maintained in RPMI 1640 medium supplemented with 10% FCS (Immuno-Biological Laboratory, Gunma, Japan), 2 mM L-glutamine, and penicillin/streptomycin (referred to as ''culture medium''). The B-LCL were transduced with retroviral vectors carrying individual HLA class I or class II cDNAs, as described previously.[5] The mAbs used in the present study were against the following antigens: pan HLA class I, HLA-DR, HLA-DQ, HLA-DP, CD4, CD8, CD45, and CD34 (all from BD Biosciences, Franklin Lakes, NJ, USA); HLA-DR8 (One Lambda, Canoga Park, CA, USA); and FITC-conjugated rabbit anti-mouse IgM (BD Biosciences). The mAb blocking experiments were performed using final concentrations of 20 μg/mL mAb. Stained cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Generation of CTL lines and clones. The CTL lines were generated from the $CD8^+$ fraction of post-HSCT PBMC after three stimulations with irradiated (33 Gy) pre-HSCT recipient PBMC. Interleukin (IL)-2 (20 U/mL; Chiron, Emeryville, CA, USA) was added on Days 1 and 5 after the second and third stimulation. The CTL clones were generated by limiting dilution and expanded as described previously[6,7] and were frozen until use. All cultures were performed in RPMI 1640 medium supplemented with 4% pooled human serum, 2 mM L-glutamine, and penicillin/streptomycin (referred to as ''CTL medium'').

Purification of $CD34^+$ leukemia cells using magnetic beads. Primary leukemic cells carrying HLA-DRB1*08:03 that had been collected and cryopreserved at the time of diagnosis were thawed and positively selected for $CD34^+$ subsets using phycoerythrine (PE)-conjugated anti-CD34 mAb (BD Biosciences) and anti-PE immunomagnetic beads through MACS MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cytotoxicity assays. Target cells were radiolabelled with 3.7 MBq ^{51}Cr for 2 h and 1×10^3 target cells/well were mixed with CTL at various effector/target (E/T) ratios in a standard 4-h cytotoxicity assay using 96-well round-bottomed plates. All assays were performed at least in duplicate. Primary dermal fibroblasts from the skin were treated with interferon (IFN)-γ (100 U/mL; Endogen, Woburn, MA, USA) and tumor necrosis factor (TNF)-α (10 ng/mL; Endogen) for 48 h or 7 days, as indicated. Percentage specific lysis was calculated as follows:

(Experimental c.p.m. − Spontaneous c.p.m.)/(Maximum c.p.m. − Spontaneous c.p.m.) × 100

Leukemic stem cell engraftment assay in immunodeficient mice. Non-obese diabetic/severe combined immunodeficient/γc-null (NOG) mice[4] were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). All mice were maintained under specific pathogen-free conditions in the Aichi Cancer Center Research Institute. The Ethics Review Committee of the Institute approved the experimental protocol. The $CD34^+$ fraction (3.0×10^6) of Philadelphia chromosome (Ph)-positive primary acute lymphoblastic leukemia (ALL) cells was preincubated for 16 h in CTL medium supplemented with 20 units/mL IL-2 at 37°C with 5% CO_2 either alone or in the presence of CTL-1H8 or a control CTL-1B9 (HLA-A*24:02-restricted, minor histocompatibility antigen-specific CTL[8]) at a T cell:ALL cell ratio of 1:1. Thereafter, the cultures were harvested, resuspended in a total volume of 300 μL CTL medium, and inoculated via the tail vein into 8–10-week-old NOG mice. Six to 7 weeks after inoculation, mice were killed, peripheral blood was aspirated from the heart, and BM cells were obtained by flushing the femora with complete medium. Nucleated cells were analyzed for the expression of human CD45, human CD34, or HLA-DR.

Limiting dilution-based CTLp frequency assay. The proportion of CTLp specific for the HLA-DRB1*08:03 of the total CTLp against potential recipient alloantigens was quantitated using a standard limiting dilution assay. Purified $CD8^+$ T cells from the PBMC obtained on specific days after HSCT, as indicated, were cultured at twofold serial dilutions with 33 Gy-irradiated 3×10^4 CD40-activated B (CD40-B) cells generated from pre-HSCT recipient PBMC in 96-well round-bottomed plates in CTL medium.[5] On Days 2 and 5, 50 U/mL IL-2 was added after each restimulation. There were at least 12 replicates for each dilution. After three rounds of stimulation, a split-well analysis was performed for HLA-DRB1*08:03-specific cytotoxicity against ^{51}Cr-radiolabeled donor B-LCL with or without HLA-DRB1*08:03 cDNA transduction or recipient B-LCL. The wells were considered to be positive if the total c.p.m. released by the effector cells was >3 SD above that in control wells (mean c.p.m. released by the target cells incubated with irradiated stimulator cells alone). In addition, $CD8^+$ cells from another recipient receiving HLA class II-mismatched HSCT were tested in a similar way. Finally, the CTLp frequency was calculated using L-Calc software (StemCell Technologies, Vancouver, BC, Canada).[9]

Results

Cytotoxicity of the $CD8^+$ CTL clone against allogeneic HLA-DRB1*08:03-positive hematopoietic cells. In all, 27 clones cytotoxic to recipient but not donor B-LCL were isolated by limiting dilution from $CD8^+$ T cells obtained on Day 207 after HSCT. Based on HLA restriction analysis using partially HLA-matched panel B-LCL, three groups of clones were identified: the first two groups (five in group 1 and 14 in group 2) were potentially restricted by HLA-A*24:02, A*33:03, B*44:03, and C*14:03 and showed lytic activity against cytokine-treated fibroblasts; the remaining eight clones in group 3 showed no lytic activity against cytokine-treated fibroblasts and were potentially restricted by HLA-A*24:02 or C*14:02. Because our primary goal was to generate CTL clones that recognized hematopoietic cells, including leukemic cells for selective GVL effect induction,[10] we omitted the group 1 and 2 clones. Of the eight group 3 clones, we chose CTL-1H8 as a representative CTL clone for further analysis owing to its superior lytic and expansion performance.

The CTL-1H8 clone was $CD8^+$ (Fig. 1a) and efficiently lysed recipient B-LCL and phytohemagglutinin-stimulated T cell lines (PHA-blasts) but not donor LCL (Fig. 1b), indicating that CTL-1H8 recognized recipient-specific alloantigen. Surprisingly, antibody-blocking experiments revealed that lytic activity against recipient B-LCL was significantly inhibited by the addition of anti-HLA-DR mAb and anti-CD8 mAb (Fig. 1c). This led us to re-examine CTL-1H8 HLA restriction using B-LCL with or without cDNA transduction of HLA-DRB1*08:03, which was mismatched between the recipient and donor. As shown in Figure 1(d), CTL-1H8 lytic activity was observed only when donor B-LCL, irrelevant B-LCL, or 721.221 B-LCL (all HLA-DRB1*08:03 deficient) were transduced with HLA-DRB1*08:03 cDNA, indicating unexpectedly that CTL-1H8 was restricted by HLA class II molecules, which are generally thought to be recognized by $CD4^+$ T cells. Because 21.221 B-LCL was deficient for HLA class I molecules, the possibility of presentation of the HLA-DBB1*0803-derived peptides to CTL-1H8 is unlikely.

Because HLA class II expression is restricted to hematopoietic cells and a fraction of activated non-hematopoietic cells, CTL recognizing HLA class II molecules could selectively mediate the GVL effect without GVHD.[11,12] Thus, we examined whether HLA-DRB1*08:03 expression on dermal fibroblasts and their susceptibility to CTL may change before and after cytokine treatment. To this end, the recipient dermal fibroblasts were incubated with IFN-γ and TNF-α for 2 or 7 days and analyzed for HLA-DR8 expression with a DR8-specific mAb. As shown in Figure 2(a), cytokine treatment for 2 days did not induce HLA-DR8 expression at all, whereas 7 days of treatment

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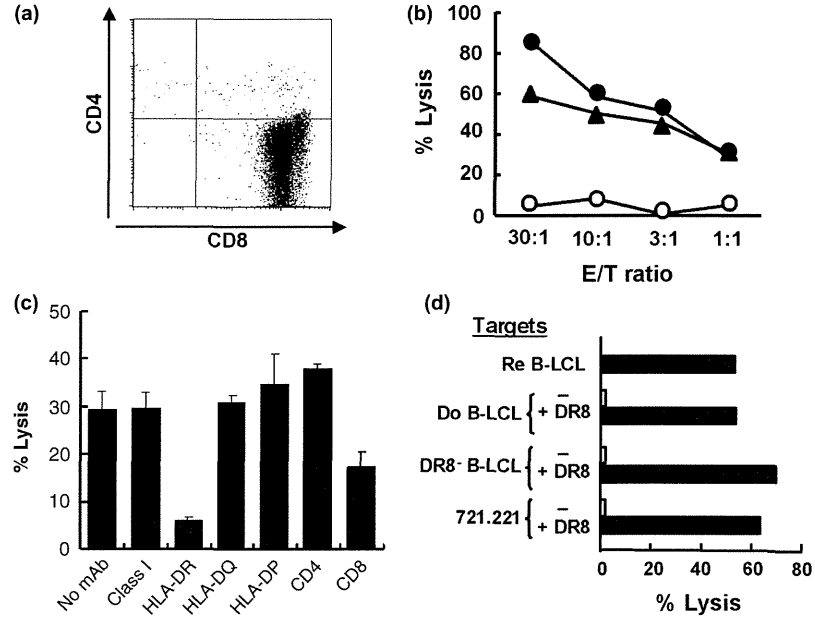

Fig. 1. Characteristics of the human leukocyte antigen (HLA)-DR8-restricted CTL clone, 1H8. (a) The cytolytic activity of CTL-1H8 was evaluated in a standard 4-h ^{51}Cr release assay. The recognition by CTL-1H8 of target cells was examined against B-lymphoblastoid cell lines (B-LCL) derived from the recipient (Re) and donor (Do), and phytohemagglutinin-stimulated T cell lines (PHA blasts). (b) Flow cytometric analysis of CTL-1H8 for CD4 and CD8. (●), Re B-LCL; (○), Do B-LCL; (▲), Re PHA blasts. (c) Antibody blocking of cytolysis was performed at an effector/target (E/T) ratio of 3:1, with the mAbs indicated at a final concentration of 10 μg/mL. (d) The HLA-DR8-negative B-LCL from the donor and an unrelated individual were transduced with mock or HLA-DRB1*08:03-encoding retroviral vector and tested with CTL-1H8 at an E/T ratio of 10:1.

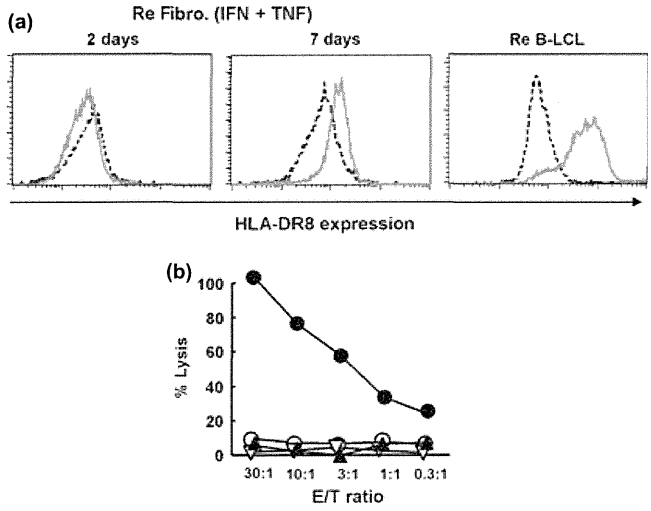

Fig. 2. Induction of human leukocyte antigen (HLA)-DR8 by cytokines and susceptibility of cytokine-treated dermal fibroblasts to CTL-1H8. (a) The recipient (Re) dermal fibroblasts (Fibro.) were incubated with 100 U/mL interferon (IFN)-γ and 10 ng/mL tumor necrosis factor (TNF)-α for 2 or 7 days and analyzed for HLA-DR8 expression with DR8-specific mAb. A recipient B-lymphoblastoid cell lines (B-LCL) was used as a positive control. (b) Recipient fibroblasts with or without 7 day cytokine treatment were tested for lysis by CTL-1H8 at indicated effector/target (E/T) ratios, in conjunction with recipient and donor (Do) B-LCL as positive and negative controls, respectively. (●), Re B-LCL; (○), Do B-LCL; (▽), Re Fibro.; (▲), Re Fibro. (IFN + TNF, 7 days.)

resulted in an approximate fourfold upregulation. However, the expression level was 1 log lower than that observed for recipient B-LCL (Fig. 2a, right panel). Despite HLA-DR8 upregulation, fibroblasts treated for 7 days were not lysed by CTL-1H8 at all (Fig. 2b), suggesting that the recognition of the HLA-DRB1*08:03 complex by CTL-1H8 may require HLA-bound antigenic peptides that are not produced in fibroblasts or that such weak upregulation may not be sufficient for recognition by CTL-1B8. The latter possibility may be less likely because primary ALL cells with similar HLA-DR8 expression were moderately lysed by CTL-1H8 (see below).

