Generation of autologous cytotoxic and helper T-cell responses against the B-cell leukemia–associated antigen HB-1: relevance for precursor B-ALL–specific immunotherapy

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Tumor relapses in patients with precursor B-cell acute lymphoblastic leukemia (B-ALL) occur frequently after primary treatment. Therefore, development of additional treatment modalities to eliminate residual tumor cells is needed. Active immunotherapy using dendritic cells (DCs) loaded with tumor-associated antigens is a promising approach to induce specific T-cell immunity in patients with cancer. In previous studies, we described HB-1 as a B-cell lineage-specific antigen that is recognized by donor-derived cytotoxic T lymphocytes (CTLs) on allogeneic B-ALL tumor cells. Here, we investigated the potential use of the HB-1 antigen as

an autologous T-cell vaccine target. To determine whether HB-1–specific CTL precursors are present within the T-cell repertoire, we induced expansion of CD8^{+} T cells using mature monocyte-derived DCs pulsed with the previously identified HB-1.B44 antigenic peptide. In 6 of 8 donors, CD8^{+} CTL lines have been generated that exert cytotoxicity against target cells exogenously pulsed with peptide or endogenously expressing the HB-1 antigen. From one of these HB-1–specific T-cell lines, we isolated a CD8^{+} CTL that produces interferon-γ on stimulation with B-ALL tumor cells. Interestingly, the HB-1 antigen also induced CD4^{+} T-helper re-

sponses on activation with protein-loaded mature monocyte-derived DCs. We identified 2 novel epitopes recognized in the context of HLA-DR4 and HLA-DR11 with the use of HB-1–specific CD4^{+} T-cell clones generated from different donors. These present data, that HB-1 induces both helper and cytotoxic T-cell responses, indicate that the HB-1 antigen is a candidate target to induce T-cell–mediated antitumor immunity in patients. (Blood. 2003;102:2885-2891)

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Introduction

Precursor B-cell acute lymphoblastic leukemia (B-ALL) is primarily a pediatric disease, as about 75% of cases occur in children.[1] Most of these children have a good prognosis, and approximately 80% will be cured by chemotherapy only. However, 25% of B-ALL cases comprise adults whose prognosis is generally worse. They usually require more intensive treatment, including high-dose chemotherapy followed by autologous or allogeneic stem cell transplantation (SCT).[2-4] Although intensive treatment has improved the overall response rate, relapse is still a major problem, indicating that not all tumor cells are eliminated by current available treatment modalities. Therefore, development of new therapeutic approaches for B-ALL remains a challenge. One such approach could be active immunotherapy that targets tumor-associated antigens (TAAs) to induce or enhance tumor-specific T-cell immunity in a setting of residual disease after primary treatment.

Proteins that are differentially (over) expressed in human tumor cells are attractive targets for T-cell–based vaccines. Various melanocyte differentiation antigens, such as tyrosinase,[5] Pmel17/gp100,[6] Melan-A/Mart-1,[7] and tyrosinase-related protein 1 and 2 (TRP1-2),[8,9] have been identified as targets for melanoma-specific cytotoxic T lymphocytes (CTLs). However, CTL responses directed against TAAs specific for leukemia have been identified rarely. Proteinase 3, a granule protein that is overexpressed in myeloid leukemia, and the Wilms tumor-1 (WT1) protein that is preferentially expressed in acute leukemia, have been shown to induce antileukemic CTL reactivity.[10-12] In previous studies, we have described a polymorphic TAA, named HB-1, which is recognized by a donor-derived CTL on allogeneic tumor cells of patients with B-ALL and Epstein-Barr virus (EBV)–transformed B cells.[13,14] This allogeneic CTL recognizes only the HB-1H variant, but CTL responses toward the HB-1Y variant can also be induced.[14,15] The HB-1 coding transcript was found differentially expressed in all B-ALL subtypes, and its normal tissue expression is restricted to low levels in testis, thymus, and tonsils with B-cell hyperplasia[14] (H. D. et al, unpublished data, 2002). This finding suggests that an HB-1–based vaccine will be tumor restricted with limited toxicities to healthy tissues.

Preclinical studies demonstrate that development of efficient antitumor immune responses requires induction of antigen (Ag)–specific CD4^{+} T-helper (Th) cells next to the generation of Ag-specific CD8^{+} T cells.[16-19] Activated CD4^{+} Th cells deliver help to the generation of CTL in 2 ways. First, they express surface ligands such as CD40L that bind to CD40 on dendritic cells (DCs),

thereby inducing expression of costimulatory molecules and the production of interleukin 12 (IL-12). Second, they amplify and sustain CD8$^+$ T-cell function by the secretion of IL-2. In the effector phase, activated CTLs exhibit direct tumor cell killing. CD4$^+$ T cells also participate in the effector phase by recruiting and activating cells with tumoricidal activity such as natural killer (NK) cells and macrophages or by exerting direct cytotoxicity. Therefore, TAA-based vaccines should elicit both CD4$^+$ and CD8$^+$ T-cell responses.

In this study, we examined immune reactivity toward the HB-1 antigen in the autologous setting and evaluated its potency as a therapeutic T-cell vaccine target. We generated in vitro autologous T-cell responses toward naturally processed epitopes using peripheral blood T lymphocytes of healthy donors. Induction of anti–HB-1 CTL responses is feasible by using mature monocyte-derived DCs pulsed with the previously identified immunogenic HB-1H.B44 peptide. Moreover, we demonstrate that mature monocyte-derived DCs pulsed with the full-length HB-1 protein also induce CD4$^+$ T-cell reactivity.

