Table II. Survival and causes of death.

	Cases (*N*)	Alive (*N*)	Death (day) <28 (*N*)	<100 (*N*)	≥100 (*N*)	Infection at CBT (*N*)	Cause of death (<day 100) Bac/Fung infection (*N*)	Viral infection (*N*)	Others (*N*)	Cause of death (≥day 100) GVHD (*N*)	Others (*N*)
Total	88	62	7	19	7	18	10	7	VOD 3 MOF1	5	PTLD 1 AI 1
SCID	40	29	6	9	2	11	2	6	1 (VOD)	1	1 (AI)
WAS	23	19	0	1	3	1	1	0	0	3	0
CGD	7	3	0	4	0	5	4	0	1 (VOD)	0	0
SCN	5	5	0	0	0	0	0	0	0	0	0
Others	13	6	1	5	2	1	3	1	1 (VOD) 1 (MOF)	1	1 (PTLD)

Bac/Fung infection, bacterial and/or fungal infection. VOD, veno-occlusive disease; MOF, multiple organ failure; AI, adrenal insufficiency; PTLD, post-transplant lymphoproliferative disorder. Cause of death total does not equal the number of deceased patients because one patient died of VOD and bacterial infection.

adrenal insufficiency (one patient). None of the other patients died of infection after day 100.

GVHD

All but five patients in the present study received either CyA- or tacrolimus-based immunosuppressant prophylaxis for GVHD. The cumulative incidence of grade 2–4 acute GVHD at day 100 was 28% (95% CI, 19–38%), and that of grade 3–4 GVHD was 8% (95% CI, 4–15%) (Fig 3A, D).

The incidence of grade 2–4 GVHD was higher in patients who underwent 2- or 3-antigen-mismatched UCBT compared with those who underwent HLA-matched or HLA-1-antigen–mismatched UCBT, but it was not statistically significant ($P = 0{\cdot}071$) (Fig 3B). On the other hand, no difference was observed in the incidence of grade 3–4 GVHD between <2-antigen-mismatched and >2-antigen-mismatched transplants (Fig 3E), although grade 3–4 GVHD was not observed by high-resolution DNA typing in patients who underwent genotypically HLA-matched transplantation.