HLA-DR8 expression in primary leukemia cells and their susceptibility to CTL-1H8. Expression of DR8 on primary leukemia cells was first examined in conjunction with CD34, which has been shown to be a stem cell marker in humans.[13] Of 51 PBMC or BM specimens from leukemia patients, five had the HLA-DRB1*08:03 or DRB1*08:02 genotype, of which three samples contained a substantial fraction of $CD34^+$ cells, all of which were from patients with ALL (Ph-ALL: HLA-DRB1*08:02; B-ALL#1 and B-ALL-#2: HLA-DRB1*08:03), and had a significant fraction of double-positive cells (Fig. 3a). We next tested whether positively selected $CD34^+$ fractions from the three ALL samples (Fig. 3b) were susceptible to CTL-1H8. As shown in Figure 3(c), the $CD34^+$ fraction from all three ALL samples was lysed by CTL-1H8 and no natural killer activity against HLA-deficient K562 cells was observed. Although the Ph-ALL sample carried the HLA-DRB1*08:02 genotype, the cells were lysed by CTL-1H8, suggesting that the single amino acid difference in the HLA-DRB1 α1 domain between *08:03 and *08:02 did not affect recognition by CTL-1H8.

Inhibition of human Ph-positive ALL cell engraftment in NOG mice by CTL-1H8. In order to determine whether HLA-DR8 recognized by CTL-1H8 is indeed expressed on leukemic stem cells and thus may have been involved in a GVL effect, we performed the leukemic stem cell (LSC) engraftment assay, as reported previously,[14] using NOG mice.[4] Because we were unable to obtain $CD34^+$ fractions of primary leukemic cells from the present patient, we selected Ph-positive primary ALL (Ph-ALL) leukemic cells (positive for HLA-A*24:02 and DRB1*08:02) for this assay because they were found to be negative for the HLA-A*24:02-restricted minor histocompatibility antigen ACC-1C and were not lysed by the ACC-1C-specific clone CTL-1B9[8] (data not shown), which was used as an irrelevant control (see Materials and Methods). Flow cytometric analysis of the harvested cells was conducted to investigate the expression of human CD45 and CD34. The BM cells of three control mice receiving Ph-ALL $CD34^+$ cells that were cultured in medium alone (n = 1) or with control CTL-1B9 (n = 2) prior to inoculation were found to contain 96.5%, 32.9%, and 10.9% human $CD45^+$ $CD34^+$ cells (Fig. 4a–c), whereas the PBMC of the same three mice contained 65.2%, 5.7%, and 9.6% human $CD45^+$ $CD34^+$ cells (data not shown). In contrast, human cells were undetectable in both BM and PBMC of mice inoculated

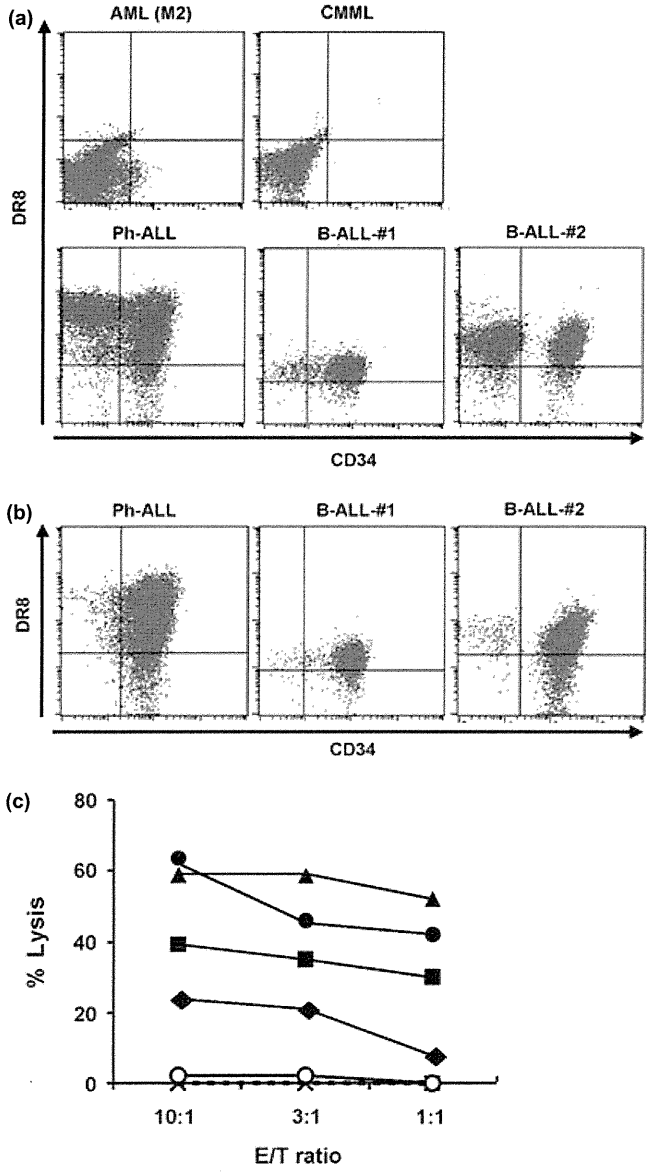

Fig. 3. Expression of human leukocyte antigen (HLA)-DR8 in primary leukemia cells and their susceptibility to CTL-1H8. (a) Expression of DR8 on five primary leukemia samples possessing the HLA-DRB1*08:02 or DRB1*08:03 genotype was examined in conjunction with CD34. (b) Purity and HLA-DR8 expression of leukemia cells after positive selection using anti-CD34-PE antibody followed by capture with anti-phycoerythrine (PE)-immunomagnetic beads. (c) Lysis of the $CD34^+$ fraction from all three acute lymphoblastic leukemia (ALL) samples by CTL-1H8, in conjunction with recipient (Re) and donor (Do) B-lymphoblastoid cell lines (B-LCL) as positive and negative controls, respectively. (●), Re B-LCL; (○), Do B-LCL; (▲), Philadelphia chromosome (Ph)-ALL; (■), B-ALL#1; (◇), B-ALL-#2; (×), K562. M2, French–American–British subtype M2; CMML, chronic myelomonocytic leukemia; E/T, effector/target. The HLA-DRB1 types of the three ALL samples were as follows: Ph-ALL, *08:02; B-ALL-#1, *08:03; and B-ALL-#2, 08:02.

with Ph-ALL cells precultured with CTL-1H8 ($n = 3$; 0.07%, 0.01%, and 0.07% human $CD45^+$ $CD34^+$ cells in BM cells; Fig. 4d–f).

After HLA-DR mismatched HSCT, HLA-DR-specific $CD8^+$ T cells are detectable in recipient post-transplant PBMC. A split-well assay was used to estimate the relative frequencies of $CD8^+$

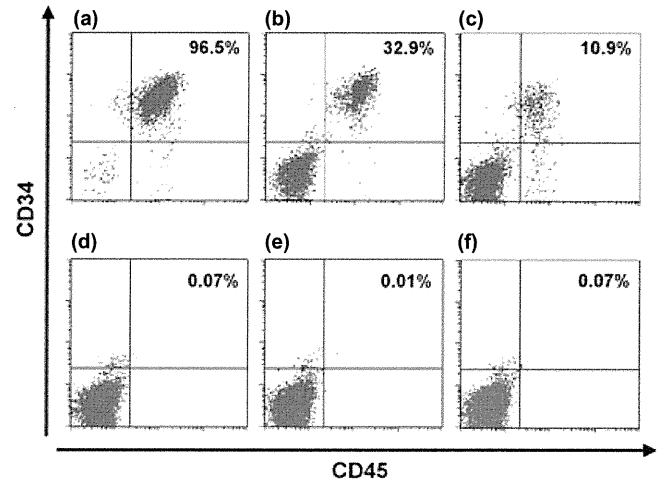

Fig. 4. Inhibition of human Philadelphia chromosome acute lymphoblastic leukemia (Ph-ALL) stem cell engraftment in non-obese diabetic/severe combined immunodeficient/γc-null (NOG) mice by CTL-1H8. The $CD34^+$ fraction (3.0×10^6) of a Ph-positive primary ALL sample was preincubated for 16 h either alone or in the presence of CTL-1H8 or a control CTL-1B9 (see Materials and Methods) and inoculated via the tail vein into 8–10-week-old NOG mice. Six to 7 weeks after inoculation, mice were killed and peripheral blood mononuclear cells and bone marrow (BM) cells were obtained. Flow cytometric profiles are shown for the expression of human CD45 and CD34 of BM cells from (a) control mice receiving Ph-ALL $CD34^+$ cells cultured in medium alone or (b,c) with control CTL-1B9 or (d–f) with CTL-1H8 prior to inoculation.

CTLp specific for HLA-DRB1*08:03 and those specific for all alloantigens expressed on the recipient's hematopoietic cells in the post-HSCT PBMC, as reported previously.[9] As shown in Figure 5 (left panel), the frequency of CTLp reactive with recipient B-LCL and HLA-DRB1*08:03-transfected donor B-LCL in peripheral blood $CD8^+$ cells obtained on Day 207 after HSCT, from which the CTL-1H8 was derived, was 1/1317 (95% confidence interval [CI] 1/906–1/1913) and 1/2689 (95% CI 1/1825–1/3961), respectively, indicating that nearly half the CTL responses to recipient alloantigens in this donor/recipient pair were directed at the mismatched HLA-DR8. On Day 355, the frequency of CTLp recognizing HLA-DRB1*08:03-transfected donor B-LCL was 1/22 580 (95% CI 1/14 241–1/35 801) and that for CTLp recognizing recipient B-LCL was 1/16 508 (95% CI 1/10 823–1/25 178), demonstrating that even at the later time point the $CD8^+$ CTL responses against HLA-DR8 continued to account for a significant fraction (73%) of the total donor CTL response in this donor/recipient pair (Fig. 5, right panel).

To explore whether our finding is a phenomenon limited to the present patient, we performed similar assays in another patient receiving cord blood HSCT mismatched by three loci (HLA-C, DR, and DQ). As indicated in Table 1, a small fraction (2.2–12.1% at three time points after HSCT) of the total donor $CD8^+$ CTL response in this donor/recipient pair was directed against the mismatched HLA-DRB1*12:01, whereas a slightly higher fraction (7.1–17.6%) was directed against the mismatched HLA-C*04:01. We were unable to examine the CTLp against the mismatched HLA-DQ molecule owing to an insufficient number of cells.

Discussion

To our knowledge, the present study is the first to demonstrate that $CD8^+$ CTL restricted by a mismatched HLA-DR molecule

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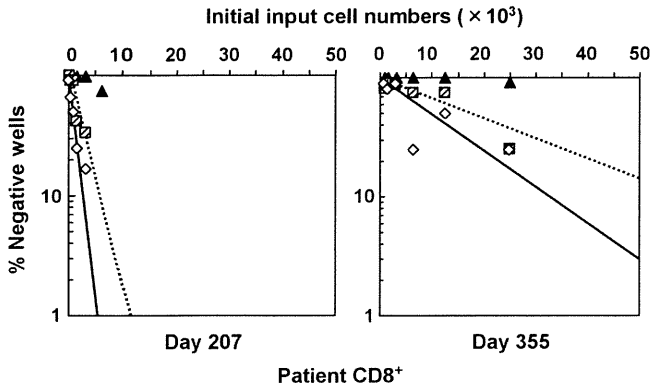

Fig. 5. Human leukocyte antigen (HLA)-DR8-specific $CD8^+$ T cells were detectable in recipient (Re) post-transplant peripheral blood mononuclear cells (PBMC) following HLA-DR-mismatched hematopoietic stem cell transplantation (HSCT). The proportion of $CD8^+$ CTL precursors specific for HLA-DRB1*08:03 among total CTL precursors (CTLp) against recipient alloantigens was quantitated using a standard limiting dilution assay with L-Calc software (StemCell Technologies, Vancouver, BC, Canada). The $CD8^+$ T cells from PBMC on Days 207 or 335 after HSCT were cultured at limiting dilution with irradiated CD40-B cells generated from pre-HSCT recipient PBMC in 96-well round-bottomed plates. After three rounds of stimulation, a split-well analysis was performed for HLA-DRB1*08:03-specific cytotoxicity against ^{51}Cr-radiolabeled recipient B-lymphoblastoid cell lines (B-LCL, -◇-) or donor B-LCL transduced with HLA-DRB1*08:03 cDNA (···▨···) or mock-transduced donor B-LCL (▲). Wells were considered positive if the total c.p.m. released by effector cells was >3 × SD above that in control wells.

