284293バリアント遺伝子並びに該遺伝子がコードする$CD8^+$細胞傷害性Ｔリンパ球mHAエピトープペプチド及びその用途」番号；特願2006-152098.

2) 葛島清隆、岡村文子、伊藤嘉規、赤塚美樹、森島泰雄.「エプスタインーバールウイルス感染細胞を特異的に攻撃する細胞傷害性T細胞エピトープペプチド及びその用途」出願日；平成18年10月28日、番号；PCT/JP2006/321479.

3) 葛島清隆、岡村文子、伊藤嘉規、赤塚美樹、森島泰雄.「エプスタインーバールウイルス感染細胞を特異的に攻撃する細胞傷害性T細胞エピトープペプチド及びその用途」出願日；平成17年10月28日、番号；特願2005-315306.

4) 葛島清隆.「HLA-A2402拘束性Ep-CAM特異的CTLが認識するエピトープ・ペプチド及びその用途」出願日；平成17年1月19日、番号；PCT/JP2005/587.

5) 赤塚美樹、高橋利忠、葛島清隆、森島泰雄.「Cathepsin H タンパク質由来の$CD8^+$細胞傷害性Tリンパ球mHAエピトープペプチドおよびその用途」：出願日；平成16年11月9日、番号；特願2004-325328.

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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
Ito Y, Demachi-Okamura, A, Ohta R, Akatsuka Y, Nishida K, Tsujimura K, Morishima Y, Takahashi T, Kuzushima K.	Full-length EBNA1-mRNA-transduced dendritic cells stimulate CTLs recognizing a novel HLA-Cw*0303 and Cw*0304-restricted epitope on EBNA1-expressing cells.	***J. Gen. Virol.***	88(Pt 3)	770-80	2007.
Morishima S, Akatsuka Y, Nawa A, Kondo E, Kiyono T, Torikai H, Nakanishi T, Ito Y, Tsujimura K, Iwata K, Ito K, Kodera Y, Morishima Y, Kuzushima K, Takahashi T.	Identification of an HLA-A24-restricted cytotoxic T lymphocyte epitope from human papillomavirus type-16 E6: The combined effects of bortezomib and interferon-gamma on the presentation of a cryptic epitope.	***Int J Cancer***	120	594-604	2007
Morishima Y, Yabe T, Matsuo K, Kashiwase K, Inoko H, Saji H, Yamamoto K, Maruya E, Akatsuka Y, Onizuka M, Sakamaki H, Sao H, Ogawa S, Kato S, Juji T, Sasazuki T, Kodera Y; Japan Marrow Donor Program.	Effects of HLA Allele and Killer Immunoglobulin-Like Receptor Ligand Matching on Clinical Outcome in Leukemia Patients Undergoing Transplantation With T-cell-Replete Marrow From an Unrelated Donor.	***Biol Blood Marrow Transplant.***	13(3)	315-28	2007
Mizuta S, Kohno A, Morishita Y, Atsuta Y, Sao H, Miyamura K, Sakamaki H, Ueda R, Morishima Y.	Long-term Follow-up of 14 Patients with Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia following Autologous Bone Marrow Transplantation in First Complete Remission.	***Int J Hematol.***	85(2)	140-5	2007
Kato K, Kanda Y, Eto T, Muta T, Gondo H, Taniguchi S, Shibuya T, Utsunomiya A, Kawase T, Kato S, Morishima Y, Kodera Y, Harada M; Japan Marrow Donor Program.	Allogeneic bone marrow transplantation from unrelated human T-cell leukemia virus-I-negative donors for adult T-cell leukemia/lymphoma: retrospective analysis of data from the Japan Marrow Donor Program.	***Biol Blood Marrow Transplant.***	13(1)	90-9	2007
Kikuchi T, Naruse TK, Onizuka M, Li S, Kimura T, Oka A, Morishima Y, Kulski JK, Ichimiya S, Sato N, Inoko H.	Mapping of susceptibility and protective loci for acute GVHD in unrelated HLA-matched bone marrow transplantation donors and recipients using 155 microsatellite markers on chromosome 22.	***Immunogenetics***	59(2)	99-108	2007
Inamoto Y, Nishida T, Suzuki R, Miyamura K, Sao H, Iida H, Naoe T, Maruyama F, Hirabayashi N, Hamaguchi M, Iseki T, Kami M, Yano K, Takeyama H, Morishita Y, Morishima Y, Kodera Y.	Significance of additional high-dose cytarabine in combination with cyclophosphamide plus total body irradiation regimen for allogeneic stem cell transplantation.	***Bone Marrow Transplant.***	39(1)	25-30	2007

Demachi-Okamura, A., Ito, Y., Akatsuka, Y., Tsujimura, K., Morishima, Y., Takahashi, T., Kuzushima, K.	Epstein-Barr virus (EBV) latent membrane protein 1-specific cytotoxic T lymphocytes targeting natural killer cell malignancies carrying EBV.	***Eur. J. Immunol.***	36(3)	593-602	2006
Ito, Y., Kondo, E., Demachi-Okamura, A., Akatsuka, Y., Tsujimura, K., Tanimoto, M., Morishima, Y., Takahashi, T., Kuzushima, K.	Three Immunoproteasome-Associated Subunits Cooperatively Generate a CTL Epitope of the EBV LMP2A by Overcoming Specific Structures Resistant to Epitope Liberation.	***J Virol***	80(2)	883-890	2006
Akatsuka Y, Torikai H, Inamoto Y, Tsujimura K, Morishima Y, Kodera Y, Kuzushima K, Takahashi T.	Bone marrow may be a reservoir of long-lived memory T cells specific for minor histocompatibility antigen.	***Br J Haematol***	135(3)	413-414	2006
Torikai H, Akatsuka Y, Miyazaki M, Tsujimura A, Yatabe Y, Kawase T, Nakao Y, Tsujimura K, Motoyoshi K, Morishima Y, Kodera Y, Kuzushima K, Takahashi T.	The human cathepsin H gene encodes two novel minor histocompatibility antigen epitopes restricted by HLA-A*3101 and -A*3303.	***Br J Haematol***	134(4)	406-416	2006
Tsujimura K, Obata Y, Matsudaira Y, Nishida K, Akatsuka Y, Ito Y, Demachi-Okamura A, Kuzushima K, Takahashi T.	Characterization of murine CD160+CD8+T lymphocytes.	***Immunol Lett,***	106(1)	48-56	2006
Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, Kuzushima K, Takiguchi M, Kaneko S.	Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma.	***Hepatology***	43(6)	1284-1294	2006
Morishima Y, Ogura M, Yoneda S, Sakai H, Tobinai K, Nishiwaki Y, Minami H, Hotta T, Ezaki K, Ohe Y, Yokoyama A, Tsuboi M, Mori K, Watanabe K, Ohashi Y, Hirashima K, Saijo N; Japan Erythropoietin Study Group.	Once-weekly epoetin-beta improves hemoglobin levels in cancer patients with chemotherapy-induced anemia: A randomized, double-blind, dose-finding study.	***Jpn J Clin Oncol.***	36(10)	655-61	2006
Asano N, Oshiro A, Matsuo K, Kagami Y, Ishida F, Suzuki R, Kinoshita T, Shimoyama Y, Tamaru J, Yoshino T, Kitamura K, Fukutani H, Morishima Y, Nakamura S.	Prognostic significance of T-cell or cytotoxic molecules phenotype in classical Hodgkin's lymphoma: a clinicopathologic study.	***J Clin Oncol.***	24(28)	4626-33	2006
Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, Bornhauser M, Christiansen F, Gratwohl A, Morishima Y, Oudshoorn M, Ringden O, van Rood JJ, Petersdorf E; International Histocompatibility Working Group.	KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy.	***Biol Blood Marrow Transplant.***	12(8)	828-36	2006
Suguro M, Tagawa H, Kagami Y, Okamoto M, Ohshima K, Shiku H, Morishima Y, Nakamura S, Seto M.	Expression profiling analysis of the CD5+ diffuse large B-cell lymphoma subgroup: development of a CD5 signature.	***Cancer Sci.***	97(9)	868-74	2006