Materials and methods

Cell culture

Cell lines were cultured in Iscove modified Dulbecco medium (IMDM; Gibco BRL, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS). EBV-transformed lymphoblastoid cell lines (LCLs) were generated from peripheral blood B cells by transformation with EBV of the B95-8 cell line and further cultured in IMDM/10% FCS. T-cell blasts were generated by stimulating peripheral blood mononuclear cells (PBMCs) with 20 μg/mL phytohemagglutinin-mucoprotein (PHA-M; Boehringer, Mannheim, Germany) in IMDM/10% human serum (HS; PAA Laboratories, Linz, Austria) for 3 days. Subsequently, T-cell blasts were washed and further cultured with 100 IU/mL IL-2 (Eurocetus, Amsterdam, The Netherlands). Leukemic cells were collected from patients with precursor B-ALL at diagnosis. B-ALL cells were preincubated with 10 ng/mL tumor necrosis factor α (TNF-α; Endogen, Woburn, MA) and 100 U/mL interferon-γ (IFN-γ; Boehringer, Zaandam, The Netherlands) for 2 days. The Burkitt lymphoma cell line Raji was transfected with either pCR3.B*4402 or pCR3.B*4403 plasmid by electroporation. Subsequently, Raji.B44 transfectants were selected with 1.5 mg/mL G418 (Gibco BRL) and cloned by limiting dilution. Raji.B*4402 and Raji.B*4403 clones were subsequently cotransfected with pcDNA3-zeo.HB-1H and selected with 0.5 mg/mL zeocin (Invitrogen, San Diego, CA).

Antibodies and immunofluorescence analysis

The following monoclonal antibodies (mAbs) were used for immunofluorescence analysis or for inhibition of T-cell reactivity: UCHT1 (CD3), MT310 (CD4), DK25 (CD8), MAB104 (CD80), HB15A (CD83), HA5.2B7 (CD86), W6/32 (anti–HLA class I), L243 (anti–HLA-DR), B7/21 (anti–HLA-DP), and Tü22 (anti–HLA-DQ). Anti–HLA class II antibodies were kindly provided by Dr G. Pawelec (Department of Internal Medicine, University of Tubingen Medical School, Germany). Clonality of generated T-cell lines was determined by using T-cell antigen receptor beta-chain variable (TCR-BV)–specific mAb (Beckman Coulter, Fullerton, CA). Immunofluorescence analysis was performed either by direct or indirect labeling. Fluorescein isothiocyanate (FITC)–conjugated goat F(ab′)$_2$ anti–mouse immunoglobulin G (IgG) and IgM (Tago Immunologicals, Camarillo, CA) was used for secondary staining. Cells were analyzed by an Epics XL flow cytometer (Beckman Coulter).

Peptide and protein synthesis

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422). Peptides were purified by reversed phase high-performance liquid chromatography (HPLC) and lyophilized. Finally, peptides were dissolved in 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), diluted in IMDM to a peptide concentration of 1 mM, and stored at −20°C before use. HB-1 protein (40 aa [amino acid]) was synthesized and purified by reversed phase HPLC (Ansynth BV, Roosendaal, The Netherlands). HB-1 protein was dissolved to a concentration of 1 mg/mL in phosphate-buffered saline (PBS) containing 10% DMSO and stored at −20°C before use.

Peripheral blood mononuclear cells of healthy donors

PBMCs were isolated from leukapheresed blood of healthy donors by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were collected and washed twice in Hanks balanced salt solution (HBSS; BioWitthaker, Verviers, Belgium) and finally resuspended in IMDM/10% FCS supplemented with 10% DMSO. Cells were cryopreserved and stored in liquid nitrogen until use.

Monocyte-conditioned medium

Monocyte-conditioned medium (MCM) was prepared by stimulating monocytes, enriched from buffy coat preparations of healthy donors by density gradient centrifugation and counterflow centrifugation, with polyclonal plastic-immobilized IgG (CLB, Amsterdam, The Netherlands) for 18 hours at 37°C.

Generation of monocyte-derived dendritic cells

DCs were used as stimulator cells and generated from thawed PBMCs. Briefly, PBMCs (5×10^6/mL) were resuspended in IMDM/5% HS and incubated for 2 hours at 37°C in 75-cm^2 tissue culture flasks. Nonadherent cells were removed, and adherent cells were subsequently cultured in IMDM/5% HS supplemented with 800 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Amstelveen, The Netherlands) and 500 U/mL IL-4 (Schering-Plough) for 6 days at 37°C. After 3 days, DCs were harvested and resuspended in IMDM/5% HS supplemented with 800 U/mL GM-CSF and 500 U/mL IL-4 at 1×10^6/well in 6-well plates. At day 6, cells were washed and further cultured for 3 days in IMDM/5% HS supplemented with 800 U/mL GM-CSF, 500 U/mL IL-4, and either 50% MCM, 1 μg/mL recombinant trimeric CD40L (kindly provided by Immunex, Seattle, WA), or 20 ng/mL TNF-α (Endogen) plus 10 μg/mL prostaglandin E$_2$ (PGE2; Pharmacia and Upjohn, Puurs, Belgium). At day 9, DCs were harvested and used for T-cell inductions. The generated DC populations expressed high levels of major histocompatibility complex (MHC) class I and MHC class II, and were more than 80% positive for CD80, CD83, and CD86 (data not shown).