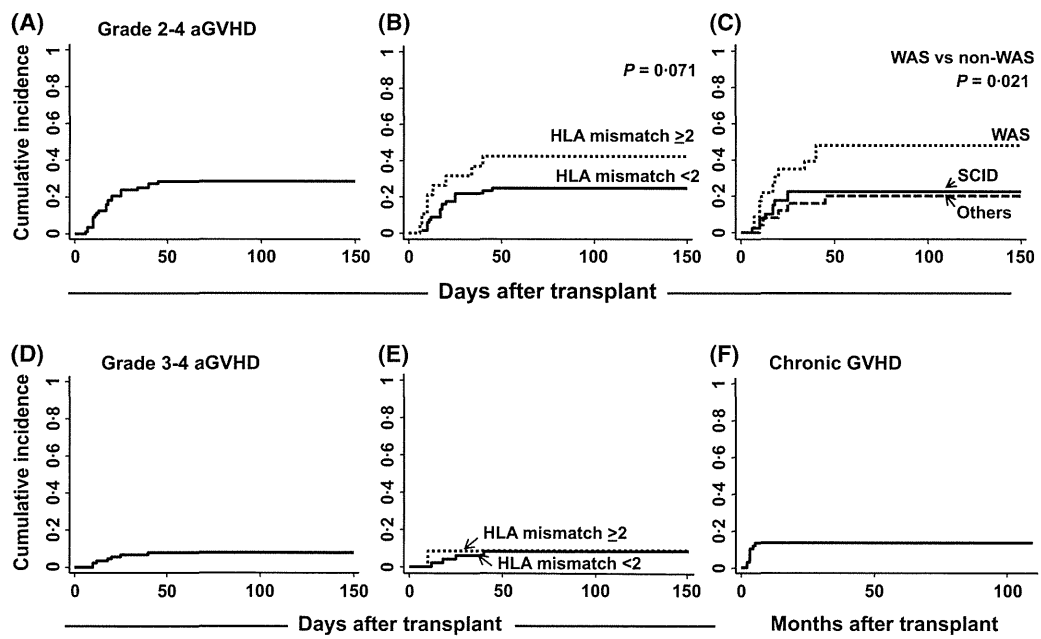

Fig 3. Cumulative probability of acute and chronic GVHD after UCBT. The cumulative incidence of grade 2–4 acute GVHD (aGVHD) at day 100 was 28% (95% CI, 19–38%) (A). The incidence was higher in transplantation mismatched for ≤2 antigens (B) and in that for WAS patients (C). The cumulative incidence of grade 3–4 acute GVHD at day 100 was 8% (95% CI, 4–15%) (D) and the incidence was not different between patients undergoing transplantation for ≥2-antigen mismatched transplant and those undergoing <2-antigen mismatched transplant (E). The cumulative incidence of chronic GVHD at day 180 was 13% (95% CI, 7–23%) (F).

When focusing on differences among the disease groups (Fig 3C), a significantly higher incidence of grade 2–4 GVHD was observed in WAS patients than in non-WAS patients, $P = 0{\cdot}021$. In addition, three of five WAS patients who developed grade 3–4 GVHD died of either GVHD (two patients) or VOD (one patient).

Chronic GVHD was observed in nine patients, and its cumulative incidence at day 180 was 13% (95% CI, 7–23%) (Fig 3F).

Infections

Twenty-eight patients (SCID, 11; WAS, eight; CGD, three and other diseases, six) developed bacterial infection after UCBT. Sixteen of the 28 patients remained alive at the time of data collection.

Fungal infection mainly caused by *Aspergillus* species was observed in eight patients (CGD, three; SCID, two; WAS, two and X-linked hyperIgM syndrome, one). Three of the eight patients died of bacterial infection, bacterial/fungal infection or GVHD.

Twenty patients (SCID, eight; WAS, four; CGD, two; SCN, two and others, four) developed CMV disease after UCBT. CMV was detected before conditioning in all eight SCID patients of which four patients died of CMV disease after transplantation. Twelve of the 20 patients remained alive at the time of analysis.

Other notable virus-related complications were respiratory syncytial virus bronchiolitis accompanied by chronic GVHD in one SCID patient and EBV-PTLD in one patient with Blau syndrome; both infections led to a fatal outcome. One WAS patient had severe haemorrhagic colitis caused by Coxsackie virus B infection, which was treated successfully by infusion of expanded CD4 T cells prepared from the infusion residual of donor cord blood (Tomizawa *et al*, 2005). Another WAS patient had persistent norovirus infection. Interstitial pneumonia not due to CMV or *Pneumocystis* was noted in three patients of which one patient had parainfluenza/rhinovirus infection, while the causative agent for infection in the remaining two patients was not identifiable.

Risk factors for overall mortality

Lastly, we analysed the factors contributing to overall survival. Using univariate analyses, the following were found to be significant contributory factors to a poor prognosis: HLA mismatch of ≥2 antigens, time to transplant >180 d, second or third transplantation, ongoing infection at the time of transplantation, no conditioning for UCBT and diagnosis other than SCID, SCN or WAS (Table III). The dose of transfused nucleated cells or CD34-positive cells did not affect the 5-year OS.

Using multivariate regression analyses, the following were found to be significant contributory factors to patient death: infection at the time of transplantation, no conditioning, HLA mismatch of >2 antigens and diagnosis other than SCID, SCN or WAS (Table IV). RIC was determined to be the favourable factor for patient survival ($P = 0{\cdot}01$) (Fig 4 and Table IV).

Table III. Univariate analyses of factors that contributed to 5-year OS.