Fukuhara N, Tagawa H, Kameoka Y, Kasugai Y, Karnan S, Kameoka J, Sasaki T, Morishima Y, Nakamura S, Seto M.	Characterization of target genes at the 2p15-16 amplicon in diffuse large B-cell lymphoma.	***Cancer Sci.***	97(6)	499-504	2006
Ogura M, Morishima Y, Kagami Y, Watanabe T, Itoh K, Igarashi T, Hotta T,Kinoshita T, Ohashi Y, Mori S, Terauchi T, Tobinai K.	Randomized phase II study of concurrent and sequential rituximab and CHOP chemotherapy in untreated indolent B-cell lymphoma.	***Cancer Sci.***	97(4)	305-12	2006
Oshiro A, Tagawa H, Ohshima K, Karube K, Uike N, Tashiro Y, Utsunomiya A, Masuda M, Takasu N, Nakamura S, Morishima Y, Seto M.	Identification of subtype-specific genomic alterations in aggressive adult T-cell leukemia/lymphoma.	***Blood.***	107(11)	4500-7.	2006
Terakura S, Murata M, Nishida T, Emi N, Akatsuka Y, Morishima Y, Kodera Y, Naoe T.	Increased risk for treatment-related mortality after bone marrow transplantation in GSTM1-positive recipients.	***Bone Marrow Transplant.***	37(4)	381-386	2006
Ogawa Y, Hotta T, Tobinai K, Watanabe T, Sasaki Y, Minami H, Morishima Y, Ogura M, Seriu T.	Phase I and pharmacokinetic study of oral fludarabine phosphate in relapsed indolent B-cell non-Hodgkin's lymphoma.	***Ann Oncol.***	17(2)	330-333	2006
Imamura M, Asano S, Harada M, Ikeda Y, Kato K, Kato S, Kawa K, Kojima S, Morishima Y, Morishita Y, Nakahata T, Okamura J, Okamoto S, Shiobara S, Tanimoto M, Tsuchida M, Atsuta Y, Yamamoto K, Tanaka J, Hamajima N, Kodera Y.	Current status of hematopoietic cell transplantation for adult patients with hematologic diseases and solid tumors in Japan.	***Int J Hematol.***	83(2)	164-178	2006
Atsuta Y, Suzuki R, Yamamoto K, Terakura S, Iida H, Kohno A, Naoe T, Yano K, Wakita A, Taji H, Hamaguchi M, Kodera Y, Sao H, Morishima Y, Hamajima N, Morishita Y.	Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation.	***Bone Marrow Transplant.***	37(3)	289-296	2006
Terakura S, Murata M, Nishida T, Emi N, Akatsuka Y, Riddell SR, Morishima Y, Kodera Y, Naoe T.	A UGT2B17-positive donor is a risk factor for higher transplant-related mortality and lower survival after bone marrow transplantation.	***Br J Haematol.***	129(2)	221-228	2005
Kasugai Y, Tagawa H, Kameoka Y, Morishima Y, Nakamura S, Seto M.	Identification of CCND3 and BYSL as candidate targets for the 6p21 amplification in diffuse large B-cell lymphoma.	***Clin Cancer Res.***	11(23)	:8265-8272	2005
Shimada K, Yokozawa T, Atsuta Y, Kohno A, Maruyama F, Yano K, Taji H, Kitaori K, Goto S, Iida H, Morishima Y, Kodera Y, Naoe T, Morishita Y.	Solid tumors after hematopoietic stem cell transplantation in Japan: incidence, risk factors and prognosis.	***Bone Marrow Transplant.***	36(2)	115-121	2005
Tagawa H, Suguro M, Tsuzuki S, Matsuo K, Karnan S, Ohshima K, Okamoto M, Morishima Y, Nakamura S, Seto M.	Comparison of genome profiles for identification of distinct subgroups of diffuse large B-cell lymphoma.	***Blood.***	106(5)	1770-1777	2005

Imai Y, Chou T, Tobinai K, Tanosaki R, Morishima Y, Ogura M, Shimazaki C, Taniwaki M, Hiraoka A, Tanimoto M, Koike T, Kogawa K, Hirai H, Yoshida T, Tamura K, Kishi K, Hotta T.	CliniMACS Study Group. Isolation and transplantation of highly purified autologous peripheral CD34+ progenitor cells: purging efficacy, hematopoietic reconstitution in non-Hodgkin's lymphoma (NHL): results of Japanese phase II study.	***Bone Marrow Transplant.***	35(5)	479-487	2005
Tagawa H, Karnan S, Suzuki R, Matsuo K, Zhang X, Ota A, Morishima Y, Nakamura S, Seto M.	Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM.	***Oncogene.***	24(8)	1348-1358.	2005
Tsuji, T., Yasukawa, M., Matsuzaki, J., Ohkuri, T., Chamoto, K., Wakita, D., Azuma, T., Niiya, H., Miyoshi, H., Kuzushima, K., Oka, Y., Sugiyama, H., Ikeda, H., Nishimura, T.	Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes.	***Blood.***	106(2)	470-476	2005
Kimura, H., Hoshino, Y., Hara, S., Sugaya, N., Kawada, J., Shibata, Y., Kojima, S., Nagasaka, T., Kuzushima, K., Morishima, T.	Differences between T Cell-Type and Natural Killer Cell-Type Chronic Active Epstein-Barr Virus Infection.	***J Infect Dis.***	191(4)	531-539	2005
Kondo, E., Akatsuka, Y., Nawa, A., Kuzushima, K., Tsujimura, K., Tanimoto, M., Kodera, Y., Morishima, Y., Kuzuya, K., Takahashi, T.	Retroviral vector backbone immunogenicity: identification of cytotoxic T-cell epitopes in retroviral vector-packaging sequences.	***Gene Ther.***	12(3)	252-258	2005
Tajima, K., Ito, Y., Demachi, A., Nishida, K., Akatsuka, Y., Tsujimura, K., Hida, T., Morishima, Y., Kuwano, H., Mitsudomi, T., Takahashi, T., Kuzushima, K.	Interferon-γ differentially regulates susceptibility of lung cancer cells to telomerase-specific cytotoxic T lymphocytes	***Int J Cancer.***	110(3)	403-412	2004
Tajima, K., Demachi, A., Ito, Y., Nishida, K., Akatsuka, Y., Tsujimura, K., Kuwano, H., Mitsudomi, T., Takahashi, T., Kuzushima, K.	Identification of an epitope from the epithelial cell adhesion molecule eliciting HLA-A*2402-restricted cytotoxic T-lymphocyte responses.	***Tissue Antigens***	64(6)	650-659	2004
Gondo, H., Himeji, D., Kamezaki, K., Numata, A., Tanimoto, T., Takase, K., Aoki, K., Henzan, H., Nagafuji, K., Miyamoto, T., Ishikawa, F., Shimoda, K., Inaba, S., Tsukamoto, H., Horiuchi, T., Nakashima, H., Otsuka, T., Kato, K., Kuroiwa, M., Higuchi, M., Shibuya, T., Kamimura, T., Kuzushima, K., Tsurumi, T., Kanda, Y., Harada, M.	Reconstitution of HLA-A*2402-restricted cytomegalovirus-specific T-cells following stem cell transplantation.	***Int J Hematol.***	80(5)	441-448	2004
Torikai, H., Akatsuka, Y., Miyazaki, M., Warren, E.H. 3rd., Oba, T., Tsujimura, K., Motoyoshi, K., Morishima, Y., Kodera, Y., Kuzushima, K., Takahashi, T.	A novel HLA-A*3303-restricted minor histocompatibility antigen encoded by an unconventional open reading frame of human TMSB4Y gene.	***J Immunol.***	173(11)	7046-7054,	2004.
Azuma, T., Otsuki, T., Kuzushima, K., Froelich, C.J., Fujita, S., Yasukawa, M.	Myeloma cells are highly sensitive to the granule exocytosis pathway mediated by WT1-specific cytotoxic T lymphocytes.	***Clin Cancer Res.***	10(21)	7402-7412	2004.