In vitro CTL induction with peptide-loaded monocyte-derived DCs

Monocyte-derived mature DCs were pulsed with 50 μM peptide for 4 hours at room temperature (RT) in the presence of 3 μg/mL human β2-microglobulin (Sigma). Subsequently, peptide-pulsed DCs were irradiated (30 Gy), washed to remove free peptide, and resuspended at 2×10^5 cells/mL in IMDM/10% HS. Cryopreserved PBMCs were thawed, and CD8$^+$ T cells were isolated by positive selection using CD8 mAb-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8$^+$ T cells (10^6/well) were cocultured with autologous peptide-pulsed DCs (2×10^5/well), and autologous-irradiated PBMCs as feeder cells (10^5/well) in 2 mL IMDM/10% HS supplemented with 1000 U/mL IL-6 (Sandoz, Basel, Switzerland) and 10 ng/mL IL-12 (Hoffman-La Roche, Nutley, NJ). At day 7 and 14, CTL cultures were harvested, washed, and restimulated with peptide-pulsed DCs at an effector-to-stimulator ratio of 10:1 in the presence of 100 IU/mL IL-2 and 5 ng/mL IL-7 (Genzyme, Cambridge, MA). From day 21 on, cultures were maintained by weekly stimulation with irradiated adherent autologous PBMCs or EBV-LCL pulsed with 10 μM peptide in IMDM/10% HS containing 100 U/mL IL-2 and 5 ng/mL IL-7.

Table 1. HLA-B44–restricted and HB-1H peptide-specific cytotoxicity of in vitro–induced CD8+ T-cell lines

| CTL donor line | B44 subtype* | HB-1 typing† | % Specific lysis‡ | | | | | |
| | | | E/T ratio 30:1 | | E/T ratio 10:1 | | E/T ratio 3:1 | |
			+HB-1H	−HB1-H	+HB-1H	−HB1-H	+HB-1H	−HB1-H
1	B*4402	HH	40	4	22	1	11	1
2	B*4402	HY	35	1	26	1	17	1
3	B*4402	HH	24	8	22	2	13	1
4	B*4402	HH	24	10	17	7	15	4
5	B*4402	HH	4	2	2	2	2	2
6	B*4403	HH	35	1	21	1	14	1
7	B*4403	HY	22	13	20	3	13	2
8	B*4403	HH	2	2	3	2	2	3

*HLA-B44 subtyping was performed by PCR and digestion analysis as described previously.[13]

†HB-1 typing was performed by PCR amplification and digestion of PCR products with restriction enzyme NlaIII, as described previously.[15]

‡Cytolytic activity of HB-1H peptide-induced CTLs was tested against Raji.B*4402 or Raji.B*4403 cells (according to the HLA-B44 subtype of the donor) either untreated or loaded with 5 μM HB-1H.B44 peptide. Specific lysis was determined by chromium release assays at different effector-to-target cell (E/T) ratios.

In vitro T-helper cell induction with protein-loaded monocyte-derived DCs

Immature monocyte-derived DCs at day 3 of culture were pulsed with 25 μg/mL HB-1 protein. Subsequently, protein-loaded DCs were further cultured and matured with 20 ng/mL TNF-α plus 10 μg/mL PGE2. Cryopreserved PBMCs were thawed, and CD4+ T cells were isolated by positive selection using CD4 mAb-coupled magnetic beads (Miltenyi Biotec). CD4+ T cells (10⁶/well) were cocultured with autologous protein-loaded DCs (2 × 10⁵/well) in 24-well plates containing 2 mL IMDM/10% HS supplemented with 1000 U/mL IL-6 and 10 ng/mL IL-12. At days 7 and 14, T-cell cultures were harvested, washed, and restimulated with protein-loaded DCs at an effector-to-stimulator ratio of 5:1 in the presence of 100 IU/mL IL-2. At day 21, T-cell cultures were tested for specific HB-1 reactivity and cloned by limiting dilution. For this procedure, autologous EBV-LCLs (2 × 10⁶) were incubated overnight at 37°C with 25 μg/mL HB-1 protein. T cells were diluted to a concentration of 1, 3, and 10 cells/well in a 96-well round-bottom microtiter plate containing 200 μL IMDM/10% HS with irradiated (80 Gy) HB-1 protein-loaded EBV-LCLs (2 × 10⁴), irradiated (60 Gy) allogeneic PBMCs (2 × 10⁴/donor) isolated from 2 healthy donors, 100 IU/mL IL-2, and 1 μg/mL PHA-M. Established T-cell clones were expanded weekly by using autologous EBV-LCLs loaded with HB-1 protein, irradiated (30 Gy) allogeneic PBMCs from 2 donors (5 × 10⁵/donor), 200 IU/mL IL-2, and 1 μg/mL PHA-M in a total volume of 2 mL IMDM/10% HS.

Chromium release assay

Chromium release assays were performed to assess cytolytic activity of CTLs. Briefly, 10⁶ target cells were incubated with 100 μCi (3.7 MBq) ⁵¹Cr (Amersham, Buckinghamshire, United Kingdom) for 1 hour at 37°C. Labeled target cells were mixed in V-bottomed microtiter plates (10³/well) with various numbers of effector cells in a total volume of 150 μL IMDM with 10% FCS. In peptide recognition assays, target cells were preincubated with various concentrations of peptide for 30 minutes at RT in a volume of 100 μL prior to the addition of effector cells. After 4 hours of incubation at 37°C, 100 μL supernatant was collected, and radioactivity was measured by a gamma counter. The mean percentage of specific lysis of triplicate wells was calculated by using the following formula: percentage of specific lysis = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