Table 1. Frequency of $CD8^+$ cells against mismatched HLA-C and DR antigens in the population of CTL precursors

	Anti-Re	Anti-HLA-C*04:01 (%)	Anti-HLA-DRB1*12:01 (%)
Day 52	1/1882	1/26 651 (7.1)	1/26 101 (7.2)
Day 102	1/7577	1/84 290 (9.0)	1/346 830 (2.2)
1.3 years	1/16 807	1/95 577 (17.6)	1/139 331 (12.1)

The patient received cord blood transplantation for her T cell acute lymphocytic leukemia. Human leukocyte antigen (HLA) typing for the recipient (Re) and donor (Do) was as follows, with mismatched alleles underlined: Re: A*24:02/11:01, B*15:01/54:01, C*01:02/04:01, DRB1*04:06/12:01, DQB1*03:01/03:02, DPB1*02:01/03:01 Do: A*24:02/11:01, B*15:01/54*01, C*01:02/–, DRB1*04:06/05:05, DQB1*03:02/04:02, DPB1*02:01/*03:01. HLA, human leukocyte antigen.

are induced physiologically and can be cytotoxic against hematopoietic cells carrying the mismatched HLA-DR allele. The HLA-DRB1*08:03-restricted $CD8^+$ CTL-1H8 clone was isolated from a patient who received an HLA-DR-mismatched HSCT. At 207 days after HSCT, CTLp frequency analysis demonstrated that nearly half the $CD8^+$ T cell responses specific for any recipient-specific alloantigen were directed against the mismatched HLA-DRB1*08:03 molecule. Although we were unable to determine the magnitude of the $CD4^+$ T cell responses against the mismatched HLA-DRB1*08:03 molecule because of a paucity of PBMC, the $CD8^+$ CTLp frequency of 1/2689 on Day 207 is high enough to conclude that the isolation of CTL-1H8 was not an artifact. (The composition of the $CD8^+$ CTLp against mismatched HLA-DPB1*02:02 could not be determined in the present study, but it is possible that the remaining CTLp would be partly restricted by the HLA-DP or minor histocompatibility antigens restricted by shared HLA alleles. In this setting, the involvement of tumor antigens could not be assessed because the stimulators used in the present analysis were recipient CD40L-activated normal B cells and not leukemia cells.) This unexpected finding is supported by data from another HLA class I- and II-mismatched HSCT recipient. Because the number of patients receiving HLA-mismatched HSCT from various donors is increasing, it would be of interest to determine the kinetics of T cell reactions to individually mismatched HLA molecules depending on the type of hematopoietic stem cell donor.

It is generally believed that class II MHC-specific TCR transgenic mice predominantly give rise to $CD4^+$ T cells, whereas class I-specific TCR transgenic mice predominantly give rise to $CD8^+$ T cells. Furthermore, CD4 and CD8 are believed to activate T cells effectively when the intrinsic affinity of the TCR or antigen expression is low,[15] and these accessory molecules can work even if the interacting MHC is not directly bound to the self TCR.[16] In line with this, it has been shown that mature $CD8^+$ T cells can develop in class II MHC-specific TCR transgenic mice when CD4 is absent[17] and that polyclonal $CD4^+$ T cells transduced with the TCR molecules cloned from a $CD8^+$ WT1-specific T cell clone can lyse and/or react with their target cells.[18] In addition, the allorecognition of MHC class II molecules by $CD8^+$ T cells prepared from class II-deficient mice,[19] by those stimulated with antigen-specific B cells,[20] and by heteroclitic $CD8^+$ T cells that also recognize a class I[21] have been described. These findings imply the flexibility of coreceptor choice under unusual conditions. Thus, HLA-mismatched HSCT could be one such unusual situation where T cells may fail to follow the lineage instruction in the thymus because of highly inflammatory and immunogenic conditions after HLA-mismatched allo-HSCT.

Leukemic stem cells have a particularly strong capacity for proliferation, differentiation, and self-renewal[22] and likely play an important role in disease relapse after HSCT. Our mouse model clearly demonstrated that at least HLA-DRB1*08:03 is expressed on such stem cells and may serve as a GVL target for CTL-1H8 *in vivo*. Unfortunately, however, we could not confirm the GVL potential of CTL-1H8 against recipient leukemia cells because of a limited number of leukemia cells cryopreserved at the time of diagnosis. Because it has been shown that AML (M6) cells do not always express either HLA-DR and CD34,[23] it would need to be determined whether a small fraction of patient stem cells coexpress both HLA-DR and CD34. Nevertheless, it is of note that targeting an HLA-DR molecule alone using a specific CTL clone was sufficient to inhibit Ph-ALL LSC engraftment, suggesting that most LSC were present in the HLA-DR strongly positive, and not weakly positive or negative, population (Ph-ALL in Fig. 3b).

Finally, GVHD is still the major cause of mortality and morbidity following allo-HSCT. Therefore, selective induction of GVL is crucial. Under less inflammatory conditions, MHC class II molecules are mainly expressed only on hematopoietic cells, including leukemia cells. Thus, targeting HLA-DR molecules could be an ideal approach for this purpose. The patient in the present study has been free of disease recurrence for more than 2 years, but developed grade II aGVHD and extensive chronic GVHD. At least in the Japanese population, it has been shown that disparity in HLA-DR is much less hazardous than that in HLA-A and -B in terms of the development of severe aGVHD and mortality.[2] It remains to be determined in future studies whether targeting a mismatched HLA-DR molecule, especially late after HSCT when inflammatory conditions have subsided, would induce detrimental GVHD. In addition, the potential targeting of an HLA-DP molecule whose disparity is almost permissive following HSCT[2] should be examined.

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Disclosure Statement

The authors declare no competing financial interests.

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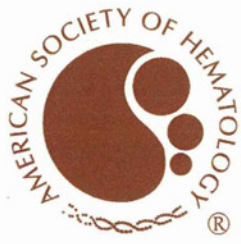

Cyclin-A1 represents a new immunogenic targetable antigen expressed in acute myeloid leukemia stem cells with characteristics of a cancer-testis antigen

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Targeted T-cell therapy is a potentially less toxic strategy than allogeneic stem cell transplantation for providing a cytotoxic antileukemic response to eliminate leukemic stem cells (LSCs) in acute myeloid leukemia (AML). However, this strategy requires identification of leukemia-associated antigens that are immunogenic and exhibit selective high expression in AML LSCs. Using microarray expression analysis of LSCs, hematopoietic cell subpopulations, and peripheral tissues to screen for candidate antigens, cyclin-A1 was identified as a candidate gene. Cyclin-A1 promotes cell proliferation and survival, has been shown to be leukemogenic in mice, is detected in LSCs of more than 50% of AML patients, and is minimally expressed in normal tissues with exception of testis. Using dendritic cells pulsed with a cyclin-A1 peptide library, we generated T cells against several cyclin-A1 oligopeptides. Two HLA A*0201-restricted epitopes were further characterized, and specific CD8 T-cell clones recognized both peptide-pulsed target cells and the HLA A*0201-positive AML line THP-1, which expresses cyclin-A1. Furthermore, cyclin-A1–specific CD8 T cells lysed primary AML cells. Thus, cyclin-A1 is the first prototypic leukemia-testis-antigen to be expressed in AML LSCs. The pro-oncogenic activity, high expression levels, and multitude of immunogenic epitopes make it a viable target for pursuing T cell–based therapy approaches. (*Blood*. 2012;119(23):5492-5501)

Introduction

It is well established that acute myeloid leukemia (AML) is organized hierarchically, initiated and maintained by a small population of cells referred to as leukemia stem cells (LSCs) that are characterized not only by unlimited reproductive capacity but also by enhanced resistance to chemotherapy and radiation. This primitive cell population, which is usually contained within a subpopulation of leukemic cells that are $CD34^+$ but lack expression of CD38 and lineage markers, is essential and adequate for long-term engraftment of primary AML cells in NOD/SCID transplantation models.[1-3] The LSC model suggests that, for a therapeutic anti-AML effect to be curative in patients, it will be necessary to identify strategies that efficiently eliminate the LSC compartment, which is often resistant to conventional chemotherapy.

In patients with intermediate-risk, high-risk, or relapsed AML, the allogeneic T cell–mediated graft-versus-leukemia effect after hematopoietic cell transplantation (HCT) or infusion of donor-derived lymphocytes in the post-HCT period has been shown to be essential for achievement of long-term remissions.[4-7] However, allogeneic HCT and unselected donor lymphocyte infusions are associated with significant toxicity because of both the conditioning regimen and the graft-versus-host activity of donor lymphocytes. An alternative strategy to provide anti-LSCs cytotoxic T lymphocytes to treat AML patients would use more targeted T-cell therapy, consisting of either adoptive transfer of T cells specific for, or vaccination against, leukemia associated antigens (LAAs).[8,9] The ability of such antigen-specific T cells to eliminate AML LSCs has been demonstrated in NOD/SCID transplantation models.[10-12]

For targeted T-cell therapy to achieve maximal efficacy against AML with minimal toxicity, identified LAAs need to have not only high expression in and presentation by leukemic cells but also lack significant expression in healthy tissues. Several AML LAAs have been described, but only Wilms tumor protein 1 (WT1), which is currently being targeted in clinical trials both with adoptive T-cell transfer and peptide vaccination, has been shown to be expressed in LSCs of the majority of AML patients at levels significantly higher than the physiologic levels in hematopoietic stem cells (HSCs).[12-15] Although objective responses/remissions have been observed in some treated patients, in many no anti-WT1 T-cell response can be elicited, and in others WT1 is not detected at levels in leukemic cells sufficiently distinct from HSCs to be targeted. Thus, additional candidate LAAs expressed in AML LSCs are greatly needed.

In this study, analyses of differential gene expression identified cyclin-A1 as a candidate new T-cell target. Cyclin-A1 is selectively expressed normally in testis, regulating progression of male germ cells through meiosis I.[16,17] *CCNA1*$^{-/-}$ mice are viable and phenotypically normal, with the exception of male infertility.[18,19] Cyclin-A1 is aberrantly expressed in AML as well as other

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malignancies.[16,20] In AML, it can sustain the malignant phenotype through pro-proliferative and antiapoptotic activities,[21-23] and overexpression of cyclin-A1 in mice causes dysplastic myelopoiesis with 15% of mice developing transplantable myeloid leukemias.[24] We now show that cyclin-A1 is a testis-leukemia-antigen that harbors a multitude of immunogenic MHC class I epitopes, which can be used to generate T cells from healthy donors that recognize and lyse leukemic cells.

Methods

Human samples

Mononuclear cells of patients with AML, chronic myeloid leukemia (CML), and myelodysplastic syndrome (MDS) from peripheral blood and bone marrow (BM) were isolated by leukopheresis or Ficoll-Hypaque (Biochrom). AML samples contained more than 60% malignant cells. Cells were collected at Fred Hutchinson Cancer Research Center and Charité Campus Benjamin Franklin. For generation of T-cell lines, leukapheresis products were obtained from 2 healthy donors at Fred Hutchinson Cancer Research Center. All samples were collected after written informed consent in accordance with the Declaration of Helsinki and with approval of the institutional review boards of both participating institutions.

Cell lines

Epstein-Barr virus–transformed lymphoblastoid cell lines (LCLs) were generated as described.[25] The T-cell/B-cell hybrid cell line T2 used to present epitopes is TAP-deficient and expresses only HLA A*0201. LCL 721.221 expresses no endogenous HLA class I because of radiation-induced deletion of the respective alleles and was stably transfected with the retroviral vector pLBPC containing the indicated HLA alleles.[26] Cell lines K562 (CML), THP-1, HL60, KG1 (AML), and U937 (monocytic cell line) were maintained in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 10% FBS, with 50μM β-mercaptoethanol (Sigma-Aldrich) also added for THP-1. T cells and dendritic cells (DCs) were maintained as described.[27]

Microarray data analysis

Two panels of microarray datasets (Affymetrix) were used: (1) 9 AML LSC samples (lineage$^-$, CD34$^+$, CD38$^-$, CD90$^-$),[14] 7 corresponding leukemic blast samples (lineage$^-$, CD34$^-$),[28] 4 HSC samples (lineage$^-$, CD34$^+$, CD38$^-$, CD90$^+$),[14] and datasets from PBMCs, CD34$^+$ BM mononuclear cells, and tissues (National Center for Biotechnology Information Gene Expression Omnibus [GEO] server GSM279585-279588, 414970, 414972, 414975, 419165-419174, 457175-457177, 483480-483496, 80576, 80582, 80602, 80615, 80619, 80653, 80689, 80712, 80734, 80738, 80739, 80759, 80792, 80824, 80826, 80867, 80869, HG U133 plus 2.0 format); and (2) 30 AML samples (> 75% malignant cells, 8 CD34$^+$ BM samples, 9 PBMCs samples and 2 testis samples (HG U133A format). This microarray data is available at the GEO database under the accession number GSE73707. Samples were normalized using the invariant set method (dChip 2.0 software[29]). Before analysis at the single probe set level, unsupervised hierarchical clustering was performed to rule out clustering because of the sample origin rather than the biologic background. Samples exceeding the mean expression plus 3 standard deviations (SDs) of the HSC samples were considered "positive." Expression values in LSCs were compared with other cell types using a 2-tailed Mann-Whitney test. Expression values from 7 LSCs with their corresponding paired leukemic blasts were compared using a 2-tailed Wilcoxon signed rank test.