Ishida, T., Iida, S., Akatsuka, Y., Ishii, T., Miyazaki, M., Komatsu, H., Inagaki, H., Okada, N., Fujita, T., Shitara, K., Akinaga, S., Takahashi, T., Utsunomiya, A., Ueda, R.	The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-Cell leukemia/ lymphoma.	***Clin Cancer Res.***	10(22)	7529-7539	2004
Storek, J., Zhao, Z., Lin, E., Berger, T., McSweeney, PA., Nash, RA., Akatsuka, Y., Metcalf, M.D., Lu, H., Kalina, T., Reindl, M., Storb, R., Hansen, J.A., Sullivan, K.M., Kraft, G.H., Furst, D.E., Maloney, D.G.	Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases).	***Clin Immunol.***	113(3)	285-298	2004
Watanabe, N., Kamachi, Y., Koyama, N., Hama, A., Liang, J., Nakamura, Y., Yamamoto, T., Isomura, M., Kudo, K., Kuzushima, K., Kojima, S.	Expansion of human CMV-specific cytotoxic T lymphocytes to a clinical scale: a simple culture system using tetrameric HLA-peptide complexes.	***Cytotherapy***	6(5)	514-522	2004
Akiyama, Y., Kuzushima, K., Tsurumi, T., Yamaguchi, K.	Analysis of HLA-A24-restricted CMVpp65 peptide-specific CTL with HLA-A*2402-CMVpp65 tetramer.	***Immunol Lett.***	95(2)	199-205	2004
Takami, A., Sugimori, C., Feng, X., Yachie, A., Kondo, Y., Nishimura, R., Kuzushima, K., Kotani, T., Asakura, H., Shiobara, S., Nakao, S.	Expansion and activation of minor histocompatibility antigen HY-specific T cells associated with graft-versus-leukemia response.	***Bone Marrow Transplant..***	34(8)	703-709	2004
Sugaya, N., Kimura, H., Hara, S., Hoshino, Y., Kojima, S., Morishima, T., Tsurumi, T., Kuzushima, K.	Quantitative analysis of Epstein-Barr virus (EBV)-specific CD8+ T cells in patients with chronic active EBV infection.	***J Infect Dis.***	190(5)	985-988	2004
Akazawa, T., Masuda, H., Saeki, Y., Matsumoto, M., Takeda, K., Tsujimura, K., Kuzushima, K., Takahashi, T., Azuma, I., Akira, S., Toyoshima, K., Seya, T.	Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice.	***Cancer Res.***	64(2)	757-764	2004
Tsujimura, K., Obata, Y., Matsudaira, Y., Ozeki, S., Taguchi, O., Nishida, K., Okanami, Y., Akatsuka, Y., Kuzushima, K., Takahashi T.	Immunity against mouse thymus-leukemia antigen (TL) protects against development of lymphomas induced by a chemical carcinogen, N-butyl-N-nitrosourea.	***Cancer Sci.***	95(11)	914-919	2004
Nishida, T., Akatsuka, Y., Morishima, Y., Hamajima, N., Tsujimura, K., Kuzushima, K., Kodera, Y., Takahash,i T.	Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant.	***Br. J Haematol.***	124(5)	629-635	2004
Kondo, E., Akatsuka, Y., Kuzushima, K., Tsujimura, K., Asakura, S., Tajima, K., Kagami, Y., Kodera, Y., Tanimoto, M., Morishima, Y., Takahashi, T.	Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles.	***Blood***	103(2)	630-638	2004

Li, S., Morishima, Y.	Association of polymorphic MHC microsatellites with GVHD, survival, and leukemia relapse in unrelated hematopoietic stem cell transplant donor/recipient pairs matched at five HLA loci.	***Tissue Antigens.***	63(4)	362-8	2004
Iida, H., Morishima, Y.	Twenty years' experience in allogeneic hematopoietic stem cell transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia in the Nagoya Blood and Marrow Transplantation Group.	***Int J Hematol.***	79(1)	79-84	2004
Izutsu, K., Morishima, Y., Japan Marrow Donor Program.	Unrelated bone marrow transplantation for non-Hodgkin lymphoma: a study from the Japan Marrow Donor Program.	***Blood.***	103(5)	1955-60	2004
Morishima, Y	Efficacy and Safety of Imatinib Mesylate for Patients in the First Chronic Phase of Chronic Myeloid Leukemia: Results of a Japanese Phase II Clinical Study.	***Int J Hematol***	80	261-266	2004
Ogura, M., Morishima, Y	Durable Response but Prolonged Cytopenia after Cladribine Treatment in Relapsed Patients with Indolent non-Hodgkin's Lymphomas: Results of a Japanese Phase II Study.	***Int J Hematol***	80	267-277	2004
Karnan, S., Morishima, Y	Analysis of chromosomal imbalances in de novo CD5-positive diffuse large-B-cell lymphoma detected by comparative genomic hybridization.	***Genes Chromosomes Cancer***	39	77-81	2004
Tagawa, H., Morishima, Y	Genome-wide array-based comparative genomic hybridization of diffuse large B-cell lymphoma: comparison between CD5-positive and CD5-negative cases.	***Cancer Res.***	64(17)	5948-55	2004
Akatsuka, Y., Morishima, Y	Major and minor histocompatibility antigens in allogeneic hematopoietic stem cell transplantation.	***Cur. Opin. Organ. Transplant***	9	64-71	2004

III. 研究成果の刊行物・別刷

Journal of General Virology (2007), 88, 770–780 DOI 10.1099/vir.0.82519-0

Full-length EBNA1 mRNA-transduced dendritic cells stimulate cytotoxic T lymphocytes recognizing a novel HLA-Cw*0303- and -Cw*0304-restricted epitope on EBNA1-expressing cells

Yoshinori Ito,[1] Ayako Demachi-Okamura,[1] Rieko Ohta,[1] Yoshiki Akatsuka,[1] Keiko Nishida,[1] Kunio Tsujimura,[1] Yasuo Morishima,[2] Toshitada Takahashi[1] and Kiyotaka Kuzushima[1]

Correspondence
Kiyotaka Kuzushima
kkuzushi@aichi-cc.jp

[1]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan
[2]Department of Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan

Epstein–Barr virus (EBV)-encoded nuclear antigen 1 (EBNA1) is an attractive target for immunotherapy against EBV-associated malignancies because it is expressed in all EBV-positive cells. Although $CD8^+$ cytotoxic T-lymphocyte (CTL) epitope presentation is largely prevented by its glycine–alanine-repeat domain (GAr), the use of mRNA-transduced dendritic cells (DCs) would offer the advantage of priming EBNA1-specific CTLs. After stimulation with GAr-containing EBNA1-transduced monocyte-derived DCs, two EBNA1-specific CTL clones, B5 and C6, were isolated successfully from a healthy donor. These CTLs recognize peptides in the context of HLA-B*3501 and HLA-Cw*0303, respectively. A novel epitope, FVYGGSKTSL, was then identified, presented by both HLA-Cw*0303 and -Cw*0304, which are expressed by >35% of Japanese, >20% of Northern Han Chinese and >25% of Caucasians. The mixed lymphocyte–peptide culture method revealed that FVYGGSKTSL-specific CTL-precursor frequencies in HLA-Cw*0303- or -Cw*0304-positive donors were between 1×10^{-5} and 1×10^{-4} $CD8^+$ T cells. Moreover, both CTL clones inhibited growth of HLA-matched EBV-transformed B lymphocytes *in vitro*, and B5 CTLs produced a gamma interferon response to EBNA1-expressing gastric carcinoma cells in the context of HLA-Cw*0303. These data demonstrate that EBNA1 mRNA-transduced DCs may be useful tools for inducing EBNA1-specific CTLs that might be of clinical interest for CTL therapy of EBV-associated malignancies.

Received 31 August 2006
Accepted 7 November 2006

INTRODUCTION

Epstein–Barr virus (EBV), a human gammaherpesvirus that establishes lifelong latency in memory B cells (Babcock *et al.*, 2000), is associated with several different lymphoid and epithelial malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and post-transplant lymphoproliferative disorder (PTLD). All EBV-positive malignant cells exhibit one of three latency types, distinguished from each other by the pattern of expressed EBV antigens. In latency type I, only EBV-encoded nuclear antigen 1 (EBNA1) is expressed, as in BL; latent membrane protein 1 (LMP1) and LMP2, as well as EBNA1, are expressed in latency type II, as in HD and NPC. In latency type III, highly immunogenic EBNA3 genes, EBNA3A, EBNA3B and EBNA3C, are expressed together with other EBV latent antigens, as in PTLD (Rickinson & Kieff, 2001).

EBNA1 is required for the maintenance and replication of the viral episome in EBV-transformed cells (Kieff & Rickinson, 2001). Because it is expressed in all EBV-associated tumours, EBNA1 is an attractive target for immunotherapy. However, $CD8^+$ cytotoxic T-lymphocyte (CTL) responses are directed preferentially toward EBNA3s among latent-cycle proteins, and EBNA1 has been believed to be immunologically invisible because of studies indicating that there has been escape from recognition by CTLs (Callan *et al.*, 1998; Khanna *et al.*, 1992; Murray *et al.*, 1992; Steven *et al.*, 1996). A glycine–alanine-repeat domain (GAr) within EBNA1 was found to prevent antigen processing for CTL recognition (Levitskaya *et al.*, 1995). Presence of this GAr was shown to prevent processing by the proteasome, the main catalytic machinery for generation of major histocompatibility complex (MHC) class I epitopes (Blake *et al.*, 1997; Levitskaya *et al.*, 1997). Moreover, the same domain was established to prevent EBNA1 mRNA translation (Yin *et al.*, 2003).