IFN-γ secretion assay

IFN-γ–producing CTLs were detected and isolated using the IFN-γ secretion assay (Miltenyi Biotec). Briefly, 1 × 10⁶ CTLs were incubated in a 24-well plate with 1 × 10⁶ irradiated (30 Gy) autologous PBMCs in a total volume of 2 mL IMDM/10% HS. Peptides were added to a final concentration of 10 μM. After 16 hours of incubation at 37°C, cells were harvested, washed with PBS/0.5% FCS/5 mM EDTA (ethylenediaminetetraacetic acid), and labeled at a concentration of 10⁸ cells/mL with 50 μg/mL Ab-Ab conjugates directed against CD45 and IFN-γ

for 10 minutes on ice. Subsequently, cells were diluted with IMDM/10% FCS at 1 × 10⁵ cells/mL and allowed to secrete IFN-γ for 45 minutes at 37°C. After the cytokine-capturing period, cells were collected, resuspended at a concentration of 10⁸ cells/mL in PBS/0.5% FCS/5 mM EDTA, and stained with 5 μg/mL phycoerythrin (PE)–conjugated anti–IFN-γ mAb and FITC-conjugated anti-CD8 mAb for 20 minutes at 4°C. Finally, cells were analyzed and isolated by cell sorting using an Epics Elite flow cytometer (Beckman Coulter).

T-cell stimulation assay

Production of IFN-γ by the generated CD8+ and CD4+ T-cell lines was determined as described.[14] Briefly, 3000 to 7000 CD8+ or 5000 CD4+ T cells were added in flat-bottom microwells containing 3 to 5 × 10⁴ stimulator cells in a total volume of 200 μL IMDM/10% FCS and 25 IU/mL IL-2. After 18 hours of incubation at 37°C, 100 μL supernatant was collected, and its IFN-γ concentration was determined by enzyme-linked immunosorbent assay (ELISA; Endogen). Peptide recognition assays were performed by using either autologous EBV-LCLs or PHA-stimulated T-cell blasts. Target cells (3 × 10⁴) were incubated with various peptide concentrations for 30 minutes at RT and tested for recognition by CD8+ and CD4+ T cells.

Results

Mature monocyte-derived DCs induce expansion of autologous CD8+ CTLs against HB-1

To determine whether CTL precursors recognizing the HB-1H.B44 antigenic peptide exist within the T-cell repertoire of HB-1H–positive
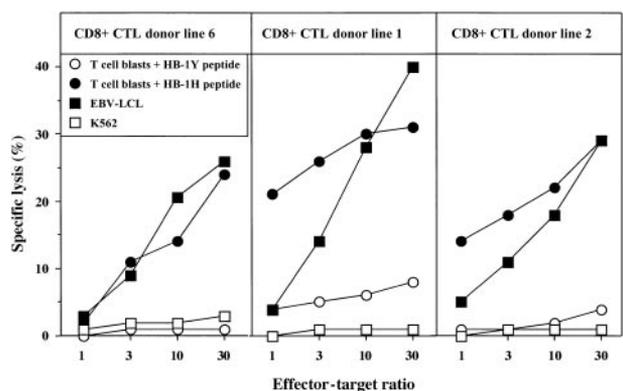

Figure 1. Specific reactivity of 3 HB-1H.B44 peptide-induced autologous CD8+ CTL lines. Cytolytic activity was tested in chromium release assays against autologous PHA-stimulated T-cell blasts either pulsed with 5 μM HB-1H.B44 or HB-1Y.B44 peptide, and autologous EBV-transformed B-cell lines that endogenously express HB-1H. K562 was used to determine aspecific lysis.
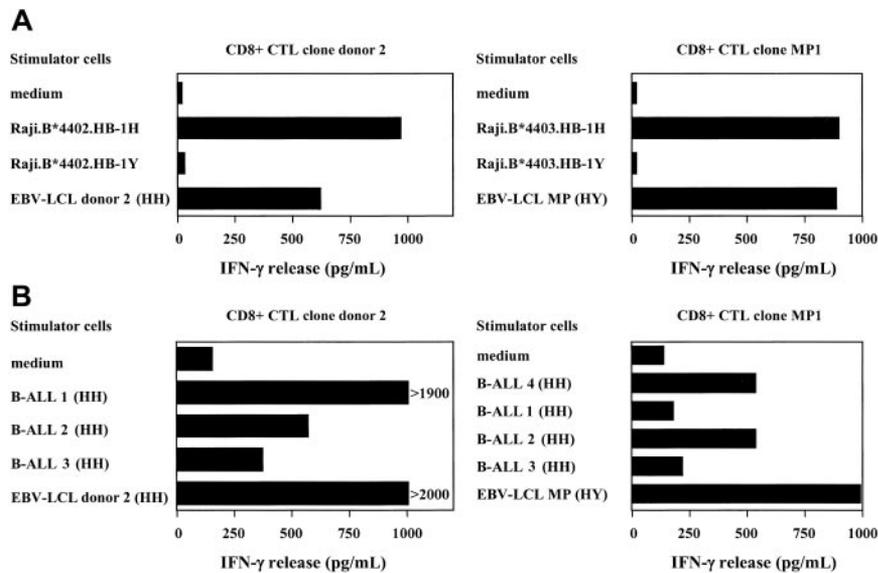
A

B

Figure 2. Specific reactivity of in vitro–induced anti–HB-1H.B44 CTL clone donor 2 in comparison with in vivo–induced CTL MP1. Production of IFN-γ of CTL clone donor 2 and MP1 was determined on stimulation with (A) Raji cells transfected with HLA-B44 (B*4402 or B*4403) plus HB-1H or HB-1Y and EBV-transformed B-cell lines endogenously expressing HB-1, and (B) B-ALL cells of HLA-B44–positive patients. HB-1 typing for each stimulator cell is shown in parenthesis. HLA-B44 subtype of patients 1, 2, and 3 is B*4402 and of patient 4 is B*4403.