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription was performed using Superscript III (Invitrogen). A panel of cDNAs from pooled healthy tissues was purchased from Clontech, and 5 samples of healthy BM were purchased from Cambrex. Quantitative 2-step real-time PCR (RT-PCR) was performed on an ABI 7500 machine (Applied Biosystems) with T_A = 60°C using the following primers/probes: glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)_fwd: GAGTCAACGGATTTGGTCGT; GAPDH_probe: 6FAM-GATATTGTTGCCATCAATGACCCCT-TAMRA; GAPDH_rev: GACAAGCTTCCCGTTCTCAG; CyclinA1_fwd: CATGAAGAAGCAGCCAGACA; cyclin-A1_probe: 6FAM-TTCGAGCAGAGACCCTGTATCTGG-TAMRA; cyclin-A1_rev: TTCGAAGCCAAAAGCATAGC. Crossing points were plotted against standard curves of pCR4-TOPO plasmids (Invitrogen) containing the respective PCR product.[30] Reactions were performed in duplicates, and expression was presented as copies per copies of GAPDH. Samples exceeding the mean expression plus 3 SDs of the BM samples were considered "positive."

Cytokines and peptides

Recombinant human IL-1β, IL-4, IL-7, IL-15, and TNF-α were obtained from R&D Systems, IL-2 and GM-CSF from Chiron, prostaglandin E_2 from MP Biomedicals, and IL-21 from PeproTech. A peptide library of a total of 103 15-mers with an overlap of 11 amino acids (AA) spanning cyclin-A1 (isoform c, NM_001111046) was purchased from Sigma-Aldrich.

Generation of cyclin-A1–specific T-cell clones

T-cell lines were generated as described with minor modifications.[27] Briefly, DCs were derived from plastic adherent PBMCs after culture for 2 days (day −2 to day 0) in DC media (CellGenix) supplemented with GM-CSF (800 U/mL) and IL-4 (1000 U/mL). On day −1, maturation cytokines TNF-α (1100 U/mL), IL-1β (2000 U/mL), IL-6 (1000 U/mL), and prostaglandin E_2 (1 μg/mL) were added. On day 0, DCs were harvested and pulsed with peptide (single peptides at 10 μg/mL, peptide pools at 2 μg/mL). T cells were isolated from PBMCs using anti-CD8 microbeads (Miltenyi Biotec) and stimulated with DCs at an E:T ratio of 1:5 to 1:10 in the presence of IL-21 (30 ng/mL). On day 3, IL-2 (12.5 U/mL), IL-7 (5 ng/mL), and IL-15 (5 ng/mL) were added.

Cells were restimulated between days 10 and 14 with the plastic adherent fraction of irradiated autologous PBMCs as antigen presenting cells after pulsing with peptide. IL-21 was added on day 0; cells were supplemented from day 1 on with IL-2, IL-7, and IL-15.

T-cell clones were generated in 2 ways: (1) unselected, by plating T cells from library-specific cell lines at limiting dilution and expanding with TM-LCLs coated with OKT3 (Ortho Biotech) and allogeneic PBMCs as feeders (REP protocol) as described[27]; and (2) selected for a single specificity, in which T cells were stimulated with autologous LCLs pulsed with the specific peptide for 4 hours at an E:T ratio of 1:2. IFN-γ secreted during the next 45 minutes was bound to the surface of the specific cells by the catch matrix reagent of the IFN-γ–secretion assay (Miltenyi Biotec). Cells were stained with IFN-γ–PE (Miltenyi) and CD8-FITC (BD Biosciences) and sorted on an Aria III instrument (BD Biosciences). T-cell clones were then generated/expanded as described.

Four T-cell clones were analyzed in this study: 2264.E30 is specific for epitope 341-351, clones 2196.D9, 2196.D11$_a$ and 2196.D11$_b$ are specific for epitope 227-235, and D11$_a$ and D11$_b$ are sister clones sharing the same TCR.

ICS and IF

For IFN-γ staining, antigen-presenting cells were pulsed with 10 μg/mL peptide overnight and washed once. Effector cells were coincubated with these cells for 6 hours in the presence of monensin. Cells were stained with anti-CD8–FITC, permeabilized using the BD Cytofix/Cytoperm kit, and stained with anti–IFN-γ–APC (BD Bioscience).

Immunofluorescence (IF) staining of AML cells and normal BM was performed after fixation with 4% formaldehyde followed by permeabilization/erythrocyte lysis with 0.1% Triton X-100. Air-dried cytospin preparations were permeabilized with 95% and 70% ethanol and immunostained with anti-cyclin-A1 (Novus Biologicals). Slides were then incubated with secondary goat anti–rabbit IgG–AlexaFluor-488 (Invitrogen), washed,

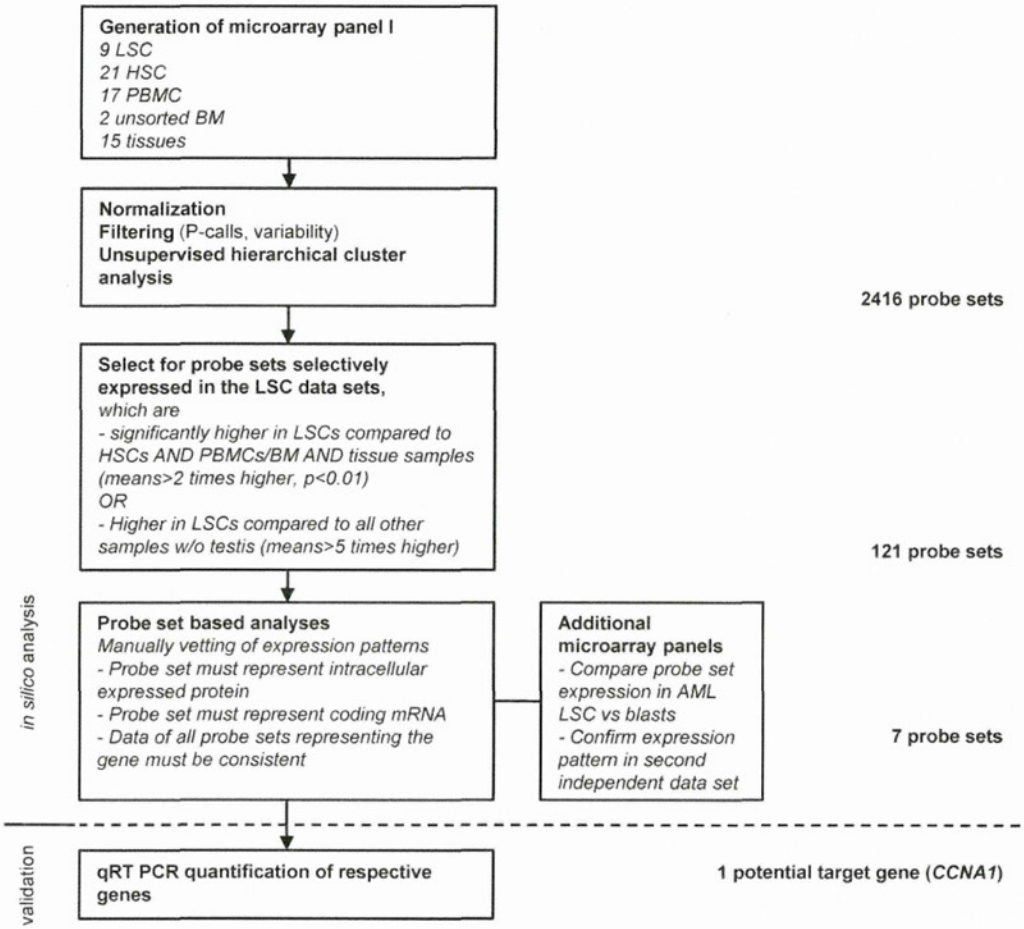

Figure 1. Systematic approach to identify potential target genes in AML LSCs based on 3′IVT expression microarray data. The microarray panel was created combining data files from 5 independent studies. Datasets were filtered for present calls in more than 10% of the samples and overall variability (reject probe sets with an SD/mean < 2). Hierarchical cluster analysis was performed to confirm clustering in accordance to sample biology. Target candidates were identified by mathematical filtering followed by visual inspection of the respective expression patterns. Targeted sequences of the probe sets were then analyzed to make sure that they actually represented a coding mRNA sequence. In case of several probe sets representing the same gene, expression patterns of all probe sets were inspected for consistency. Finally, target candidates, which were known to be membrane bound or secreted, were rejected.

dried, and mounted with 4,6-diamidino-2-phenylindole Vectashield (Vector Laboratories).

For FACS intracellular staining (ICS) staining of cyclin-A1, BM cells from AML patients were stained with anti-CD45–V450 and either anti-CD34–APC or anti-CD33–PE-Cy5 (BD Biosciences). Cells were fixed and permeabilized in lysis/permeabilization buffer (0.233% Triton X-100 in PBS, calcium, and magnesium free). Cyclin-A1 was detected using human-specific anti-cyclin-A1 antibody (Novus Biologicals) followed by indirect detection with AlexaFluor-480–conjugated goat anti–rabbit $F(ab')_2$ fragment (Invitrogen).

HLA stabilization assay

T2 cells were pulsed with 100 μg/mL peptide in serum-free RPMI containing 1 μg/mL β_2-microglobulin (Sigma-Aldrich) for 16 hours. Cells were then incubated for 4 hours in the presence of brefeldin A (Sigma-Aldrich), and stained with anti-HLA A/B/C-FITC (W6/32; BD Biosciences).

Caspase-3 assay

Target cells were membrane-labeled with PKH26 (Sigma-Aldrich). T-cell clones were used at the end of the REP cycle (day 12 or later). Targets and T cells were incubated at an E:T ratio of 3 to 5:1 for 4 hours. As a negative control, targets were incubated without effectors; as a positive control, targets were incubated in the presence of 4μM camptothecin or 1μM staurosporine (Sigma-Aldrich). Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit and stained with anti-active caspase-3–antibody conjugated to FITC or AlexaFluor-647 (C92-625; BD Biosciences).

^{51}Chromium release assay

Standard ^{51}Cr release assays were performed as described[27] using 5000 targets cells at E:T ratios of 10 to 1.25:1 in triplicates. Spontaneous release was assessed by incubating targets in the absence effectors. Percentage specific lysis was calculated using the formula: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Results

Cyclin-A1 is selectively expressed in AML LSCs, leukemic blasts, and testis

To systematically screen for candidate genes selectively expressed in the AML LSC compartment for targeting with T cells, we analyzed 9 LSC microarray datasets with samples of different hematopoietic cell subsets and nonhematopoietic tissues. Suitable candidate genes were identified by mathematical filtering and manual vetting of the model-based expression values (Figure 1). Based on the microarray expression data, as well as published data

on oncogenicity and cellular location, and after validating that the probe sets were homologous to coding mRNA, 7 candidate probe sets were identified (Figure 2A-B). For all of the probe sets, the levels detected in LSCs were not statistically different from the levels detected in the corresponding more differentiated leukemic blast samples (Figure 2C; data not shown). The overexpression of the probe sets in AML samples was confirmed in a second independent panel of microarrays for all probe sets, with the exception of 233734_s_at and 1554298_a_at, which were not represented on the HG U133A format (data not shown).

To confirm the microarray data, all 7 candidates were quantified by quantitative RT-PCR in a third independent sample set (Figure 2D; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). All but 1 of the candidates were eliminated because of either lack of correlation between microarray data and quantitative RT-PCR results (1 gene), significant expression in healthy tissues and/or HSCs (5 genes), or low total copy number less than 10^{-2} copies/copies GAPDH (1 gene).

Only cyclin-A1 displayed selective expression in LSCs and/or AML blasts in all 3 datasets. The respective probe set had been validated by quantitative RT-PCR in an earlier study.[20] In the first microarray panel, cyclin-A1 was overexpressed in 6 of 9 analyzed LSC samples (Figure 2B). No overexpression was found in any non-AML sample except testis. Expression values of cyclin-A1 were significantly higher in LSCs than in all other samples ($P < .001$, Figure 2B), although this P value has to be considered exploratory because the statistical testing was performed on the array set used for target selection. As no significant difference in cyclin-A1 expression was observed between the LSCs and leukemic blasts derived from the same patients ($P = .297$, Figure 2C), the expression pattern of cyclin-A1 was then confirmed in an additional panel that included AML cells not selected for LSCs, BM normal $CD34^+$, and PBMCs, parts of which have already been published.[20] We now analyzed these AML datasets with 2 datasets of testis tissue. Using a cut-off value of mean plus 3 SDs of the BM samples, no BM $CD34^+$ or PBMC sample achieved positivity, whereas 21 of 30 AML samples displayed cyclin-A1 expression. In both testis samples, the cyclin-A1 expression was higher than the average expression in the AML samples (data not shown).

To confirm the expression detected in arrays with probe set 205899_at, cyclin-A1 was quantified in AML samples, other hematopoietic cell subsets, and nonhematopoietic tissues using quantitative RT-PCR. Cyclin-A1 over-expression was detected in 24 of 44 analyzed AML samples not selected for LSCs (55%). No over-expression of cyclin-A1 was detected in BM, G-CSF mobilized $CD34^+$ cells or proliferating T cells (Figure 2D).

Next, we analyzed the frequencies and expression levels of cyclin-A1 in different French-American-British (FAB) AML subtypes and BM samples from patients with CML and MDS. Highest expression levels were observed in acute promyelocytic leukemia (APL), as previously described.[31] Moreover, the frequencies of cyclin-A1 positivity did vary with FAB subtype, with 100% positivity in APL (3/3) and M4 (5/5), > 50% positivity in M0 (3/5) and M5 (7/13), and < 50% in M1 (3/8), M2 (1/3) and unspecified and/or secondary AML (2/7, Figure 2D). No over-expression was found in patients with MDS or CML. The median copy number of cyclin-A1 per GAPDH in the AML samples was approximately 10-fold higher than the copy number of WT1 in the same sample set (supplemental Figure 2).