To explore the possibility of targeting EBNA1, EBV-specific $CD4^+$ T-cell responses have been examined and

EBNA1-specific $CD4^+$ T-cell responses have been shown to be mainly T helper type 1 in nature (Bickham *et al.*, 2001), with direct recognition of EBV-transformed cells (Khanna *et al.*, 1997; Munz *et al.*, 2000). Several MHC class II-restricted EBNA1 epitopes have been identified (Khanna *et al.*, 1995; Kruger *et al.*, 2003; Leen *et al.*, 2001; Paludan *et al.*, 2002; Voo *et al.*, 2002), implying that EBNA1-specific $CD4^+$ T cells may play a role in controlling tumour growth *in vivo*. Surprisingly, recent studies demonstrated that EBNA1-specific $CD8^+$ CTLs moderately lyse EBV-transformed lymphoblastoid cell lines (LCLs) and suppress LCL outgrowth *in vitro* (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). Defective ribosomal products of EBNA1 were shown to be the sources of EBNA1 CTL epitopes presented on the cell surfaces.

In this study, we induced $CD8^+$ EBNA1-specific CTL clones from peripheral blood mononuclear cells (PBMCs) by using GAr-containing EBNA1 mRNA-transfected dendritic cells (DCs) as antigen-presenting cells (APCs). Antigen-transduced DCs have been demonstrated to prime antigen-specific CTLs efficiently *in vitro* (Grunebach *et al.*, 2003; Heiser *et al.*, 2000; Muller *et al.*, 2004; Nair *et al.*, 1998) and *in vivo* (Heiser *et al.*, 2002; Nair *et al.*, 2000; Su *et al.*, 2003; Zeis *et al.*, 2003). Furthermore, we identified a novel human leukocyte antigen (HLA)-C-restricted CTL epitope and confirmed EBNA1 recognition by CTL clones using tetrameric MHC–peptide complexes (tetramer). Finally, we estimated frequencies of EBNA1-specific CTL precursors (CTLp) in PBMCs of healthy EBV-seropositive donors and assessed effects of the CTL clones on EBNA1-expressing cells *in vitro*.

METHODS

Donors and cell lines. The study design and purpose, approved by the institutional review board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all blood donors and informed consent was obtained. CD40-activated B (CD40-B) cells were generated from PBMCs of blood donors as described previously (Kondo *et al.*, 2002; Schultze *et al.*, 1997). Briefly, PBMCs were cultured with irradiated human CD40L-transfected NIH3T3 cells (referred as to t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA), recombinant interleukin-4 (IL-4) (Genzyme) and cyclosporine A (Sandoz) in the culture medium. Expanding CD40-B cells were stimulated twice a week. LCLs were prepared by transforming PBMCs with B95-8 cell-culture supernatant as described previously (Kuzushima *et al.*, 1999) and cultured in RPMI 1640 medium (Sigma) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as complete culture medium). HLA-Cw*0303-expressing gastric carcinoma cells [MKN45 (referred to as MKN45-Cw0303) cells] were generated from MKN45 cells by retroviral transduction using Phoenix GALV cells (kind gifts from Dr Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, and Dr Nolan, Stanford University, Stanford, CA, USA). Retroviral transduction of HLA genes was performed as described previously (Akatsuka *et al.*, 2002; Kondo *et al.*, 2002). EBNA1 (without GAr)-expressing MKN45-Cw0303 cells (referred to as MKN45-Cw0303-ΔGA-EBNA1) were then generated from MKN45-Cw0303 cells by lentivirus transduction (Bai *et al.*, 2003). For this, an EBNA1-coding sequence without GAr (EBNA1 codons 92–323) was inserted into the lentivirus self-inactivating vector (CSIICMV-MCS; kindly provided by Dr Hiroyuki Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) (Bai *et al.*, 2003). MKN45-Cw0303 cells expressing full-length EBNA1 (referred to as MKN45-Cw0303-full-EBNA1) were also generated from MKN45-Cw0303 cells by mRNA transduction. Transduction of *in vitro*-transcribed full-length EBNA1 mRNA was performed as described above. MKN45-Cw0303 and MKN45-Cw0303-ΔGA-EBNA1 cells were cultured in complete culture medium with 1.0 μg puromycin ml^{-1}.

Preparation of DCs. DCs were prepared as described previously (Dauer *et al.*, 2003; Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994). Briefly, $CD8^+$ T cells were isolated from PBMCs by using CD8 MicroBeads (Miltenyi Biotec) and stored at −135 °C. The CD8-depleted PBMCs were suspended in 4 ml RPMI 1640 medium supplemented with 5 % human serum (MP Biomedicals), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as DC medium) and incubated for 2 h in six-well plates at 37 °C. Non-adherent cells were removed by gentle pipetting and adherent cells were cultured in DC medium in the presence of 50 ng granulocyte–macrophage colony-stimulating factor ml^{-1} (GM-CSF; Osteogenetics) and 10 ng IL-4 ml^{-1} (Osteogenetics). On days 2 and 4, half of the medium was replaced with fresh DC medium containing GM-CSF and IL-4. On day 6, DCs were collected and electroporated for mRNA transduction.

Production and transduction of *in vitro*-transcribed mRNA. To generate *in vitro*-transcribed EBNA1 mRNA, a pcDNA/EBNA1 vector was constructed. The coding sequence for EBNA1 was obtained by extraction of total RNA from B95-8-transformed LCLs using an RNeasy kit (Qiagen) and, after reverse transcription, EBNA1 cDNA was amplified by PCR with specific primers as follows: EBNA1 forward primer, 5′-AAGCTTGCCACCATGTCTGACGAGGGGCCAGGTACAG; reverse primer, 5′-GAATTCTCACTCCTGCCCTTCCTCACCCTC. The full-length EBNA1 fragment was then ligated into pcDNA3.1(+) (Invitrogen) using its *Hin*dIII and *Eco*RI sites (pcDNA/EBNA1). Clones were sequenced to verify their identity. Resulting plasmid DNA was linearized and transcribed *in vitro* by using an mMESSAGE and mMACHINE kit (Ambion) according to the manufacturer's instructions. A 3′-poly(A) tail was added by using poly(A) polymerase (Ambion) followed by purification with an RNeasy kit. The resulting mRNA was visualized by using the Reliant RNA gel system (Cambrex). DCs and CD40-B cells were transfected with mRNA by electroporation. First, they were washed twice with serum-free RPMI 1640 medium and suspended at a final concentration of 2.5×10^7 cells ml^{-1}. After mixing with 20 μg mRNA in 40 μl RPMI 1640 medium, they were then electroporated in a 2 mm cuvette by using an Electro Square Porator ECM 830 (Harvard Apparatus), under conditions of 450 V and 500 μS for DCs and 350 V and 350 μS for CD40-B cells. DCs were subsequently cultured in DC medium supplemented with GM-CSF and IL-4 for 3 h, followed with tumour necrosis factor alpha (PeproTech), IL-1β (PeproTech) and prostaglandin E_2 (Cayman Chemical) for maturation. CD40-B cells were seeded immediately onto irradiated t-CD40L cells and, after 36–48 h, these cells were used as APCs.

EBNA1 staining. EBNA1 mRNA-transfected CD40-B cells were collected and fixed with 4 % paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized with PBS containing 0.5 % Tween 20 and reacted with anti-EBNA1 rabbit polyclonal antibodies (kindly provided by Dr Tatsuya Tsurumi, Aichi Cancer Center Research Institute, Nagoya, Japan) for 30 min at 4 °C. After washing with PBS, cells were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Beckman Coulter) for 30 min at 4 °C. The stained cells were analysed by FACSCalibur (BD Biosciences) using CELLQUEST software (BD Biosciences).

CTL induction. The stored $CD8^+$ T cells were thawed, washed and co-cultured with irradiated (33 Gy) autologous EBNA1 mRNA-transfected DCs in 200 µl RPMI 1640 medium supplemented with 10 % human serum, 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 µg streptomycin ml^{-1} and 50 µg kanamycin ml^{-1} (referred to as CTL medium) in the presence of 5 ng IL-7 ml^{-1} (R&D Systems) and 5 ng IL-12 ml^{-1} (R&D systems) at 5 % CO_2 in a humidified incubator. On days 8, 16 and 23, T cells were restimulated with EBNA1 mRNA-transfected and irradiated DCs. One day after each restimulation, IL-2 (Shionogi) was added to a final concentration of 20 U ml^{-1}. To establish T-cell clones, limiting dilution of polyclonal CTLs was performed (Kuzushima *et al.*, 2001). In brief, polyclonal $CD8^+$ T cells were seeded at 1 cell per well in round-bottomed 96-well plates containing CTL medium with a monoclonal antibody (mAb) specific to CD3 (30 ng ml^{-1}; Ortho Biotech), 1×10^5 irradiated (33 Gy) PBMCs and 2×10^4 irradiated (55 Gy) LCLs. The next day, IL-2 was added to each well (50 U ml^{-1}). After 2 weeks culture, growing wells were split into two replicates and used as effectors in ELISPOT assays against either autologous EBNA1 mRNA-transfected CD40-B cells or autologous LCLs. Positive wells were transferred into flasks and expanded with anti-CD3 mAb, irradiated feeder cells and IL-2.