individuals, we stimulated CD8+ T cells isolated from healthy donors 3 times with autologous peptide-loaded mature DCs. Specificity of the initiated T-cell cultures was tested by chromium release assays. Six of 8 in vitro–induced CTL lines significantly lysed peptide-pulsed Raji cells transfected with either HLA-B*4402 or HLA-B*4403 according to the donor HLA-B44 subtype (Table 1). Cytolytic activity of 3 HB-1H–specific CTL lines (CTL donor lines 1, 2, and 6) against unpulsed and peptide-pulsed autologous target cells is shown in Figure 1. All 3 CTL lines significantly lysed HB-1H–pulsed autologous PHA-stimulated T-cell blasts, whereas HB-1Y–pulsed T-cell blasts were not recognized. Furthermore, all 3 CTL lines recognized autologous EBV-transformed B cells endogenously expressing the *HB-1H* allele (Figure 1). Nonspecific lysis of K562 cells was not observed. These results clearly demonstrate that anti–HB-1H CTLs can be generated in vitro using peptide-loaded mature DCs and exert specific cytolytic activity. This finding implicates that their precursors have not been deleted from the T-cell repertoire of HLA-B44–positive individuals positive for the *HB-1H* allele.

Autologous CD8+ CTLs recognize the naturally presented HB-1H.B44 epitope

Next, we characterized in more detail HB-1–specific autologous CTLs generated in vitro and compared reactivity with that of in vivo–induced allogeneic anti–HB-1H CTL MP1. To isolate HB-1–specific CTLs from bulk cultures, we used the IFN-γ secretion assay. CTL donor line 2 contained 20% IFN-γ–producing CD8+ T cells (data not shown). These IFN-γ–secreting cells were sorted by

flow cytometry and were restimulated weekly with autologous peptide-pulsed EBV-LCLs. TCR repertoire analysis of the resulting culture revealed that more than 95% were positive for TCRBV2 and CD8 (data not shown). CTL clone donor 2 recognizes specifically the endogenously processed HB-1H epitope but not the HB-1Y epitope, like the allogeneic CTL MP1 (Figure 2A).

To characterize the avidity of CTL clone donor 2, we tested for IFN-γ release on stimulation with Raji.B*4402 cells pulsed with different peptide concentrations, ranging from 0.01 to 10 μM. The in vitro–induced CTL clone donor 2 started to recognize Raji.B*4402 cells pulsed with approximately 300 nM (data not shown). In contrast, the in vivo–induced CTL MP1 started to recognize Raji.B*4403 cells pulsed with very low concentrations of peptide (approximately 3 nM; data not shown). Autologous EBV-LCLs naturally expressing HB-1H clearly stimulated CTL clone donor 2 to release IFN-γ, despite the lower avidity compared with CTL MP1 (Figure 2A).

We have shown previously that the in vivo–induced CTL MP1 mediates cytokine release and cytotoxicity against allogeneic B-ALL cells expressing HB-1.[13,14] To determine whether the in vitro–induced HB-1–specific CTL clone donor 2 also recognizes B-ALL cells, we have tested tumor cells obtained from patients at diagnosis for their ability to induce cytokine production. As shown in Figure 2B, significant amounts of IFN-γ are released by CTL clone donor 2 when stimulated with allogeneic HLA-B*4402–positive B-ALL tumor cells. Furthermore, B-ALL cells of the B*4403-positive patient 4 and 1 of 3 B*4402-positive patients were recognized by CTL MP1 (Figure 2B). This finding confirms
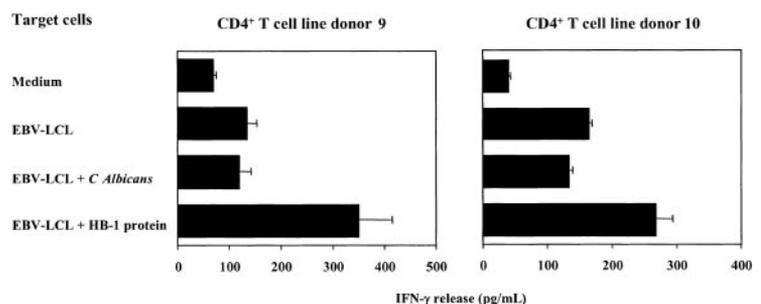

Figure 3. Specific reactivity of CD4+ T cells stimulated 3 times with autologous DCs loaded with the HB-1 protein. IFN-γ production of the established T-cell lines was tested on stimulation with autologous EBV-LCLs either untreated or pulsed with 25 μg/mL HB-1 protein or *Candida albicans* protein extract. Error bars represent 1 SD of triplicates.
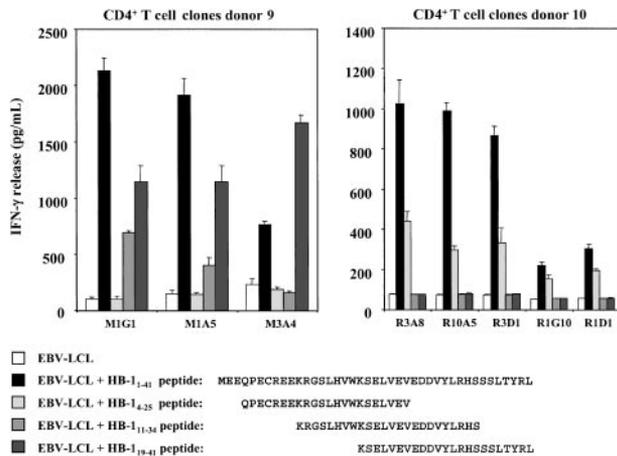
Figure 4. Localization of the Th epitopes recognized by the CD4+ T-cell clones generated from the HB-1–specific bulk cultures. Production of IFN-γ by the CD4+ clones was tested on stimulation with autologous EBV-LCLs pulsed with the HB-1 protein or HB-1–derived peptides. Error bars represent 1 SD of triplicates.

our previous results that CTL MP1 recognizes the HB-1 peptide much more efficiently in the context of the HLA-B*4403 subtype (data not shown).