Factors	Hazard ratio	95% CI	*P*-value
Age: ≥12 months	1·73	(0·78–3·83)	0·175
Diagnosis			
WAS and SCN	1·00		
SCID	2·34	(0·75–7·36)	0·145
Other diseases	5·39	(1·70–17·0)	0·004*
Nucleic cell dose: ≥$8{\cdot}2 \times 10^7$/kg	1·51	(0·69–3·29)	0·299
CD34 cell dose: ≥$2{\cdot}1 \times 10^5$/kg	0·86	(0·36–2·08)	0·744
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·68	(0·58–4·83)	0·337
4/6 matched	3·78	(1·23–11·60)	0·020*
3/6 matched	3·24	(0·63–16·74)	0·160
4/6 or 3/6 matched	2·64	(1·20–5·83)	0·016*
Time to transplant: ≥180 d	1·89	(0·85–4·17)	0·117
Infection at transplant	6·24	(2·61–14·9)	<0·0001*
Second or third transplantation	3·37	(1·26–9·02)	0·016*
Conditioning			
MAT	1·00		
RIC	0·41	(0·13–1·23)	0·111
No conditioning	2·89	(1·21–6·93)	0·017*

*Significant contributory factors to the poor prognosis.

Discussion

This paper reports the outcome of UCBT for 88 PID patients, the largest cohort of PIDs to receive UCBT to date. The overall survival rate for PID patients undergoing UCBT was comparable to that previously reported for 46 Japanese PID patients undergoing BMT from either HLA-identical siblings or unrelated donors (Sakata *et al*, 2004), and also to that reported by the European Society of Immunodeficiency and other stem cell transplantation centres for PID patients receiving BMT from HLA-matched related donors, HLA-mismatched related donors or unrelated donors (Antoine *et al*, 2003; Rao *et al*, 2005; Dvorak & Cowan, 2008). The time for haematopoietic recovery was comparable to or better than the median recovery time observed in a large cohort of UCBT in children with haematopoietic disorders (Thomson *et al*, 2000; Michel *et al*, 2003) and in adults with leukaemia (Laughlin *et al*, 2004; Atsuta *et al*, 2009). The incidence of grade 2–4 GVHD (28%) in UCBT was lower compared with that reported in unrelated donor BMT in PID patients in Japan (47%) (Sakata *et al*, 2004), with that reported in BMT in 90 SCID patients (34%) (Neven *et al*, 2009) and with that observed in the studies of UCBT for childhood haematological malignancies (Thomson *et al*, 2000; Michel *et al*, 2003; Sawczyn *et al*, 2005). The incidence of chronic GVHD (13%) after UCBT was slightly

Table IV. Multivariate analyses of factors that contributed to 5-year OS.

Factors	HR	95% CI	*P*-value
Diagnosis			
WAS and SCN	1·00		
SCID	1·71	(0·39–7·38)	0·475
Other diseases	7·50	(2·06–27·19)	0·002*
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·53	(0·50–4·66)	0·454
4/6 matched	5·64	(1·66–19·14)	0·006*
3/6 matched	1·04	(0·68–23·96)	0·124
4/6 or 3/6 matched	3·87	(1·63–9·19)	0·002*
Infection at transplant	4·61	(1·74–12·16)	0·002*
Conditioning			
MAT	1·00		
RIC	0·20	(0·06–0·69)	0·011†
No conditioning	4·87	(1·79–13·3)	0·002*

*Significant contributory factors to an unfavourable prognosis.
†Significant contributory factors to a favourable prognosis.