ELISPOT assays. ELISPOT assays were performed as described previously (Kuzushima *et al.*, 2003). Briefly, $CD8^+$ T cells were co-cultured with various stimulators in wells of Multiscreen-HA plates (Millipore) coated with anti-human gamma interferon (IFN-γ) mAb (Pierce Biotechnology). As stimulators, (i) autologous EBNA1 mRNA-transfected CD40-B cells or non-transfected CD40-B cells and (ii) autologous or allogeneic LCLs (1×10^5 cells per well) were seeded into each well. For peptide-titration assays and overlapping-peptide assay, serial concentrations of synthetic peptides were pulsed to autologous CD40-B cells for 1 h at room temperature. After probing with anti-human IFN-γ rabbit polyclonal antibodies (Pierce Biotechnology) followed by exposure to horseradish peroxidase-labelled anti-rabbit IgG antibody (Genzyme) and substrate, the plates were washed and dried. IFN-γ spots were enumerated under a dissecting microscope. The numbers of spots were confirmed by three investigators.

Peptides. Peptides were purchased from Bio-Synthesis Inc. The whole EBNA1 protein, excluding the GAr, deduced from the prototype B95-8 DNA sequence (GenBank accession no. V01555) was covered by 20 aa long synthetic peptides overlapping by 13 aa (total of 56 peptides). In addition, potential epitope peptides VYGGSKTSL (509–517), FVYGGSKTSL (508–517) and VFVYGGSKTSL (507–517) were synthesized, as predicted by the program SYFPEITHI (http://www.syfpeithi.de/) (Rammensee *et al.*, 1999). The known EBNA1 epitope peptide HPVGEADYFEY (Blake *et al.*, 1997) was also synthesized.

Tetramer production and staining. HLA-Cw*0303 and -Cw*0304 cDNA clones were used as templates to amplify sequences encoding the extracellular domains of HLA-Cw*0303 and -Cw*0304 heavy chains with primers C03F (5′-AACCATGGGCAGCCATTCTATGCGCTATTTTTACACCGCTGTGTCCCGGCC-3′) and C03R (5′-AAGGATCCTGGCTCCCATCTCAGGGTGAGG-3′). C03F contains several base changes designed to optimize protein expression in *Escherichia coli* BL21 (DE3) pLysS. The PCR product was digested with *Nco*I and *Bam*HI and cloned into a vector containing a BirA biotinylation site in frame with the 3′ end of the HLA sequence. Recombinant HLA-B*3501 protein was produced by using pGMT7-B35 (a kind gift of Dr McMichael, Weatherall Institute of Molecular Medicine, Oxford, UK). Recombinant MHC molecules were folded *in vitro* with β2-microglobulin and epitope peptides FVYGGSKTSL or VFVYGGSKTSL. Soluble complexes, purified by gel filtration, were biotinylated by using the BirA enzyme (Avidity LCC). Phycoerythrin (PE)-labelled tetramers were produced by mixing these biotinylated complexes with PE-labelled streptavidin (Molecular Probes). Tetramer staining was performed as follows. CTL clones (2×10^5) were incubated with tetramers at a concentration of 0.1 mg ml^{-1} and FITC–anti-CD8 mAb (Caltag) at 4 °C for 15 min. After washing twice, stained cells were fixed in 0.5 % paraformaldehyde and analysed by flow cytometry.

Mixed lymphocyte–peptide culture. Mixed lymphocyte–peptide culture was performed as described by Coulie *et al.* (2001) with modifications. PBMCs were isolated by density-gradient centrifugation and suspended in CTL medium. An aliquot was used for enumeration of $CD8^+$ cells. The cells were then distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in 200 µl medium in the presence of epitope peptide (1 µg ml^{-1}) and IL-2 (20 U ml^{-1}). Thirty-two cultures of each PBMC density were set up. On day 7, half of the medium was replaced by fresh medium containing the peptide and IL-2. Tetramer staining was performed on day 14. We counted the tetramer-positive microcultures and estimated the anti-EBNA1 CTLp frequency by limiting-dilution evaluation using the L-Calc program (Stem Cell Technologies).

Outgrowth-inhibition assay. Outgrowth-inhibition assays were performed as described by Lee *et al.* (2004) with modifications. Briefly, target LCLs were seeded into round-bottomed 96-well plates at 2×10^4 cells per well in triplicate. EBNA1-specific CTL clones (1×10^4 cells per well) or CTL medium alone (as a control) were added to target-cell cultures. All cultures were maintained weekly by changing half of the medium. After 4 weeks culture, the number of LCLs in the culture at each setting was counted. Cell growth (percentage of control) was calculated as [no. LCLs from the culture with CTLs (clone B5 or clone C6)]/[no. LCLs from the culture without CTLs (medium)] × 100. B-cell identity of grown cells was confirmed by staining with PE–cyanin 5-labelled anti-CD19 and PE-labelled anti-CD8 mAbs (Beckman Coulter) and analysis by flow cytometry.

Detection of IFN-γ-producing $CD8^+$ T cells by flow cytometry. EBNA1-specific CTL recognition was measured as described previously (Kuzushima *et al.*, 2001) with slight modifications. Briefly, 5×10^5 B5 CTLs were incubated with 2×10^6 MKN45-Cw0303, MKN45-Cw0303-ΔGA-EBNA1 or MKN45-Cw0303-full-EBNA1 cells in 1 ml complete culture medium in a culture tube at 37 °C for 6 h, in the presence of brefeldin A (Sigma) during the last 5 h. After the incubation, the cell suspensions were fixed with 4 % paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with IC Perm (BioSource International) and stained with PE–cyanin-5.1-labelled anti-CD8 (Beckman Coulter), PE-labelled anti-CD69 (Immunotech Coulter) and FITC-labelled anti-human IFN-γ (BD Biosciences) mAbs. Stained cells were analysed by flow cytometry. Live gating of the $CD8^+$ subset was performed and 50 000 events were acquired for each analysis.

RESULTS

EBNA1 expression in full-length EBNA1 mRNA-transfected CD40-B cells

To generate EBNA1-expressing APCs, we first produced full-length EBNA1 mRNA with a poly(A) tail from the pcDNA/EBNA1 plasmid by using an *in vitro* transcription system. The yield of capped mRNA was low, probably due to the presence of GAr (comprising GC-rich sequences), and this was not overcome fully by the change of reaction temperature or by adding single-stranded DNA-binding protein to the reaction mixture (data not shown). However,

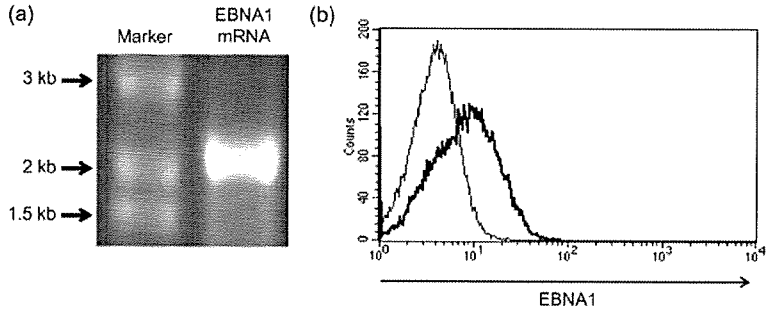

Fig. 1. EBNA1 expression in cells transfected with *in vitro*-transcribed full-length mRNA. (a) *In vitro*-transcribed full-length EBNA1 mRNA produced successfully from an EBNA1-cDNA plasmid. The quality of the EBNA1 mRNA was assessed by gel electrophoresis followed by staining with ethidium bromide. (b) EBNA1 protein expression in EBNA1 mRNA-transfected CD40-B cells. CD40-B cells were transfected with full-length EBNA1 mRNA by electroporation and intracellular staining of EBNA1 protein was performed and analysed by flow cytometry.

the amount of mRNA was sufficient and seen as a single band on the gel (Fig. 1a). Transfection was then performed by electroporation and EBNA1 expression was detected in most CD40-B cells, although the mean fluorescent intensity appeared to be low (Fig. 1b).