To confirm a uniform cyclin-A1 expression within the malignant blast population, IF was performed in 3 AML samples, which expressed cyclin-A1 in quantitative RT-PCR and in healthy BM. Whereas the BM showed no fluorescence, we observed a strong homogenous fluorescence in all 3 AML samples (Figure 2E; data not shown). In addition, cyclin-A1 ICS was performed in 4 primary AML samples. Again, we observed a uniform bright staining in the blasts in all samples tested, both gated on blasts in the CD45/side scatter (SSC) plot and after staining CD33 or CD34 (Figure 2F; data not shown).

Mapping of multiple immunogenic oligopeptides on cyclin-A1

For identification of MHC class I-restricted T-cell epitopes, a reverse immunology approach was used. Three different isoforms for cyclin-A1 have been described with isoform C distinguishable by having a shorter N-terminus. As no functional domains have been identified on the longer N-termini of isoforms A and B, and the respective transcripts for these isoforms could not be amplified by nested PCR either from testis or AML samples (data and primer sequences not shown), we constructed a peptide library representing the shorter isoform C so that immune escape from targeting epitopes in the N-termini could not occur if cells express only this shorter isoform.

T-cell lines were generated from donors 2196 and 2264, who both express HLA A*0201, the most common class I allele found in whites, by 4 stimulations with autologous antigen presenting cells pulsed with the peptide library. Using autologous LCLs pulsed with the library as stimulators for an ICS assay, more than 60% of cells in both T-cell lines appeared specific for cyclin-A1 based on IFN-γ production (Figure 3A). To identify the immunogenic 15-mers within the library, the 2 T-cell lines were stimulated with 20 peptide pools containing 10 to 13 15-mers, with every 15-mer being contained in 2 peptide pools (Figure 3B) and tested for IFN-γ production. We identified 18 peptides for donor 2196 and 10 peptides for donor 2264 that were present in corresponding pools and recognized by the respective T-cell lines, and these 15-mers were tested individually (Figure 3C). After identifying the immunogenic 15-mers, the minimal immunogenic AA sequence of each epitope was determined by stimulating with shorter peptides. With this approach, 8 immunogenic oligopeptides were mapped (Table 1).

To identify epitopes that are HLA A*0201-restricted, the T-cell lines were tested for recognition of K562 directly, which does not express any endogenous HLA class I molecules on the surface, or K562 stably transfected with HLA A*0201 as APCs pulsed with the respective peptide (supplemental Figure 3A; data not shown). Epitopes 218-226, 227-235, and 341-351 stimulated responses in the context of HLA A*0201. Both donors expressed HLA A*0201, which was the only shared MHC class I allele; by regenerating T-cell lines to each epitope from fresh cells obtained from both donors, we subsequently confirmed both the immunogenicity and the HLA A*0201-restriction of these epitopes (data not shown). The HLA restriction of other epitopes was determined using 721.221 cells transfected with a single HLA allele or allogeneic LCLs sharing defined HLA class I alleles with the respective donor (supplemental Figure 3B; data not shown).

Characterization of 2 HLA A*0201 restricted epitopes and generation/analysis of T-cell clones

The minimal immunogenic sequence of the 15-mer 341-355 was determined in the T-cell line to be the 11-mer SLIAAAAFCLA (Figure 4A), which was surprising because of both its length and the absence of a characteristic AA residue in the carboxy-terminal HLA A*0201 anchor position, particularly because one of the

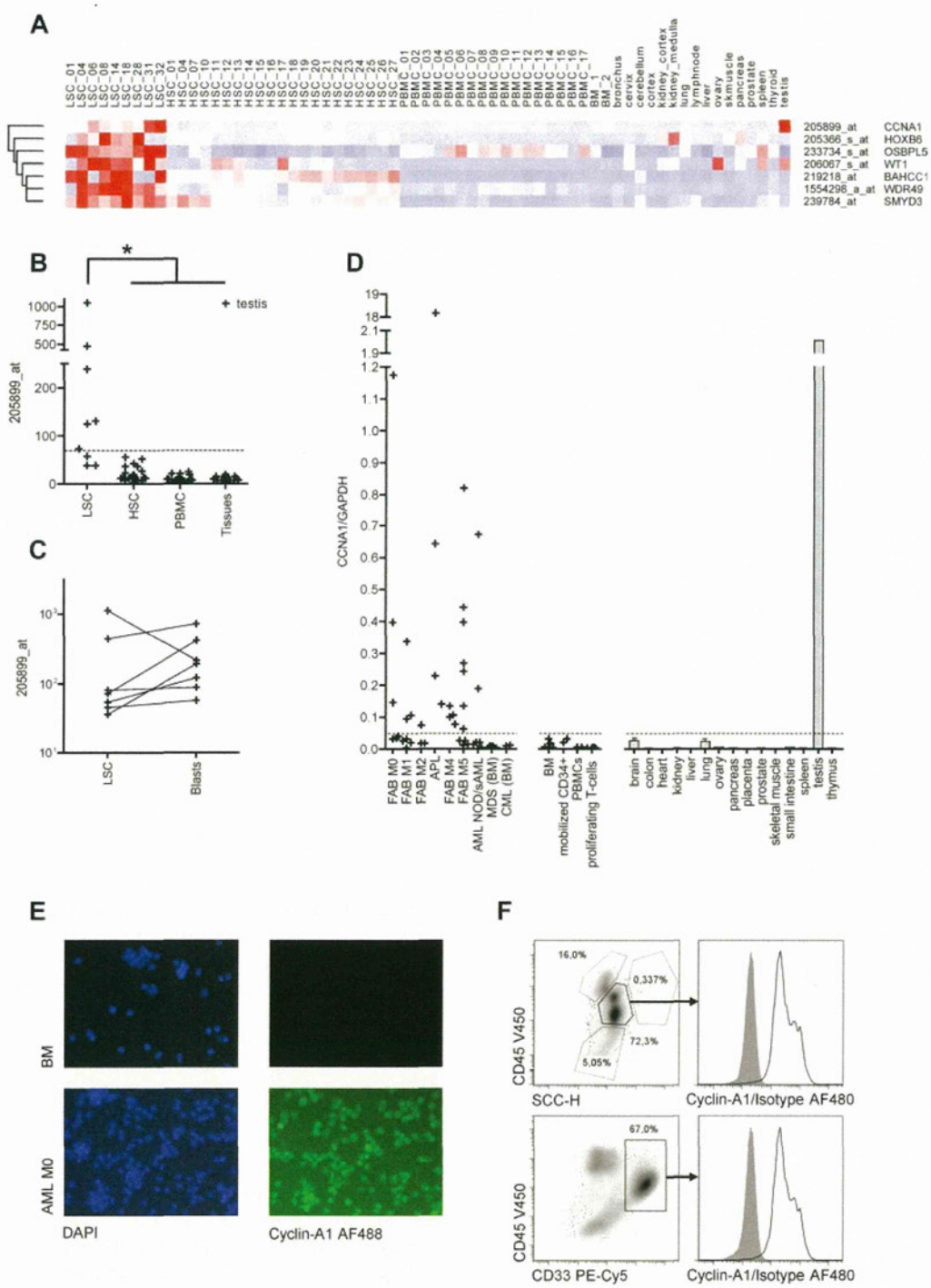

Figure 2. Identification of cyclin-A1 as target candidate by in silico expression analysis and RT-PCR quantification. (A) Seven candidate target genes predicted in silico: Heatmap of normalized probe sets. Each row represents one probe set, and each column represents one sample dataset. Red represents increased levels of expression; and blue, decreased levels of expression. The darkest shade of red/blue represents average expression ± 3 SDs. (B-C) Model-based expression of probe set 205899_at representing cyclin-A1. (B) Expression in AML LSCs compared with HSCs/CD34$^+$ BM mononuclear cells, PBMCs, and nonhematopoietic somatic tissues. The dashed line represents the cut-off value of 69.2 (mean plus 3 SDs of the HSC samples). *$P < .001$ (explorative). (C) Expression in AML LSCs and corresponding blasts ($P = .297$). (D) Cyclin-A1 expression quantified by quantitative RT-PCR. The dashed line represents the cut-off value of 0.048 (mean plus 3 SDs of the BM samples). (E) Immunofluorescence staining of cyclin-A1 in primary AML samples (representative example). Shown is a healthy BM (top row) and primary leukemic blasts (bottom row). Cells were stained with 4,6-diamidino-2-phenylindole (DAPI; left) and indirectly for cyclin-A1 (AlexaFluor-488, right). Negative controls without primary antibody and nonspecific isotype showed no fluorescence in both samples (not shown). Images were captured using a Nikon Eclipse E800 microscope. (F) Intracellular FACS staining of cyclin-A1 in primary AML blasts (representative example). BM mononuclear cells of a patient with cyclin-A1-expressing AML after gating out doublets in forward scatter (FSC)-area/height (A/H) and SSC-A/H plots. Top row: gating on blasts based on SSC and CD45 expression. Bottom row: gating on CD33-positive cells. Histograms show cyclin-A1 (black line) and isotype control (shaded) in the blast population.

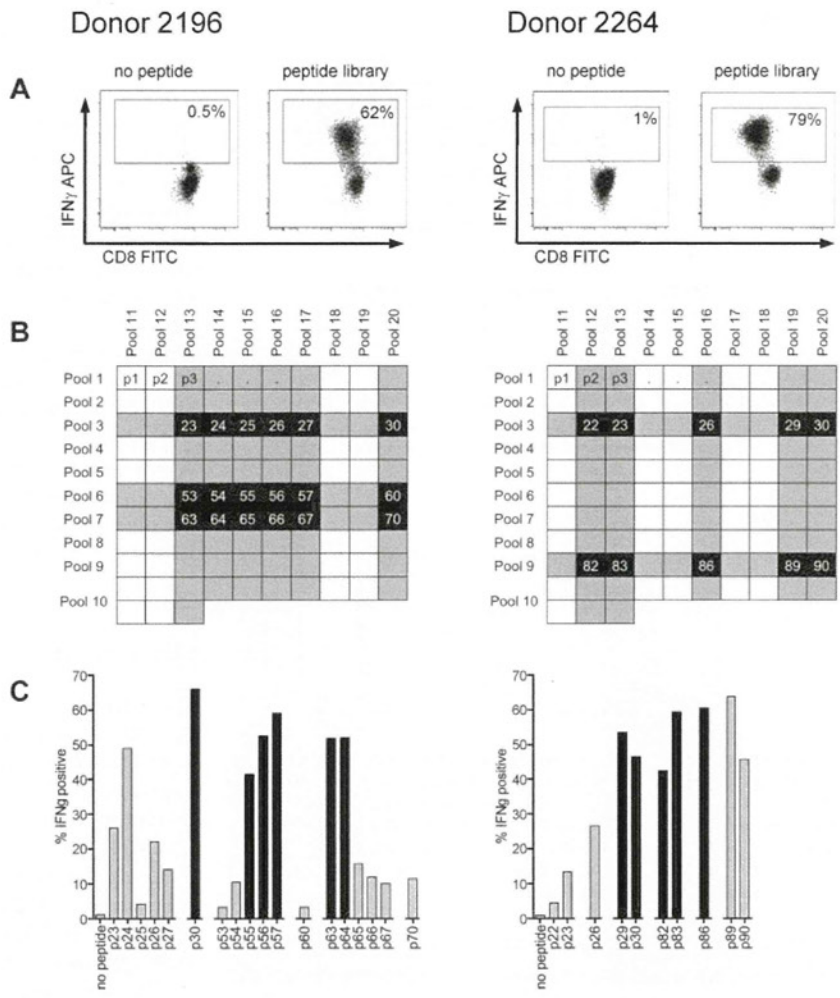

Figure 3. Reverse immunology strategy for epitope mapping in cyclin-A1 using ICS for IFN-γ. (A) After 4 stimulations with the peptide library, T-cell lines from both donors consisted of more than 60% specific cells (gated on $CD8^+$ cells). (B) The cell lines were subsequently tested against 20 peptide pools with each 15-mer being represented in 2 different pools in the peptide matrix. Shaded rows/columns indicate pools with more than 10% IFN-γ-positive cells from each donor. (C) Peptides, which tested positive in both of its pools (> 10% specificity, marked black), were further analyzed as individual peptides. Black columns represent the peptides for which the minimal immunogenic AA sequence from the initial 15-mer was determined.

10-mers (SLIAAAAFCL, 10-mer 1) had the appropriate AA residues in both anchor positions. We therefore tested whether the 11-mer could form stable peptide-MHC complexes by performing an HLA stabilization assay. For this, T2 cells were pulsed with 100 μg/mL of an irrelevant peptide or the 11-mer, 10-mer 1, or 10-mer 2 (LIAAAAFCLA) overnight. After washing and incubation with brefeldin for 4 hours, HLA surface expression was assessed by FACS. An increase in MFI was observed for both the 11-mer and 10-mer 1, but the 11-mer/MHC complex did not appear as stable as the 10-mer 1/MHC complex (Figure 4B).