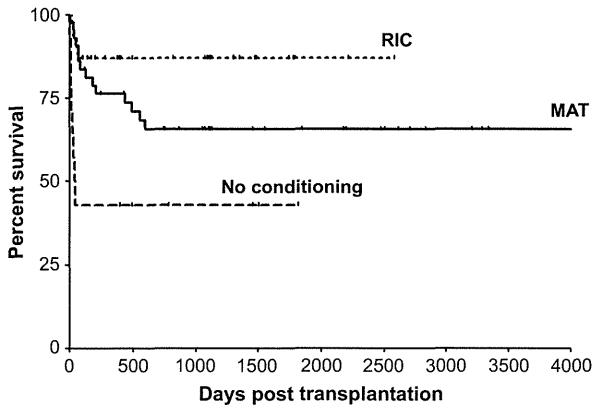

Fig 4. Kaplan–Meier estimates of overall survival after umbilical cord transplantation. Comparison of overall survival between reduced intensity conditioning (RIC), myeloablative therapy (MAT), and no conditioning is shown. For 5-year OS, MAT *versus* RIC, $P = 0{\cdot}111$, MAT *versus* no conditioning, $P = 0{\cdot}017$ in univariate analysis.

lower than that after URBMT in PID patients in Japan (20%) (Sakata *et al*, 2004), and was lower compared to that in UCBT studies for childhood leukaemia (Michel *et al*, 2003; Sawczyn *et al*, 2005). Thus, UCBT in PID patients in the present study was associated with a good survival rate, good engraftment rate, rapid haematological recovery and a lower incidence of acute and chronic GVHD.

Given that the 5-year OS for SCID patients (71%) was better than that for SCID patients receiving bone marrow from HLA-mismatched related donors in both Japan (5-year OS, 36%, Imai, Morio, Kamachi, Kumaki, Ariga, Nonoyama, Miyawaki, and Hara, unpublished observations) and Europe (5-year OS, 52%, Antoine *et al*, 2003), UCBT would be particularly beneficial for patients requiring rapid access to donor units yet lacking a matched related donor.

The present study found that several key risk factors were associated with overall mortality. First, infection was the major cause of mortality during the first 100 d after UCBT in PID patients, and the frequency was much higher than that observed in other disorders following UCBT (Rocha & Gluckman, 2006; Kurtzberg *et al*, 2008, Szabolcs *et al*, 2008). As predicted and reported in previous studies (Antoine *et al*, 2003; Cuvelier *et al*, 2009), infection at the time of transplantation was associated with poor survival ($P < 0{\cdot}0001$), suggesting that the control of pre-existing infection at the time of UCBT is critically important.

Eight of 11 SCID patients who had active infection, mainly CMV pneumonia, died before day 50, while 26 of 28 patients without infection at the time of UCBT remained alive at the time of data collection. UCBT without conditioning was selected for 12 patients, of which seven had CMV infection and one had *Pneumocystis* pneumonia at the time of transplantation. Six out of the seven patients died of CMV infection; and one patient with *Pneumocystis* pneumonia did not survive UCBT.

UCBT in WAS patients achieved a good 5-year OS, as reported in a previous study of 15 cases (Kobayashi *et al*, 2006). One of the key factors would have been the time from diagnosis to transplantation. In our WAS patients, UCBT was performed at a median age of 14 months (range, 4–84 months), when most patients were thrombocytopenic, but did not yet have uncontrolled infection or autoimmunity.

Four CGD patients died of bacterial or fungal infection without engraftment. Although these patients were not categorized as those with active infection at the time of transplantation, they required intravenous administration of antimicrobial and antifungal agents before and after transplantation.

Second, HLA disparity was a risk factor associated with overall mortality. Lower survival was observed in UCB recipients transplanted with a ≥2 antigen-mismatched graft compared with those transplanted with a <2 antigen-mismatched graft [Hazard Ratio (HR) = 3·87, $P = 0{\cdot}002$]. Although no difference was observed in 5-year OS between recipients of HLA-matched and those of HLA 1-antigen mismatched UCBT in the present study, we would need data from a larger number of patients with information on more extensive and sensitive HLA typing to discuss the impact of fully matched HLA on transplant outcome.