Induction of EBNA1-specific CTL lines and clones by using EBNA1 mRNA-transfected APCs

To explore the capacity for T-cell stimulation, autologous $CD8^+$ T lymphocytes were co-cultured with CD40-B cells transfected with full-length EBNA1 mRNA, and IFN-γ-producing cells were enumerated by ELISPOT assay. As shown in Fig. 2(a), $CD8^+$ T lymphocytes of donor Y01 produced IFN-γ spots without *in vitro* stimulation. As $CD8^+$ T lymphocytes of other donors did not produce significant spots on *ex vivo* analysis, the T cells were stimulated weekly with irradiated CD40-B cells that had been transfected with the full-length EBNA1 mRNA. After two rounds of stimulation, $CD8^+$ T lymphocytes of another donor (K04) produced IFN-γ spots upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay (Fig. 2a). These data indicate that the full-length EBNA1 mRNA was translated and that $CD8^+$ T-lymphocyte epitopes are processed and presented on APCs.

Next, EBNA1-specific CTL clones were established by using donor Y01 monocyte-derived DCs transfected with full-length EBNA1 mRNA. The transduced DCs were distributed in 96-well plates and used to stimulate autologous $CD8^+$ T lymphocytes in the presence of IL-7 and IL-12. After three rounds of stimulation, aliquots of each microculture were tested for their ability to secrete IFN-γ specifically upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay. Thirty-two microcultures out of 36 wells were scored as EBNA1-specific (data not shown), and lymphocytes from two well-growing microcultures were cloned by limiting dilution. CTL clones B5 and C6 were thus established, recognizing EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs, but not mock-transfected autologous CD40-B cells or HLA-mismatched allogeneic LCLs (Fig. 2).

Identification of the presenting HLA molecules

The donor was typed genetically as HLA-A*2402, -A*3101, -B*1507, -B*3501 and -Cw*0303. To identify the antigen-presenting HLA molecule, a panel of partially HLA-matched LCLs was used to stimulate clones B5 or C6 to produce IFN-γ. In addition to autologous LCLs, allogeneic LCLs expressing HLA-B*3501 were recognized by CTL clone C6 (Fig. 3a), and one LCL with HLA-Cw*0303 and one with -Cw*0304 were recognized by clone B5 (Fig. 3b), demonstrating that HLA-B*3501 is the putative restriction element for clone C6 recognition, whilst both HLA-Cw*0303 and -Cw*0304 act for clone B5.

Identification of EBNA1 antigenic peptides

To identify the epitope region, clones B5 and C6 were stimulated with autologous CD40-B cells incubated with sets of peptides of 20 aa length, overlapping by 13 aa and covering the complete EBNA1 protein sequence without GAr. Because the primary structure of GAr is not likely to be contained in MHC class I epitopes, we did not include this part as an epitope source. Peptide 24 was recognized by clone C6 (Fig. 4). Regarding the HLA-B*3501-restricted epitope, HPVGEADYFEY has been reported previously (Blake *et al.*, 1997). As this epitope sequence is located in the centre of peptide 24 (aa 402–421) (Fig. 5a), we tested whether clone C6 might recognize HPVGEADYFEY-pulsed autologous CD40-B cells and confirmed a response to HPVGEADYFEY-pulsed stimulation (data not shown).

In the case of clone B5, two overlapping peptides, 38 (aa 500–519) and 39 (aa 507–526), were recognized (Fig. 4), sharing the 13 aa sequence VFVYGGSKTSLYN [underlined in Fig. 5(b)]. To predict the optimal epitope binding to HLA-Cw*0303, the program SYFPEITHI was applied. Because the anchor leucine at the C terminus and the auxiliary anchors valine and tyrosine at the third position of epitopes were predicted by the program, we examined the 11mer (VFVYGGSKTSL) and the 10mer (FVYGGSKTSL) (Fig. 5b). Half-maximal recognition of the peptide-pulsed target cells was obtained with 5–10 nM of the 10mer peptide and 1–5 nM of the 11mer (Fig. 5c), suggesting that these two peptides may be the optimal epitopes. The 9mer,

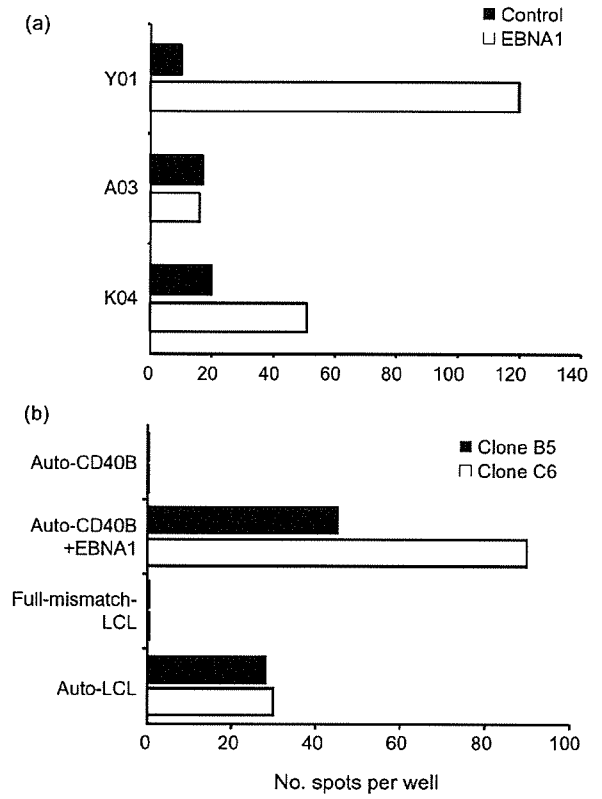

Fig. 2. Presence of anti-EBNA1 T cells in cultures primed with EBNA1 mRNA-transfected APCs. (a) Numbers of IFN-γ-producing cells from $CD8^+$ T lymphocytes without *in vitro* stimulation (Y01) or after two rounds of stimulation (A03 and K04) in ELISPOT assays. Aliquots of 100 000 cells were cultured in single wells with autologous CD40-B cells transduced with full-length EBNA1 mRNA for 20 h. Data from one representative experiment of two are shown. (b) $CD8^+$ T cells from one selected donor were stimulated with autologous DCs transfected with *in vitro*-transcribed EBNA1 mRNA. After three stimulations at weekly intervals, polyclonal $CD8^+$ T cells from two positive cultures were cloned by limiting dilution. Established clones B5 and C6 were then tested for recognition of EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs by ELISPOT assay. Five thousand CTLs were seeded in each well. Data from one representative experiment of two are shown.

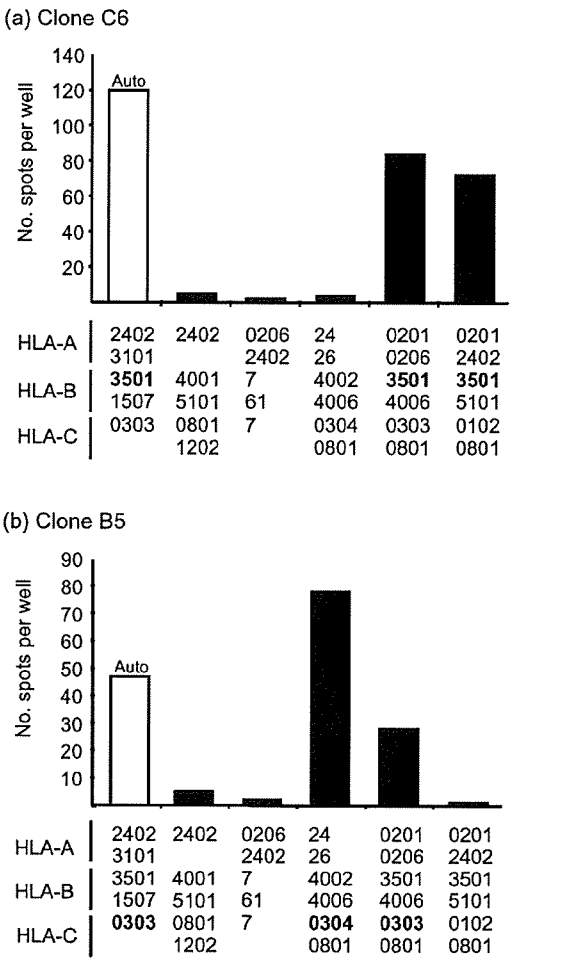

Fig. 3. Identification of presenting HLA molecules for EBNA1-specific CTL clones. (a) HLA-B*3501 molecules function as restriction elements for CTL clone C6. (b) HLA-Cw*0303 and -Cw*0304 molecules function as restriction elements for CTL clone B5. Autologous and allogeneic LCLs were used to stimulate clones B5 or C6 to produce IFN-γ spots. Single allele-matched LCLs were included and cultured with the CTLs (5×10^3) for 20 h. Each bar represents the mean number of spots in duplicate wells.