Table 1. Immunogenic peptides on cyclin-A1 isoform c

AA position	AA sequence	Donor	HLA restriction	Clone available
118-127	YEVDTGTLKS	2264	B*4001	Yes
120-131	VDTGTLKSDLHF	2196	B*5701	Yes
167-175	YAEEIYQYL	2264	B*4001	Yes
218-226	AETLYLAVN	2196	A*0201	No
227-235	FLDRFLSCM	2196	A*0201	Yes
253-261	ASKYEEIYP	2196	ND	No
330-339	LEADPFLKYL	2264	B*4001	No
341-351	SLIAAAAFCLA	2264	A*0201	Yes

The HLA restriction of the HLA B epitopes was determined using ICS for IFN-γ production with 721.221 stimulator cells transfected with a single HLA allele or allogeneic LCLs sharing one or 2 HLA class I alleles with the respective donor.

ND indicates not determined.

To analyze lysis of AML samples, we initially generated T-cell clones by limiting dilution from both of the poly-specific T-cell lines and screened the derived clones for specificity. This approach only yielded clones specific for epitopes not restricted to A*0201. Therefore, to facilitate isolation of clones specific for the A*0201 epitopes, we had identified from analysis of the poly-specific CD8 T-cell lines the lines were first enriched for reactive T cells by stimulation with the A*0201-binding peptides and sorting of the cells secreting IFN-γ with an IFN-γ–capture reagent before cloning. This approach produced several clones specific for epitope 227-235 from donor 2196 and 341-351 from donor 2264.

To identify a suitable cyclin-A1–expressing leukemic cell line that could be used as a target cell to assess processing and presentation of cyclin-A1 epitopes, we quantified cyclin-A1 in 5 myeloid leukemia cell lines (Figure 5A). THP-1, a HLA A*0201-positive M5b AML line, expressed the highest levels of cyclin-A1. Therefore, THP-1 cells were coincubated with HLA-A*0201-restricted T-cell clones, and lysis from recognition of presented cyclin-A1 epitopes assessed by measuring caspase-3 activation/cleavage. Using an E:T ratio of 3:1, no significant caspase-3 cleavage was detected in THP-1 cells cultured without effectors or after 4-hour coincubation with clone 2264.A1, which is specific for the B*4001-restricted epitope 118-127. However, caspase-3 cleavage was observed in THP-1 cells incubated with clone 2196.D9 or 2196.$D11_a$ (both specific for epitope 227-235)

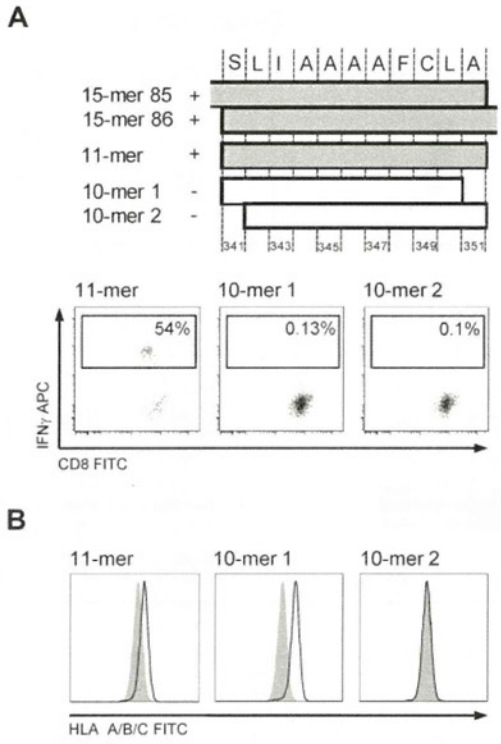

Figure 4. HLA A*0201-restricted epitope 341-351. Mapping the minimal immunogenic AA sequence and HLA stabilization. (A) The position of the different peptides in cyclin-A1. IFN-γ ICS of T-cell line (donor 2264) stimulated by autologous LCLs pulsed with the 10-mer 1, 10-mer 2, and 11-mer. Only pulsing with the 11-mer results in IFN-γ production in the T-cell line. (B) HLA stabilization assay: both 10-mer 1 and the 11-mer peptide stabilize HLA A*0201 on T2 cells. Negative controls are T2 cells pulsed with an irrelevant 15-mer (shaded).

and 2264.E30 (specific for epitope 341-351; Figure 5B). Thus, both epitopes are processed from endogenously expressed protein and presented in the context of HLA A*0201.

CD8 T cells specific for epitope 227-235 recognize and lyse primary AML cells

To determine whether cytotoxic T lymphocytes specific for a cyclin-A1 epitope recognize primary AML cells, we tested cyclin-A1–expressing blasts from 2 A*0201-positive and 2 A*0201-negative patients. Because of very limited amounts of cells available, the analysis was performed only with clone 2196.D11$_b$, which recognized epitope 227-235. The clone 2196.D11$_b$ was first tested for induction of apoptosis in a 4-hour caspase-3 assay. For maximal apoptosis of these targets, staurosporine was used rather than camptothecin because it induced apoptosis in a higher percentage of cells. As the different AML samples showed different rates of spontaneous apoptosis, the data were normalized by calculating specific caspase-3 cleavage as: 100 × (experimental − spontaneous)/(staurosporine − spontaneous). Using an E:T ratio of 5:1, 2196.D11$_b$ induced significant apoptosis of the A*0201-positive AML specimens, but not A*0201-negative ones (Figure 6A). To determine whether the observed caspase-3 cleavage reflected classic lytic activity, we performed a standard 4-hour ^{51}Cr release assay over a range of E:T ratios. Significant lysis of the A*0201-positive specimens was observed at an E:T as low as 1.25:1, whereas no specific lysis was detectable in the A*0201-negative targets. Thus, primary AML cells were killed in an HLA-restricted fashion.

Discussion

In the present study, we found that cyclin-A1 is an LAA expressed in AML stem cells that appears to be an attractive target for T cell-based therapy. To identify target candidates selectively expressed in LSCs, a systematic analysis of microarray data was performed, including from sorted LSC specimens. Using a reverse immunology approach, several class I epitopes were characterized, including 3 HLA-A*0201–restricted epitopes, and T-cell responses generated to 2 of these epitopes were capable of killing an AML cell line in vitro. Moreover, a CD8 clone specific for at least one of these epitopes effectively killed primary AML cells.

The identification of an appropriate target antigen is a critical and commonly limiting step for development of T cell–based immunotherapy. AML stem cell microarray data were used to identify probe sets with a suitable expression pattern. Although more than 100 probe sets passed the mathematical selection step, only one gene was found as a suitable target candidate after quantitative RT-PCR. The selection process displayed some of the

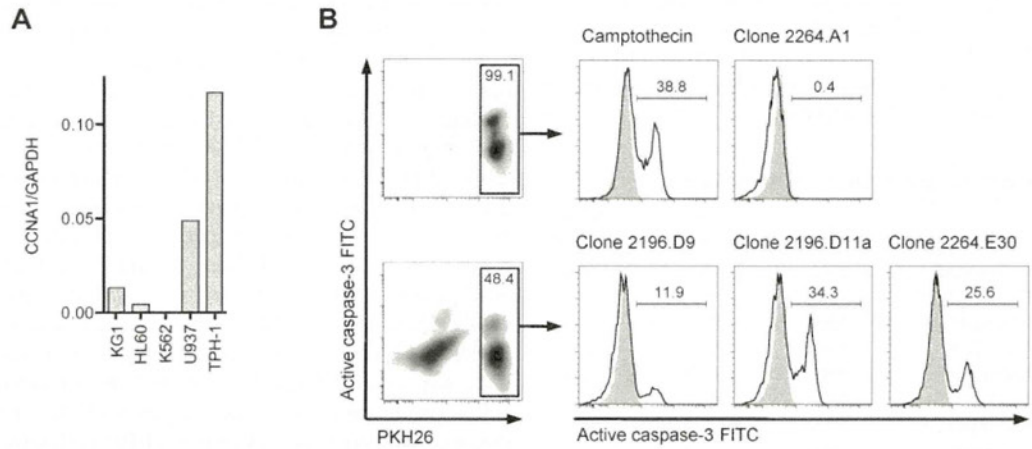

Figure 5. T-cell clones recognize endogenous processed epitopes 227-235 and 341-351. (A) Expression of cyclin-A1 in several myeloid cell lines quantified by quantitative RT-PCR. (B) Clones 2196.D9 and D11$_a$ (both specific for epitope 227-235) and clone 2264.E30 (specific for epitope 341-351) were tested for apoptosis induction in the THP-1 cell line using a caspase-3 assay. As negative controls, targets alone (shaded) and clone 2264.A1 specific for epitope 118-127 (HLA B*4001 restricted, THP-1 is B*4001-negative) were used. As a positive control, targets were incubated with 4μM camptothecin. The histograms correspond to the gates on the PKH26-prelabeled target cells.

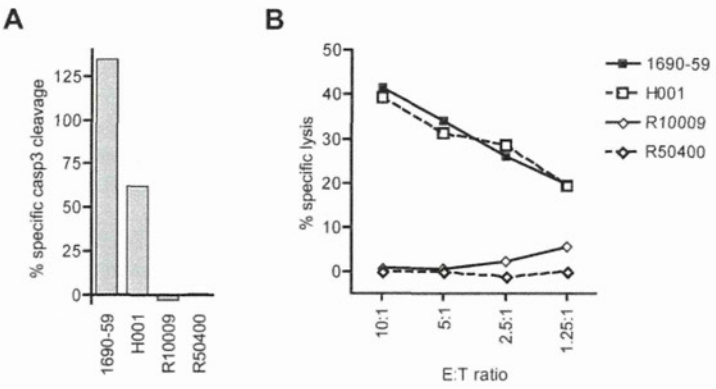

Figure 6. CD8 T-cell clone 2196.D11$_b$ exhibits specific cytotoxic activity for cyclin-A1–expressing primary AML samples. (A) Four-hour caspase-3 assay. Specific caspase-3 cleavage is shown after subtraction of spontaneous caspase-3 activation, and determined relative to staurosporine-induced cleavage as 100%. (B) Four-hour ^{51}Cr release assay at a range of E:T ratio with the same effector and target cells. H001 and 1690-59 are HLA A*0201 positive, and R10009 and R50400 are A*0201-negative.

drawbacks of a pure in silico approach. First, not all probe sets identified by the mathematical selection steps displayed the desired expression pattern. Second, after revising the target sequences of the probe sets, we found a high percentage of sequences to not represent a coding transcript. Finally, we found large differences between in silico data predictions and quantitative RT-PCR results. This might be because, as opposed to quantitative RT-PCR, microarrays do not provide linear data. Furthermore, the absence of a standardized protocol for sample processing and hybridization impacts the comparability of the different datasets.

Cyclin-A1 was highly expressed in AML cells, including the stem cell compartment of more than 50% of patients, and cyclin-A1 expression was highly restricted to malignant cells, a fact that was predescribed only based on semiquantitative data.[31] We previously noted that both WT1 and cyclin-A1 are expressed at significantly higher levels in LSCs than in HSCs,[14] but WT1 can also be detected in several nonhematopoietic organs, such as kidney, spleen, and ovary at levels potentially higher than in leukemic blasts (supplemental Figure 1C). In the present study, which is the first providing linear expression data of cyclin-A1 in AML, healthy hematopoiesis, and tissues in direct comparison, we now found that, except for AML, cyclin-A1 is expressed only in germ cells from testis, which is considered to be an immune-privileged site.[32] Minimal cyclin-A1 staining in healthy BM as described in immunohistochemistry in an earlier study[31] was not observed in our IF staining and might be the result of unspecific binding of the polyclonal antibodies used. Consequently, toxicities from recognition of normal cells while targeting cyclin-A1 appear unlikely.

Based on the expression pattern, cyclin-A1 falls into a group of antigens called cancer-testis-antigens (CTAs), which are characterized by expression in normal tissues restricted to immature germ cells in testis and by aberrant expression in cancers. There have been at least 2 systematic genome-wide studies to detect new cancer-testis genes (CTGs), but neither identified *CCNA1* as such a gene.[33,34] This is probably because hematologic malignancies, compared with solid tumors, have been found to infrequently express any of the known CTGs[35] and were therefore either not included or underrepresented in the cancer panels used in the 2 studies. Recently, a classification of CTGs based on a systematic analysis of in silico expression data differentiating CTGs as testis-restricted and testis-selective CTGs was proposed, with the latter displaying minimal expression in non-immune privileged tissues. Interestingly, testis-selective CTGs had a high probability of being autosomal, not belonging to a multigene family, and being associated with a known function in gametogenesis and/or meiosis.[33] Most of these characteristics of a testis-selective CTA apply to *CCNA1*, as it is located on chromosome 13q, is a single-copy gene, and has a known function in meiosis. The only predescribed leukemia-associated CTA PRAME was neither expressed in any LSC sample of our microarray datasets nor found overexpressed by quantitative RT-PCR in any of the AML samples (not shown). Thus, we think that cyclin-A1 can now be classified as the first described CTA associated with AML LSCs.