Together, these data demonstrate that autologous anti–HB-1 CTLs secrete IFN-γ on stimulation with the endogenously processed epitope on HB-1–expressing cell lines and B-ALL tumor cells.

Autologous CD4+ Th responses against HB-1 can be induced by mature monocyte-derived DCs

To investigate the potential of the HB-1 protein to induce Th responses, we stimulated CD4+ T cells isolated from 2 healthy donors (donors 9 and 10) with autologous protein-loaded mature monocyte-derived DCs. After 3 rounds of stimulation, the induced T-cell lines were tested for HB-1 specificity in cytokine release assays. As shown in Figure 3, we obtained 2 T-cell lines that on stimulation with autologous EBV-LCLs pulsed with the HB-1 protein produced higher levels of IFN-γ than on stimulation with untreated EBV-LCLs or EBV-LCLs pulsed with *C albicans* protein extract. Next, HB-1–specific CD4+ T-cell lines were cloned by limiting dilution. Three and 5 CD4+ clones recognizing the full-length HB-1 protein were obtained from donor 9 (M1G1, M1A5, and M3A4) and donor 10 (R3A8, R10A5, R3D1, R1G10, and R1D1), respectively (Figure 4). To determine which part of the HB-1 protein is recognized by these T-cell clones, 3 overlapping peptides were generated: designated as HB-1$_{4-25}$, HB-1$_{11-34}$, and HB-1$_{19-41}$. T-cell clones M1G1 and M1A5 of donor 9 recognized EBV-LCLs loaded with the peptides HB-1$_{11-34}$ and HB-1$_{19-41}$, whereas clone M3A4 recognized only the HB-1$_{19-41}$ peptide (Figure 4). Interestingly, all HB-1–specific CD4+ clones isolated

from donor 10 were specific for the N-terminal HB-1$_{4-25}$ peptide. These data clearly demonstrate that CD4+ Th responses can be induced against the HB-1 protein by using mature monocyte-derived DCs. We isolated several CD4+ Th clones specific for different epitopes within the HB-1 protein.

HB-1–specific CD4+ Th clones recognize HLA-DR–restricted epitopes

Next, we determined the MHC class II restriction molecule for clones M3A4 and R3A8 recognizing distinct HB-1 epitopes. Recognition of autologous EBV-LCLs pulsed with the appropriate peptide was inhibited by anti–HLA-DR mAb, whereas anti–HLA-DP and -DQ mAbs had no effect (Figure 5). To further identify the HLA-DR molecule involved in presentation of the HB-1 epitopes, we tested a panel of EBV-LCLs sharing one or more HLA-DR molecules with the autologous EBV-LCLs. Clone M3A4 recognized 2 individuals sharing DR4 and DR53 with the autologous EBV-LCLs (Table 2). However, EBV-LCLs of 4 individuals positive for DR53 but negative for DR4 were not recognized, indicating that the epitope recognized by clone M3A4 is presented by DR4. Clone R3A8 recognized EBV-LCLs sharing both DR11 and DR52 with the autologous EBV-LCLs (Table 3). Four individuals sharing only DR52 were not recognized, indicating that this epitope is presented by DR11. These data demonstrate that the HB-1 antigen contains distinct Th epitopes presented by either HLA-DR4 or HLA-DR11.

Identification of DR4- and DR11-restricted HB-1 Th epitopes

To define the minimal HB-1 epitopes recognized by CD4+ clones M3A4 and R3A8, a set of overlapping peptides were pulsed at various concentrations on autologous EBV-LCLs and tested for recognition. CD4+ clone M3A4 recognized peptide EDDVYLRH-SSSLTYRL (HB-1$_{26-41}$) very efficiently (Figure 6A). Truncation of 3 amino acids, 26 to 28 or 39 to 41, resulted in a significant decrease in recognition, whereas deletion of one amino acid, 26 or 41, resulted only in a minor decrease. This finding indicates that the shortest peptide well recognized by CD4+ clone M3A4 is the 16-mer HB-1$_{26-41}$ peptide that is also predicted to bind efficiently to HLA-DRB1*0401 molecules by the epitope binding program SYFPEITHI (http://syfpeithi.bmi-heidelberg.com/scripts/MHC-Server.dll/home.htm; data not shown). Half-maximal IFN-γ release was obtained by incubating EBV-LCL stimulator cells with approximately 40 nM of this peptide, indicating high avidity of CD4+ clone M3A4 for the epitope. This finding is concordant with results obtained for other HLA class II–restricted epitopes.[20-22]

Three peptides spanning the amino acid sequence EQPECREEKRGSLHV (HB-1$_{3-17}$) were recognized by CD4+ clone R3A8 (Figure 6B). Truncation of the glutamic acid at position 3 resulted in a decrease in recognition, which could be
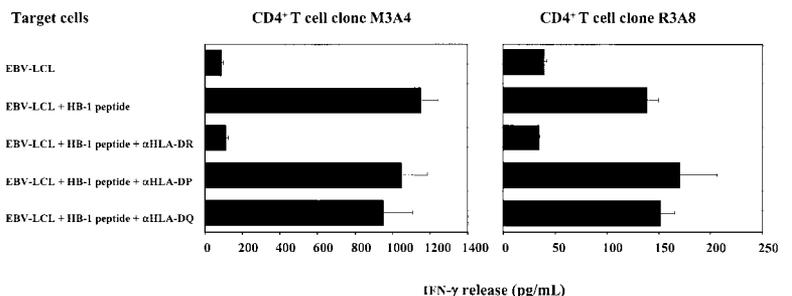
Figure 5. Inhibition of IFN-γ release by anti–HLA class II mAb. HB-1–specific CD4+ clones M3A4 and R3A8 were stimulated with autologous EBV-LCLs pulsed with the HB-1$_{19-41}$ peptide or the HB-1$_{4-25}$ peptide, respectively. Blocking studies were performed by using purified anti–HLA-DR (L234), anti–HLA-DP (B7/21), or anti–HLA-DQ (Tü22) mAb (10 μg/mL) which were present throughout the assay. Error bars represent 1 SD of triplicates.