VYGGSKTSL, was not recognized, even at much higher concentrations.

Tetramers bind to the EBNA1-specific clone B5

As the peptide-dilution assay provided two optimal epitope candidates, we made fluorescently labelled tetramers incorporating the 10mer peptide FVYGGSKTSL or the 11mer VFVYGGSKTSL for further experiments. As shown in Fig. 5(d), these tetramers bound specifically to CTL clone B5. However, the tetramer incorporating the 10mer demonstrated higher avidity for the B5 clone than that incorporating the 11mer, suggesting the 10mer peptide FVYGGSKTSL to be the minimal and optimal epitope for the CTL. Moreover, clone B5 bound strongly to the HLA-Cw*0304 tetramer incorporating the 10mer, showing concordance with the results shown in Fig. 3(b). In addition, we characterized *in vitro*-expanded T cells from two donors by co-staining with MHC–peptide tetramer and CD62L. A proportion of 9.8% of HLA-Cw*0303-FVYGGSKTSL tetramer-positive lymphocytes were CD62-positive in one donor, and 6.5% in the other.

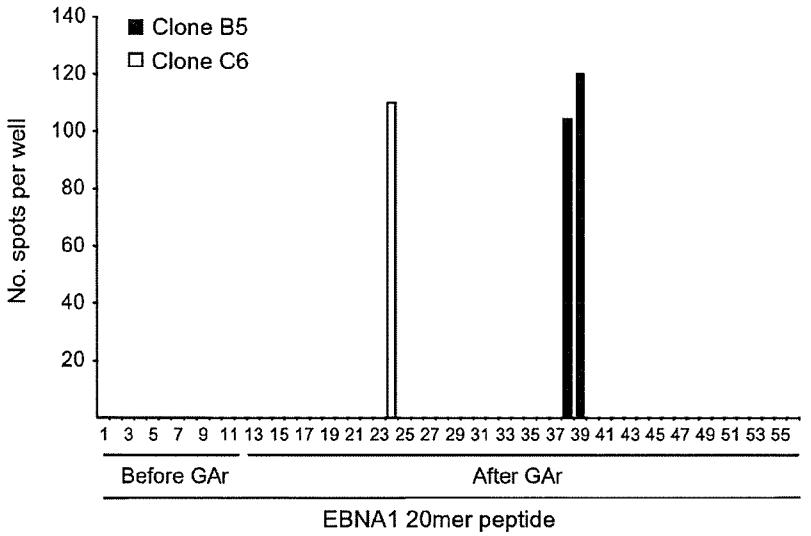

Fig. 4. Identification of overlapping peptides recognized by EBNA1-specific CTL clones. Autologous CD40-B cells (1×10^5 per well) were pulsed with 10 μg ml^{-1} of each of a set of 20mer overlapping peptides encompassing the EBNA1 protein, excluding GAr, and co-cultured with 5×10^2 CTL clone B5 or C6. Production of IFN-γ spots was then measured by ELISPOT assay.

Frequencies of EBNA1 epitope-specific $CD8^+$ T cells in PBMCs of healthy EBV-seropositive donors

We estimated frequencies of EBNA1 epitope-specific $CD8^+$ T cells in healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method. PBMCs from two donors with HLA-B*3501, one donor with HLA-Cw*0303 and three donors with HLA-Cw*0304 were tested. We could compare the anti-EBNA1 CTLp frequency in two donors with both HLA-B*3501 and HLA-Cw*0303 (HLA-Cw*0304). Representative tetramer staining of negative and positive microcultures from mixed lymphocyte–peptide culture wells is shown in Fig. 6. EBNA1-specific CTLp frequencies of HLA-B*3501-positive donors were 7.2×10^{-6} and 1.8×10^{-4}, and for HLA-Cw*0303 and -Cw*0304 were from 2.5×10^{-5} to $> 2.1 \times 10^{-4}$. We did not find any hierarchy between the two EBNA1 epitopes in either of the donors with both HLA-B*3501 and HLA-Cw*0303 (Cw*0304) molecules (Table 1).

Effect of EBNA1-specific CTLs on EBV-infected B-cell growth

Clones B5 and C6 did not lyse autologous LCLs in the chromium-release assay (data not shown). Here, a final set of experiments was performed to ask whether these EBNA1-specific CTLs could affect the long-term growth and survival of EBNA1-expressing LCLs. Autologous and allogeneic LCLs with or without the restricting HLA molecules were seeded in 96-well plates in the presence or absence of responding CTLs. Cultures were then assayed for LCL outgrowth after 4 weeks. At the end, LCL outgrowth was assessed by microscopic inspection and confirmed by CD19 expression by flow cytometry. As shown in Fig. 7(a), both CTL clones clearly inhibited outgrowth of not only autologous LCLs, but also allogeneic LCLs with restricting HLA, suggesting that these CTL clones have the ability to inhibit outgrowth of EBV-positive cells with latency type III.

Recognition by EBNA1-specific CTL clone B5 of HLA-Cw*0303-transduced gastric carcinoma cells expressing EBNA1

Because naturally EBV-positive gastric cell lines are difficult to establish, we generated EBNA1-expressing gastric carcinoma cells designated MKN45-Cw0303-ΔGA-EBNA1 and MKN45-Cw0303-full-EBNA1 to verify that EBV-positive gastric cancer cell lines present the FVYGGSKTSL epitope. To investigate recognition by clone B5, we applied flow cytometry to detect EBNA1-specific CTLs producing IFN-γ. As shown in Fig. 7(b), 2.55 % of B5 clone cells produced IFN-γ when co-cultured with MKN45-Cw0303-ΔGA-EBNA1 cells, demonstrating specific recognition of the FVYGGSKTSL epitope on cells transduced with GAr-deleted EBNA1. Otherwise, B5 clone cells did not produce IFN-γ when co-cultured with MKN45-Cw0303-full-EBNA1 cells.

DISCUSSION

Recently, EBNA1-specific CTLs were shown to recognize and lyse HLA-matched LCLs (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). In previous studies, such CTLs were generated from PBMCs of healthy donors after *in vitro* stimulation with autologous LCLs or EBNA1 peptide-pulsed PBMCs. As EBNA1 is expressed in all EBV-associated tumours and might be an important target for immunotherapy, we have explored the efficient induction of EBNA1-specific CTLs. Of the different methods used to obtain HLA class I-restricted epitopes on APCs for stimulation, we chose to employ full-length EBNA1 mRNA-transfected DCs. This strategy offers the following advantages: (i) the method is not dependent on knowledge of the HLA haplotype of each donor; (ii) there is complete deletion of antigenicity of the vector-backbone sequence; and (iii) the yield of mRNA *in vitro* transcription is highly reproducible and transduction is very efficient (Van