Cyclin-A1 is an alternative CDK2-associated type-A cyclin with an essential role in male gametogenesis, positively regulating the G_2/M phase transition in meiosis I.[17,36-38] The expression of meiotic genes in nonmeiotic non–germ cell malignancies has been observed with other CTGs, such as *SYCP-1* and *SPO11*, and expression of meiosis-associated genes has been hypothesized to contribute to chromosomal aberrations and therefore support the initiation and further cytogenetic clonal evolution of a malignancy.[39,40] In the case of cyclin-A1 in AML, this mechanism may not apply because, even though expression of cyclin-A1 has been shown to sustain the malignant phenotype,[21-23] our recently published data indicated higher cyclin-A1 expression in AML specimens with normal rather than aberrant karyotypes.[20] Furthermore, in case of APL, cyclin-A1 appears to be a target of the RAR-α fusion proteins rather than causal for the translocation.[41] Several lines of evidence suggest that cyclin-A1 can play a role in the cell cycle regulation of mitotic and especially malignant cells, with cyclin-A1 positively regulating the G_1/S transition independent of the particular cell type.[37,42] Therefore, the major leukemogenic mechanism resulting from overexpression of cyclin-A1 in myeloid cells might be accelerated S-phase entry rather than induction of chromosomal instability.[37]

Besides the expression pattern and physiologic function of a gene, another important characteristic of a broadly useful T-cell target is having a large number of available epitopes, and overexpressed LAAs would be predicted to have a much larger number of targetable AA sequences than the very limited distinct sequences present in unique leukemia antigens generated by translocations or mutations. Using 2 normal donors, we identified 8 immunogenic peptides restricted to at least 3 different HLA class I molecules. Such broad immunogenicity is probably facilitated by limited tolerance induction to CTAs.

The characterization of epitope 341-351 demonstrates one limitation of silico epitope prediction approaches for identifying immunogenic peptides. Whereas the observed binding to A*0201 of the 10-mer(341-350) from the immunogenic 15-mer could be predicted retrospectively by 3 of 3 tested prediction algorithms (SYFPEITHI,[43] BIMAS,[44] IEDB analysis resource[45]), no binding was predicted for the 11-mer; therefore, this epitope would have never been identified without the use of a peptide library. A possible

explanation for the fact that all clones generated to the larger 15-mer peptide were reactive exclusively against the 11-mer might be that T cells reactive with the 10-mer had been eliminated by negative selection because of cross-reactivity to a similar epitope on a high-abundance self-protein. Indeed, for the shorter epitope 341-350, which did bind to A*0201 more stably, similar though not identical epitopes were identified in cyclin-A2 and cyclin-B1, which are broadly expressed self-antigens.

The efficient cytotoxic activity of cyclin-A1-specific T-cell clones demonstrated in this study may reflect several factors: Cyclin-A1 expression appears significantly higher than other AML associated antigens such as WT1, PRAME, HMMR, and PRTN3 (Li et al[46]; S.O., unpublished data, May 2011). Moreover, the dramatic periodicity of cyclin-A1 expression is accompanied by high degradation rates regulated by the ubiquitin-proteasome-mediated pathway.[47] This suggests that epitopes are likely being made available for presentation. Finally, although the testis appears to provide peripheral immune privilege to germline cells,[32] little is known about negative selection during development of T cells reactive with gametogenesis-associated antigens, especially CTAs. However, reduced deletion and/or central tolerance for testicular antigens would explain both the high incidence of autoimmune mediated infertility observed in patients after traumatic disruption of the blood-testis barrier[32] and the high cytotoxic activity of the T-cell clones generated in this study. Further studies analyzing both the functionality and the TCR affinity of spontaneous T-cell responses in patients with cyclin-A1 expressing diseases are initiated to further investigate this assumption.

Recently, a National Cancer Institute pilot project to prioritize cancer antigens made an effort to not only develop a priority-ranked list of the known cancer antigens but to also provide a list of weighted "ideal" antigen criteria/characteristics for evaluating other antigens.[13] According to these criteria, cyclin-A1 would appear to be a highly suitable antigen for targeting AML based on: a very selective oncofetal expression pattern with uniformly high expression in leukemia in a large fraction of patients, being leukemogenic when overexpressed in mice, expression in the LSC compartment, having many epitopes available, and having an intracellular location. Thus, applying the weighted criteria of the National Cancer Institute pilot project to cyclin-A1 resulted in a ranking for cyclin-A1 between 3 to 5 among the 75 antigens that were ranked.[13] Therefore, cyclin-A1 appears to now be the first described non–X-linked leukemia-testis-antigen with optimal features for targeting of AML LSCs by T cell–based therapy approaches, such as vaccination and/or adoptive T-cell transfer.

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Authorship

Contribution: S.O. designed and performed the experiments and prepared the final manuscript; R.M. and I.L.W. provided and analyzed microarray data; T.S. performed FACS assays; D.S. provided and analyzed microarray data and provided patient samples; U.K., M.B., E.H.W., and B.W. provided patient samples; K.R.L. and Y.E.C. performed IF and FACS assays; M.H. performed FACS sorting; Y.A. performed molecular cloning and retroviral transfection; P.D.G. designed the concept, reviewed the data, and prepared the final manuscript; and all authors reviewed and edited the manuscript.

Conflict-of-interest disclosure: A patent application for the use of cyclin-A1 as a T-cell target antigen has been submitted by S.O., P.D.G., R.M., and I.L.W. The remaining authors declare no competing financial interests.

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Autophagy Creates a CTL Epitope That Mimics Tumor-Associated Antigens

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Abstract

The detailed mechanisms responsible for processing tumor-associated antigens and presenting them to CTLs remain to be fully elucidated. In this study, we demonstrate a unique CTL epitope generated from the ubiquitous protein puromycin-sensitive aminopeptidase, which is presented via HLA-A24 on leukemic and pancreatic cancer cells but not on normal fibroblasts or EBV-transformed B lymphoblastoid cells. The generation of this epitope requires proteasomal digestion and transportation from the endoplasmic reticulum to the Golgi apparatus and is sensitive to chloroquine-induced inhibition of acidification inside the endosome/lysosome. Epitope liberation depends on constitutively active autophagy, as confirmed with immunocytochemistry for the autophagosome marker LC3 as well as RNA interference targeting two different autophagy-related genes. Therefore, ubiquitously expressed proteins may be sources of specific tumor-associated antigens when processed through a unique mechanism involving autophagy.

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Introduction

Exploring the mechanisms underlying cancer-specific CTL recognition is important in the establishment of safe and effective immunotherapy. The discrimination of normal and malignant cells by CTLs depends on the repertoire of antigenic peptides displayed via the MHC class I molecules of these cells. As both normal and malignant cells possess antigen-processing machinery, the repertoire displayed depends on the expression level of the target proteins. These target proteins are degraded in the cytoplasm by the proteasome, with the resulting short peptides being translocated into the endoplasmic reticulum, where they bind to MHC class I molecules [1]. Therefore, tumor antigens are bascially determined by their expression pattern, not by the machinery responsible for the processing. If malignant cells possess unique antigen-processing machinery, they may create cancer-specific antigenic peptides, even from ubiquitously expressed proteins.

Autophagy is equally as important for peptide degradation as is proteasomal lysis of cytoplasmic proteins and organelles [2]. In macroautophagy (referred to as autophagy hereafter), autophagosomes are formed and then fused with lysosomes to produce autolysosomes, where proteins are degraded by lysosomal proteases. Upregulation of autophagy is an adaptation to stresses such as starvation, oxidant injury and genomic damage. Although autophagy normally functions under physiological conditions, degradation by the autophagosome is also important under aberrant conditions, such as cancer [3]. Thus, autophagy plays a pivotal role not only in suppressing tumorigenesis but also in promoting tumor progression. Autophagy is also known to affect both innate and adaptive immunity [4,5]. Particularly in the latter, autophagy participates in MHC class II antigen presentation [6,7], although reports regarding MHC class I presentation via autophagy are sparse.

Herein, we provide evidence of a cancer-specific CTL epitope created through both autophagy and proteasomal action, derived from the ubiquitously expressed protein puromycin-sensitive aminopeptidase (PSA). The data suggest that unique processing accounts for differential epitope liberation between normal and cancer cells.

Results

Artificial Antigen Presenting Cells (aAPCs) Effectively Induce Tumor-specific CTLs

Generating tumor-specific CTLs generally requires autologous tumor cell lines. To bypass the substantial difficulties in establishing such lines, we have sought to use aAPCs to express endogenous tumor-associated peptides on given HLA molecules. The K562 cell line is an ideal platform for this use due to the absence of HLA expression on their cell surface; thus, K562 cells

could serve as APCs when an HLA needs to be exogenously expressed [8]. To establish the K562-based aAPCs, K562 cells were stably transduced with HLA-A24, CD86 and 4-1BBL with lentiviral vectors, and a positive population was isolated (Figure 1A) to generate tumor-specific T cells from HLA-A24-positive donors. After 2 rounds of stimulation, polyclonal T cells secreted IFN-γ in an antigen-specific manner. Using a limiting dilution culture of the bulk CTL line, we established a T-cell clone, designated as 16F3, that produced IFN-γ against HLA-A24-expressing K562 cells (referred to as A24-K562) but not against dermal fibroblast cells, normal bronchial epithelial cells or EBV-transformed B lymphocytes (B-LCLs) (Figure 1B, 1C). Moreover, 16F3 recognized three out of five pancreatic cancer cell lines (Figure 1D) in an effect that was blocked by anti-HLA class I Abs (data not shown). These cell lines were positive for HLA-A24 expression (data not shown). However, 16F3 did not produce IFN-γ in response to T2-A24 cells pulsed with the 22 HLA-A24-restricted peptides derived from previously reported tumor antignes (data not shown).

PSA-derived Epitope Induces a Specific CTL

As 16F3 recognized antigens specifically expressed on cancer cells (Figure 1B–D), we explored its nature with a cDNA library derived from K562 cellular mRNA. The library was cloned into an expression vector and divided into 960 pools, each containing 100 cDNA clones. Plasmid DNA was extracted from each pool and then transfected into HLA-A24-expressing HEK-293T (referred to as A24-293T) cells. In the first screening, one of the pools induced 16F3 to produce IFN-γ (data not shown). The positive pool was subcloned into individual cDNA clones and screened (Figure S1A). The single clone, 8G, that induced 16F3 to produce IFN-γ proved to be a variant cDNA of PSA (NM_006310). This variant PSA cDNA is generated via intronic polyadenylation [9] and consists of exons 1–12 with part of an intron located directly downstream of exon 12 (Figure S1B). To identify the epitope recognized by 16F3, A24-293T cells were transfected with plasmids encoding truncated forms of PSA. Upon analysis, the antigenicity disappeared with the following mutations: C-terminal truncation between amino acid residues 200 and 300 and N-terminal truncation between 241 and 251 (Figure S1C). To define the N- and C-terminal ends more precisely, further truncation was performed within this region. As inclusion of isoleucine at position 261 was essential for the stimulation of the clone (Figure S1D), this amino acid was indicated to be located in the C-terminus. Exclusion of aspartic acid at position 250 abolished antigenicity; thus, we considered an unusually long 12–mer peptide, DYFNVPYPLPKI (residues 250–261), having tyrosine at the second position as a primary anchor for HLA-A24 binding [10], to be the minimal epitope. A synthesized 12-mer peptide (residue 250–261) was demonstrated to bind HLA-A24 molecules more efficiently than a CMV pp65 peptide (Figure S1E) and to be recognized by 16F3 (Figure S1F). Failure of recognition of KP-2 and SUIT-2 cells by 16F3 was attributable to the absence of epitope presentation because both are recognized by 16F3 when pulsed with the peptide (Figure S2). A 9–mer peptide, DYFNVPYPL (residue 250–258), which was highly scored for HLA-A24 binding by the BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/), was not recognized by 16F3 (Figure S1D).

PSA has Two Variant Forms to be Presented

The PSA protein is expressed ubiquitously [11]. Thus, we investigated the expression levels of both full-length and variant PSA mRNA with RT-PCR. The primers for detecting the full-length forms corresponded to exon 11 and exon 13, and those for the variant forms corresponded to exon 11 and part of the intron located directly downstream of exon 12 (Figure 2A, top). Both forms of PSA mRNA were detected in all of the samples (Figure 2A, bottom). To assess protein expression, Western blot analysis was performed using monoclonal antibodies against full-length PSA. The PSA protein was also expressed in all samples (Figure 2B). As the expression pattern of PSA was not concordant with that of sensitivity to 16F3, we speculated that 16F3 might recognize a distinct peptide derived from a separate protein that is antigenically close to the PSA-derived peptide. To examine this hypothesis, we investigated the CTL response against cells treated with PSA-specific siRNAs. The target sequences of three siRNAs were chosen outside of the PSA variant coding regions using BLOCK-iT™ RNAi Designer (Invitrogen). Because two pancreatic cancer cell lines, KP-3 and MIA PaCa-2, were able to accept siRNA efficiently, these cells were used in these experiments. The expression of the PSA protein was reduced in the cells treated with siRNAs specific for PSA (Figure 2C), which led to much lower capacities to stimulate 16F3 than with a negative control siRNA (Figure 2D), indicating that 16F3-mediated IFN-γ production occurs through recognition of PSA.