Table 2. Presentation of the HB-1 epitope to CD4$^+$ T-cell clone M3A4 by HLA-DR4

EBV-LCLs	HLA-DR specificity				IFN-γ production, pg/mL*	
					−Peptide	+Peptide
Donor 9	DR3$^+$	DR4$^+$	DR52$^+$	DR53$^+$	<	748
No. 11	DR3$^+$	DR4$^-$	DR52$^+$	DR53$^-$	<	<
No. 12	DR3$^+$	DR4$^-$	DR52$^+$	DR53$^-$	<	<
No. 13	DR3$^+$	DR4$^-$	DR52$^-$	DR53$^-$	<	<
No. 14	DR3$^-$	DR4$^+$	DR52$^-$	DR53$^+$	<	895
No. 15	DR3$^-$	DR4$^+$	DR52$^-$	DR53$^+$	<	544
No. 16	DR3$^-$	DR4$^-$	DR52$^+$	DR53$^+$	<	<
No. 17	DR3$^-$	DR4$^-$	DR52$^+$	DR53$^-$	<	<
No. 18	DR3$^-$	DR4$^-$	DR52$^+$	DR53$^-$	<	<
No. 19	DR3$^-$	DR4$^-$	DR52$^-$	DR53$^+$	<	<
No. 20	DR3$^-$	DR4$^-$	DR52$^-$	DR53$^+$	<	<
No. 21	DR3$^-$	DR4$^-$	DR52$^-$	DR53$^+$	<	<

*EBV-LCLs were incubated for 18 hours with 25 μg/mL HB-1$_{19-41}$ peptide and washed. CD4$^+$ T-cell clone M3A4 was then incubated for 20 hours with 30 000 untreated and peptide-pulsed EBV-LCLs. IFN-γ released in the supernatant was measured by ELISA. < indicates < 70 pg/mL IFN-γ.

Figure 6. Identification of the HLA-DR–restricted Th epitopes recognized by CD4$^+$ clones M3A4 and R3A8. Autologous EBV-LCLs were pulsed at various concentrations with HB-1–derived peptides and tested for recognition by the CD4$^+$ T-cell clones M3A4 (A) and R3A8 (B).

improved by the addition of the valine at position 17. Peptide EQPECREEKRGSLHV is also predicted to bind to HLA-DRB1*1101 molecules by the epitope-binding program SYFPEITHI (data not shown). These data indicate that the HLA-DR11–restricted epitope recognized by CD4$^+$ clone R3A8 is the 15-mer HB-1$_{3-17}$ peptide. High concentrations of peptide were needed to stimulate clone R3A8 to release IFN-γ, indicating low avidity of this CD4$^+$ clone for the HB-1.DR11 epitope (Figure 6B).

Discussion

Effective cellular immunotherapy for leukemia needs identification of specific tumor antigens that are recognized by the immune system. A potential group of antigens are those proteins that are differentially (over) expressed in hematopoietic tumor cells. So far, only a few of these leukemia-associated antigens (proteinase 3 and WT1) have been shown to induce autologous CTL responses.[10-12] Previously, we have identified HB-1 as a polymorphic leukemia-associated antigen that is recognized by donor-derived CTLs on allogeneic tumor cells of patients with B-ALL.[13,14] Its B-cell–restricted expression pattern strongly suggests that the HB-1 protein could serve as an autologous vaccine target to induce

Table 3. Presentation of the HB-1 epitope to CD4$^+$ T-cell clone R3A8 by HLA-DR11

EBV-LCLs	HLA-DR specificity			IFN-γ production, pg/mL*	
				−Peptide	+Peptide
Donor 10	DR11$^+$	DR13$^+$	DR52$^+$	<	92
No. 22	DR11$^+$	DR13$^-$	DR52$^+$	<	89
No. 23	DR11$^+$	DR13$^-$	DR52$^+$	<	196
No. 24	DR11$^-$	DR13$^+$	DR52$^+$	<	<
No. 25	DR11$^-$	DR13$^-$	DR52$^+$	<	<
No. 26	DR11$^-$	DR13$^-$	DR52$^+$	<	<
No. 27	DR11$^-$	DR13$^-$	DR52$^+$	<	<
No. 28	DR11$^-$	DR13$^-$	DR52$^-$	<	<
No. 29	DR11$^-$	DR13$^-$	DR52$^-$	<	<