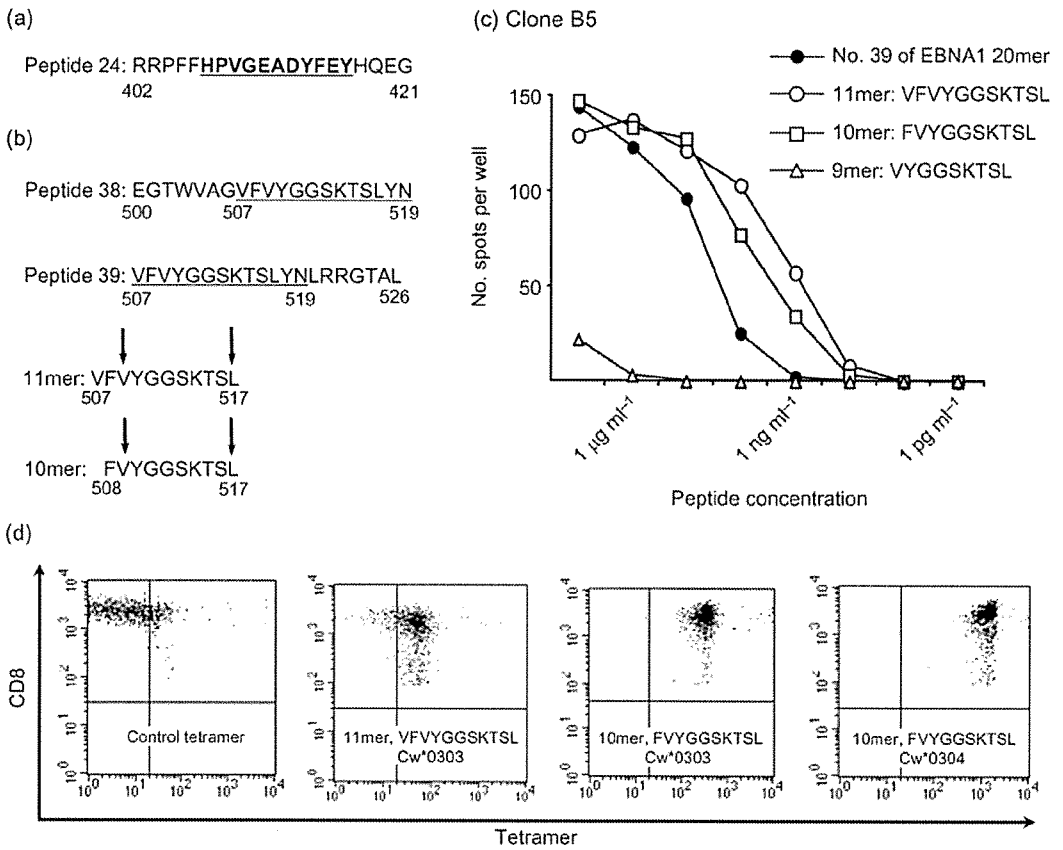

Fig. 5. Identification of optimal EBNA1 antigenic peptides recognized by EBNA1-specific CTL clones. (a) Amino acid sequence of the overlapping peptide recognized by the HLA-B*3501-restricted clone C6. The known epitope HPVGEADYFEY is indicated in bold and underlined. The numbers of the amino acid position in EBNA1 are shown. (b) Amino acid sequences of two consecutive overlapping peptides recognized by clone B5 and the potential optimal epitope sequences. The overlapping sequence between peptides 38 and 39 is underlined. Arrows indicate the primary and auxiliary anchors for HLA-Cw*0303 predicted by the program SYFPEITHI. The numbers of the amino acid positions in the EBNA1 protein are shown. (c) Titration of EBNA1-derived synthetic peptides. Autologous CD40-B cells were incubated for 1 h with 10-fold serial dilutions of synthetic peptides 507–526 (no. 39, 20mer), 507–517 (11mer), 508–17 (10mer) and 509–517 (9mer). CTL clone B5 (200 cells per well) was subsequently added and cultured for 20 h. Each symbol indicates the mean number of spots in duplicate wells. (d) The HLA-Cw*0303-restricted EBNA1-specific CTL clone B5 was stained with PE-conjugated HLA-Cw*0303–FVYGGSKTSL, HLA-Cw*0303–VFVYGGSKTSL or HLA-Cw*0304–FVYGGSKTSL tetrameric complexes and FITC-labelled anti-CD8 antibodies, and analysed by flow cytometry.

Tendeloo *et al.*, 2001). As an antigen, we used GAr-containing full-length EBNA1 instead of a GAr-deleted example to selectively activate CTL populations capable of reacting with epitopes that escape from the inhibitory mechanism governed by EBNA1 encoding GAr. Of note, even a low level of antigen delivery into DCs could induce antigen-specific CTL responses (Grunebach *et al.*, 2003), suggesting that this strategy has the potential to induce a CTL response even when a low density of EBNA1 epitopes is presented on DCs. In this study, we generated HLA-B- and -C-restricted EBNA1-specific CTLs successfully from a single donor, demonstrating that this method is a useful tool for generating EBNA1-specific CTLs, allowing investigation of the contribution of EBNA1 to cell-mediated immune responses in EBV-associated malignancies. Moreover, EBNA1 may have antigenicity when expressed on APCs, even if containing GAr *in vivo*. Finally, this induction method may be applicable for preparing EBNA1-specific CTLs for immunotherapy.

EBNA1 is generally immunologically invisible and only a small number of CTL epitopes have been identified (Blake *et al.*, 1997, 2000; Voo *et al.*, 2004). Of these, five epitopes are HLA-B-restricted and one is presented in the context of HLA-A. We detected two, one from HLA-B and another from HLA-C, from a single donor. To our knowledge, this is the first demonstration of an HLA-C-restricted EBNA1 epitope. To determine the minimal epitope, we compared

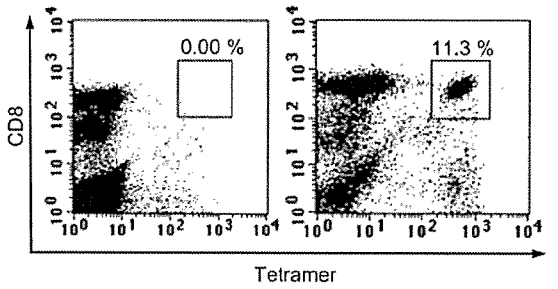

Fig. 6. Mixed lymphocyte–peptide culture analysis to estimate frequencies of C6 CTLp in $CD8^+$ lymphocytes of healthy EBV-seropositive donors. PBMCs from healthy EBV-seropositive donors with HLA-Cw*0303 were distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in CTL medium with FVYGGSKTSL peptide (1 μg ml^{-1}) and IL-2 (20 U ml^{-1}). Half of the medium was replaced by fresh medium containing the relevant epitope peptide and IL-2 on day 7 and tetramer staining was performed on day 14. The plots show only data for $CD8^+$ lymphocytes, corresponding to 20–40 % of the cells in representative positive and negative cultures. The proportions of $CD8^+$ lymphocytes labelled specifically with the HLA-Cw*0303–FVYGGSKTSL tetramer are indicated.

the 11mer (VFVYGGSKTSL) and 10mer (FVYGGSKTSL) in a peptide-titration assay and found peptide concentration with half-maximal recognition of the target cells to be almost the same. However, clone C6 bound more strongly to tetramers incorporating the 10mer and we speculate that the N-terminal valine of the 11mer might be trimmed efficiently to yield 10mer in ELISPOT assay medium containing FCS. Moreover, the 10mer FVYGGSKTSL epitope was presented by HLA-Cw*0303 and -Cw*0304 molecules. As these two HLA-C alleles are possessed by >35 % of Japanese, >20 % of Northern Han Chinese (Hong *et al.*, 2005) and >25 % of Caucasians, this new epitope should enable us to analyse cellular immunity to EBNA1 in a broad population. Indeed, we estimated $CD8^+$ T-cell frequencies specific to either FVYGGSKTSL or HPVGEADYFEY in PBMCs of healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method followed by tetramer staining and found that EBNA1-specific CTLp frequencies of HLA-B*3501- or HLA-Cw*0303 (and -Cw*0304)-positive donors were between 1×10^{-5} and 1×10^{-4}. These data provide useful information for understanding cellular immunity to EBNA1. For determination of frequencies of EBNA1 epitope-specific CTLs, the *ex vivo* ELISPOT assay (Blake *et al.*, 2000) is simple and readily applicable, because frequencies can be predicted at the level of 1×10^{-4} $CD8^+$ lymphocytes.

Adoptive immunotherapy with CTLs has proved feasible for preventing and treating EBV-associated PTLD, HD and NPC (Bollard *et al.*, 2004; Gottschalk *et al.*, 2005; Straathof *et al.*, 2005). With respect to the targets for EBV-specific CTLs, EBNA3s and LMP2 are major EBV latent antigens; EBNA3s are immunodominant and LMP2 is recognized frequently, but is subdominant. In contrast, CTL responses to other antigens (EBNA2, EBNA-LP, LMP1 and EBNA1) seem to be less frequent (Rickinson & Moss, 1997), although EBNA1 can be immunodominant in some EBV-seropositive donors (Blake *et al.*, 2000). Indeed, LCL-activated EBV-specific CTL lines from NPC patients for adoptive immunotherapy demonstrate stronger responses to the immunodominant EBNA3s than against LMP1 and LMP2 (Straathof *et al.*, 2005). In addition, tetramer and functional

Table 1. Frequencies of anti-EBNA1 CTL precursors

Donor	HLA-B*3501				HLA-Cw*0303/0304			
	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)
Y01*	38	2×10^5	32/32	$>1.8 \times 10^{-4}$	32	1×10^5	20/32	3.5×10^{-5}
		1×10^5	32/32			5×10^4	15/32	
		5×10^4	32/32			2.5×10^4	13/32	
T02†	32	2×10^5	10/32	7.2×10^{-6}	32	2×10^5	32/32	$>2.1 \times 10^{-4}$
		1×10^5	7/32			1×10^5	32/32	
		5×10^4	5/32			5×10^4	32/32	
A03†		NA‡			23	2×10^5	20/32	2.5×10^{-5}
						1×10^5	13/32	
						5×10^4	12/32	
K04†		NA			19	2×10^5	18/32	2.7×10^{-5}
						1×10^5	13/32	
						5×10^4	11/32	

*HLA-Cw*0303.
†HLA-Cw*0304.
‡NA, Not available.