The Processing of the PSA Epitope with the MHC Class I Involves the Vacuolar Pathway

Next, we addressed the mechanisms underlying the epitope generation using specific inhibitors, as no difference in the mRNA expression of TAP1, TAP2, LMP2 or LMP7 was evident between 16F3-sensitive and 16F3-insensitive cells (data not shown). For this purpose, we prepared a CMV pp65-expressing aAPC [12]. Fixed APCs are typically used to examine the effects of drugs on antigen-processing mechanisms [13]. However, 16F3 did not recognize paraformaldehyde- or glutaraldehyde-fixed A24-K562 cells (Figure S3A), and further treatment of quenching with glycine did not recover the epitope recognition (Figure S3B). Therefore, IFN-γ production was measured in the presence of reversible inhibitors, such as BFA, CQ and BafA, to avoid the restoration of the epitope following inhibitor removal during co-culture with T-cells (Figure 3A–C). IFN-γ production was detected via intracellular staining with BFA, as this drug causes the accumulation of IFN-γ within the cytosol (Figure 3A), and via secretion assays with other inhibitors (Figure 3B, 3C). IFN-γ production by 16F3 was decreased after treatment with both a proteasome inhibitor, lactacystin and with BFA, inhibiting the endoplasmic reticulum to Golgi transport (Figure 3A, 3B). This effect was also observed for another HLA-A24-restricted CTL clone specific to CMV pp65 [14], indicating the involvement of the classical MHC class I presentation pathway. Unexpectedly, CQ, an inhibitor of acidification inside the endosome/lysosome, decreased epitope processing from PSA, but not from the CMV protein (Figure 3B). In addition, KP-3 cells treated with either CQ or BafA, another acidification inhibitor, decreased PSA epitope presentation (Figure 3C). CQ and BafA did not affect T-cell function during co-culture, as demonstrated by intact IFN-γ secretion by cognate peptide-pulsed target cells in the presence of the inhibitors (Figure 3B, 3C). These data indicate that a vacuolar pathway mediates the processing of the PSA epitope, contrasting with the classical MHC class I pathway for the CMV epitope, although both utilize the same HLA-A24 molecules for presentation.

Two examples have been reported with regard to MHC class I molecules loading epitopes via CQ-sensitive vacuolar pathways. One example indicates that recycled MHC class I molecules, as with to MHC class II, can assemble with peptides in the acidic environment of the late endosome [15]. The other example involves the intracellular trafficking of peptides to the endosome mediated by specific signals within the cytoplasmic domain of class

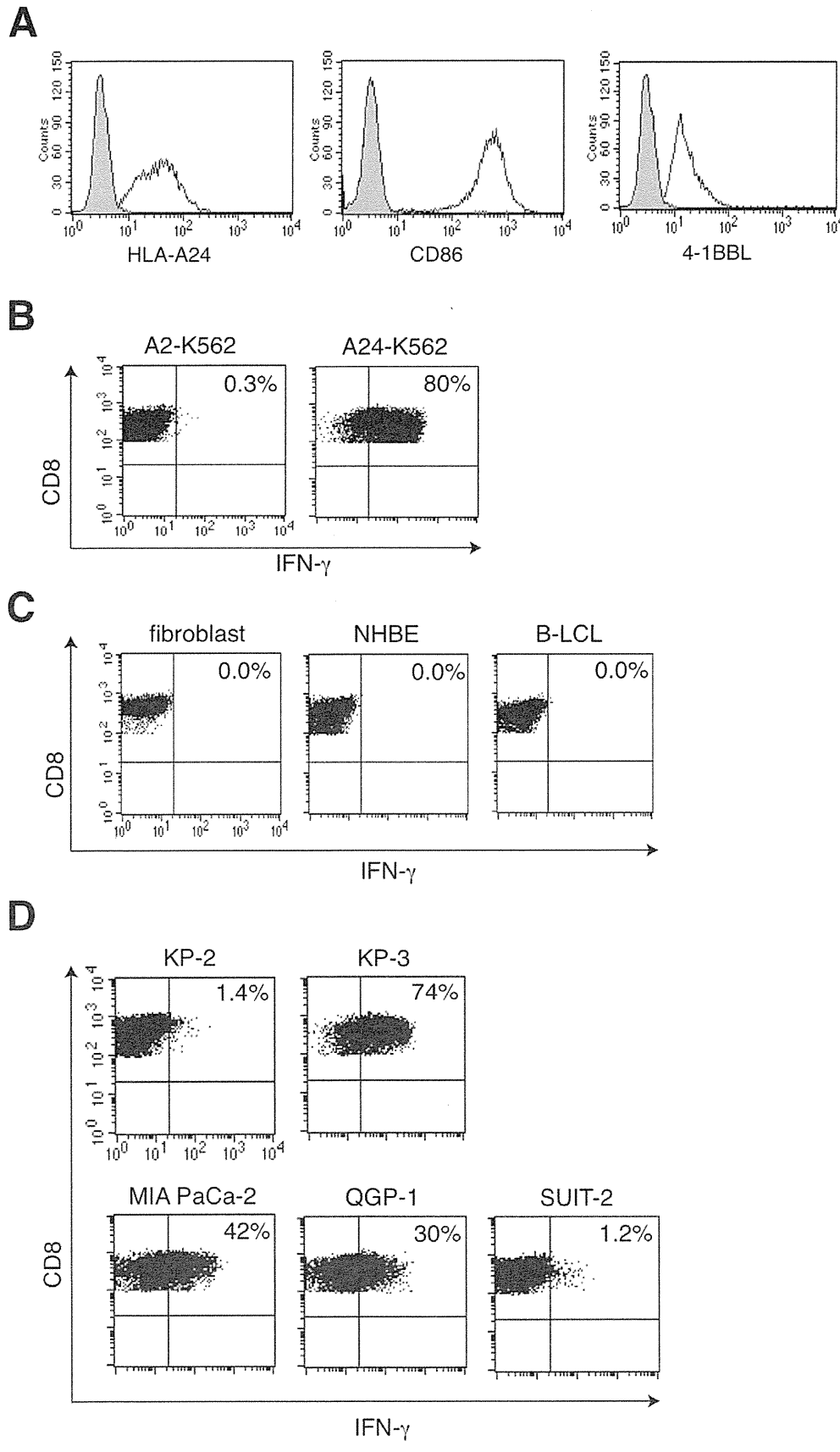
A
Counts
HLA-A24
CD86
4-1BBL
B
A2-K562
A24-K562
0.3%
80%
CD8
IFN-γ
C
fibroblast
NHBE
B-LCL
0.0%
0.0%
0.0%
CD8
IFN-γ
D
KP-2
KP-3
1.4%
74%
MIA PaCa-2
QGP-1
SUIT-2
42%
30%
1.2%
CD8
IFN-γ

Figure 1. Characterization of a CTL clone, designated as 16F3, from in vitro culture with aAPCs. A, Surface expression of HLA-A24, CD86 and 4-1BBL molecules on the K562 cells used for stimulation. The shaded and solid area show non-transduced and lentiviral-transduced cells, respectively. B–D, IFN-γ secretion of 16F3 upon incubation with various cells. The 16F3 cells were incubated with K562 cells expressing either HLA-A2 or HLA-A24 (B) for 4 h, and the IFN-γ secreting cells were detected and analyzed. The frequency is shown as the percentage of the total living $CD8^+$ T cells. HLA-A24-positive fibroblast cells, normal human bronchial epithelial cells and B-LCLs were used as representative non-cancerous cells (C). Five HLA-A24-positive pancreatic carcinoma cell lines were also used as stimulator cells (D). The data are representative of three independent experiments.
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I molecules [16]. To examine the above possibilities, we generated mutant HLA-A24 molecules that were reported to lack any capacity for internalization and endosomal/lysosomal trafficking [16–18]. HLA-A24-YA contains a single point mutation replacing a tyrosine of exon 6 with alanine, and HLA-A24-YAΔ7 has a deletion of exon 7 and the same tyrosine substitution (Figure S4A). CMV pp65-expressing K562 was stably transduced with lentiviral vectors expressing those mutated HLA-A24 forms. All of the constructs similarly expressed HLA on the cell surfaces (Figure S4B). Both HLA mutants were recognized via either 16F3 or CMV clones as efficiently as wild type HLA-A24 (Figure S4C). In addition, primaquine, an inhibitor of the recycling of HLA class I from the endosome to the cell surface [19], did not inhibit PSA epitope presentation (data not shown). These data indicate that both MHC class I molecule recycling and an endosomal trafficking pathway of MHC class I molecules are irrelevant to the generation of the epitope.

Constitutively Active Autophagosomes are Indispensable for the PSA Epitope Generation

As CQ and BafA are known to inhibit lysosomal function, we evaluated the level of autophagy using an immunofluorescence assay to detect the autophagosomal marker LC3. KP-3 and MIA PaCa-2 cells, which process and present the PSA epitope, had strong punctate LC3 staining, whereas SUIT-2, KP-2 and normal fibroblast cells that were not sensitive to CTL recognition showed weak staining (Figure 4A). Moreover, colocalization of autophagosomes and PSA was observed with BafA treatment for 4 h (Figure 4B), indicating that the protein might become a substrate of autophagic digestion in KP-3 and MIA PaCa-2 cells. To examine whether autophagic puncta could progress to autolysosomes in these cells, the autophagic flux was measured using a difference in the sensitivity of GFP and mRFP to the lysosomal environment [20]. When mRFP-GFP-LC3 tandem-tagged fluorescent protein entered the autolysosome from the autophagosome, the GFP fluorescence signals would be diminished, but the mRFP would remain fluorescent. KP-3 and MIA PaCa-2 cells transfected with the plasmid had many mRFP puncta in autolysosomes, with few colocalized signals of GFP/mRFP in autophagosomes (Figure 4C), implying that these cells have a high autophagic flux state and that the LC3 puncta were not mere aggregates. Furthermore, we measured the flux status by evaluating an autophagosomal marker, LC3-II, which digests itself in the autolysosome. The status of the autophagosome is accurately represented by differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors, such as CQ, that block the degradation of the autolysosome [21]. KP-2 cells and B-LCLs expressed LC3-II at low levels, and changes in the LC3-II band were limited on the addition of CQ (Figure 4D). However, LC3-II levels were markedly increased in K562, KP-3 and MIA PaCa-2 cells that were recognized efficiently by 16F3 (Figure 4D). Fibroblasts and SUIT-2 cells had low or moderate autophagic flux. These data suggest that the epitope presentation requires constitutively active autophagosomes and autophagic flux.

PSA Epitope Processing and Presentation in Pancreatic Cancer Cells Engages the Autophagic Pathway

Recently, autophagy has been shown to deliver cytosolic proteins continuously to endosomes/lysosomes for antigen loading onto MHC class II molecules [22]. To address the involvement of an active autophagic pathway in antigen presentation onto MHC class I molecules, we first treated CMV pp65-expressing A24-K562 cells with 3-MA, a commonly used inhibitor of autophagy. We observed a decrease in IFN-γ production by 16F3 (Figure 5A) but not by CMV-specific CTLs. To confirm that autophagic processing is critical for epitope generation, siRNAs specific for two atgs, atg5 and atg7, were independently transfected into KP-3 and MIA PaCa-2 cells to suppress the protein expression and resultant autophagosome formation [21,23]. After the siRNA treatment, atg5 and atg7 mRNA expression levels were reduced in KP-3 and MIA PaCa-2 (Figure 5B). Knockdown of each gene in both cells resulted in a decreased IFN-γ production by 16F3 (Figure 5C). HLA-A24 expression was not significantly changed on the surfaces of either 3-MA- or siRNA-treated cells compared with the control (Figure S5, S6). These results suggest that constitutively active autophagosomes play a significant role in epitope generation with MHC class I in cancer cells that have an elevated autophagic activity.

Degradation of Full-length PSA Protein is Inhibited by CQ but Not by Lactacystin

To provide an insight into the hierarchy of autophagy and proteasomal digestion to process PSA, K562 cells were treated with CQ, and the expression levels of the full-length PSA protein were examined by Western blot analysis. As shown in Figure 6A and C, the level of the PSA protein increased in K562 following CQ treatment. The increase was not observed in KP-2 cells with less autophagy. The increase in p62 protein, an efficient substrate of autophagy [24], was comparable in K562. Next, these cells were treated with lactacystin, and the levels of PSA along with HIF-2α protein, a substrate of proteasomal digestion [25], were examined. The level of PSA in K562 cells was not changed, whereas that of HIF-2α was increased (Figure 6B, 6C).

Autophagy-induced Fibroblast Cells can Not Generate the PSA Epitope

Finally, we tested whether rapamycin, an m-TOR inhibitor being well documented as an inducer of autophagy, or low nutrient culture conditions could induce normal fibroblast cells to create the epitope. As shown in Figure 7A, autophagosomes increased by the drug treatment and by low nutrient culture conditions. However, these cells ere not recognized by 16F3 (Figure 7B). The same treatments did not induce B-LCLs to present the epitope to 16F3 (data not shown). These data demonstrate that, in normal cells, induced autophagy can not create the epitope, suggesting that constitutively active autophagy in cancerous cells is required for the epitope creation.