*EBV-LCLs were incubated for 18 hours with 25 μg/mL HB-1$_{4-25}$ peptide and washed. CD4$^+$ T-cell clone R3A8 was then incubated for 20 hours with 30 000 untreated and peptide-pulsed EBV-LCLs. IFN-γ released in the supernatant was measured by ELISA. < indicates < 40 pg/mL IFN-γ.
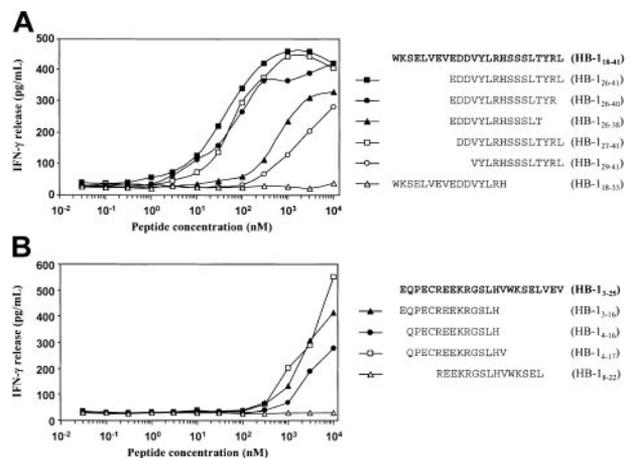
antitumor immunity in patients with B-ALL after primary treatment. In the autologous setting, HB-1 represents a B-cell–specific "self-antigen" inducing T-cell tolerance. However, it has been shown that toward many tissue-specific differentiation antigens autologous T-cell immunity can be induced by using professional antigen-presenting dendritic cells by breaking immunologic tolerance.[19] Here, we addressed whether autologous HB-1–specific T-cell responses can be induced by in vitro stimulation with antigen-pulsed monocyte-derived mature DCs. We observed that HB-1–specific CTL responses against the previously identified HLA-B44–restricted peptide EEKRGSLHVW could be generated in 6 of 8 healthy donors. This finding indicates that HB-1–specific T-cell precursors have not been deleted from the T-cell repertoire of healthy individuals. Apparently, HB-1 is immunologically ignored because of its relatively low expression as suggested for other differentially expressed tumor antigens.[19]

For 3 of the in vitro–induced HB-1–specific CTL lines, we showed specific lysis of autologous EBV-transformed B-cell lines naturally expressing and presenting the HB-1.B44 epitope as well as peptide-loaded target cells. From one of these CD8$^+$ T-cell lines, we isolated CTL clone donor 2 that produces IFN-γ on stimulation with Raji cells transfected with the HB-1 cDNA in the context of HLA-B*4402. Like the in vivo–induced HB-1–specific CTL MP1, CTL clone donor 2 releases significant amounts of IFN-γ when stimulated with B-ALL tumor cells obtained from HLA-B44–positive patients. From these findings, we can conclude that DCs pulsed with the HLA-B44–restricted HB-1 peptide induce autologous CTLs with the ability to recognize B-ALL tumor cells.

Because of its capacity to mediate direct killing of tumor cells, much attention has been focused on CD8$^+$ T-cell–mediated antitumor immunity. However, preclinical data clearly demonstrate that CD4$^+$ Th cells are important in inducing effective antitumor responses.[16-18] For instance, Ossendorp et al[17] showed that tumor protection against a highly aggressive MHC class II–negative T-lymphoma cell line was enhanced when a tumor-specific CTL epitope was injected simultaneously with a tumor-specific CD4$^+$ Th epitope. The requirement of CD4$^+$ T-cell help during the induction of antitumor CTL responses was also demonstrated in the human setting.[23] It has been shown that many TAAs contain CD4$^+$ Th epitopes besides CTL epitopes.[24] Here, we demonstrate that HB-1 induces CD4$^+$ Th responses. We identified 2 HB-1–derived

Th epitopes presented by different HLA-DR subtypes. Peptide HB-1[26-41] (EDDVYLRHSSSLTYRL) was recognized by CD4+ clone M3A4 in the context of HLA-DR4. This epitope is not only predicted to bind to HLA-DR4 but also to HLA-DR3 and HLA-DR11 as determined by the SYFPEITHI peptide-binding program. It is known that Th peptides can be promiscuous in binding to MHC class II molecules.[25,26] Therefore, peptide HB-1[26-41] could serve as a widely applicable Th epitope in HB-1–based vaccination. Interestingly, the peptide HB-1[3-17] (EQPECREEKRGSLHV), recognized by CD4+ clone R3A8 in the context of HLA-DR11, overlaps almost completely the HLA-B44–restricted CTL epitope EEKRGSLHVW. In conclusion, our present data show that the leukemia-associated HB-1 antigen contains both CD4+ and CD8+ T-cell epitopes as described for many other human tumor antigens.[24]

DCs pulsed with short MHC class I–binding synthetic peptides have been used for inducing CD8+ T-cell immunity against cancer.[27-30] The observed immunologic and clinical responses were limited and require further development to improve efficacy. Several other formulations of tumor antigens can be used to load DCs such as tumor cell lysates, apoptotic bodies, purified proteins, or nucleic acids.[31] An even more interesting approach is the use of long synthetic peptides of 30 to 40 amino acids containing both Th and CTL epitopes.[32] The advantage of these long peptides may be more efficient cellular uptake and processing, resulting in a high-epitope density at the cell surface. It has been shown that the physical linkage of MHC class I– and class II–restricted epitopes within long peptides potentiates specific CTL responses.[32-34] DCs pulsed with the 40-aa synthetic HB-1 protein can be recognized by both HB-1–reactive CD4+ as well as CD8+ T-cell clones (data not shown). Currently, we are analyzing whether this 40-aa long protein induces more vigorous CTL responses in vitro.

In this report, we demonstrate that the B-cell leukemia-associated antigen HB-1 is able to induce both CD4+ Th and CD8+ CTL responses, implicating that HB-1 is a suitable target for B-ALL–specific immunotherapy. On the basis of the results of our previous and present studies, a clinical pilot study has been initiated to use autologous HB-1 protein-pulsed DCs in patients with residual disease after primary treatment. A major challenge of such vaccination studies will be the correlation between immunologic responses and the disappearance of residual tumor cells.

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