Finally, non-SCID/SCN/WAS patients showed a significantly lower survival rate (HR = 5·40, $P < 0{\cdot}0001$ by multivariate analyses). Although a previous large-scale study showed that results of HSCT according to disease did not show obvious disease-specific findings (Antoine *et al*, 2003), it is not yet known if UCBT is suitable for all types of PIDs. This may indicate donor source other than UCB is preferable for certain types of PID. Although the success of UCBT noted for X-linked hyperIgM syndrome, bare lymphocyte syndrome and

X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (Tono *et al*, 2007) is encouraging, optimization of transplantation procedures and determination of suitable timing for UCBT may be necessary for this group of patients. Alternatively, this may simply indicate an expansion of transplantation to less favourable clinical conditions or to less favourable transplantation conditions. Studies on a larger cohort are necessary for drawing any conclusion on whether diagnosis is significant overall.

Recent studies suggest improved survival after BMT for PID with the RIC regimen; however, to date, comparison of CBT using RIC *versus* MAT has not been made. In our study, 87% of patients on the RIC regimen and 66% on the MAT regimen remained alive at the latest follow-up. Multivariate analyses revealed that the RIC regimen is associated with a higher 5-year OS than the MAT regimen (HR = 0·20, $P = 0·011$). Although it is premature to conclude that RIC provides an equal or superior outcome to MAT for all PID patients, non-myeloablative treatment may be beneficial at least for certain types of PID. RIC was selected preferentially in SCID and CGD patients, with good survival rates: 17 of 18 SCID patients and three of four CGD patients remain alive. As a result of this, we are in the process of initiating a clinical trial of UCBT with RIC in SCID patients. On the other hand, only two of 23 WAS patients received RIC. Our previous data showed that a conditioning regimen other than BU/CY or BU/CY/ATG was the only independent factor associated with failure in HSCT for WAS patients (Kobayashi *et al*, 2006). However, whether this holds true for UCBT in younger WAS patients should be determined.

Notably, although the outcome of UCBT for WAS in this cohort was excellent compared with that from previously reported HSCT results using different donor sources (Kobayashi *et al*, 2006; Friedrich *et al*, 2009), UCBT in WAS patients was associated with a high rate of grade 2–4 acute GVHD (11 of 23 patients) and a post-transplant infectious episode (13 of 23 patients). Eight patients experienced bacteraemia/sepsis and six suffered a viral infection (CMV pneumonia, four; Coxsackie virus enterocolitis, one and persistent norovirus infection, one). The high rate of serious infections and GVHD in WAS patients after transplantation warrants further study in search of preventive measures that might include RIC for severe, transplantation-related toxicities.

Long-term follow-up of the clinical and immunological status is necessary when considering the lifespan of PID patients. Recent studies on the long-term outcome after HSCT for SCID revealed the presence of relatively late complications, such as chronic GVHD, autoimmune events, severe or recurrent infections, chronic human papilloma virus infection, nutritional problems and late rejection in 50% of patients (Mazzolari *et al*, 2007; Neven *et al*, 2009). Similarly, long-term follow-up of HSCT in WAS patients revealed that 20% of patients developed chronic GVHD-independent autoimmunity (Ozsahin *et al*, 2008). One possible measure that might be taken to avoid the chronic problems associated with CBT would be to select a HLA-matched UCB unit, as HLA disparity was a risk factor for both overall survival and the development of GVHD in our study. The advantage of RIC over MAT in preventing late complications needs careful assessment, together with data on mortality, engraftment and early post-transplant complications.

Finally, the issue of SCID patients who died before or without receiving SCT, most likely due to uncontrolled infection, still remains unresolved. This suggests that the early diagnosis of SCID and prevention of opportunistic infection within a protected environment and the administration of appropriate prophylactic drugs is critically important for the improvement of survival in SCID patients in general. To that end, neonatal screening with the employment of T cell receptor excision circles should be beneficial for an improved outcome in SCID patients (McGhee *et al*, 2005; Morinishi *et al*, 2009).

We report the results of UCBT for 88 PID patients in Japan. Despite the limitations of a retrospective, non-randomized study, our study suggests that unrelated umbilical cord blood can be considered as a promising stem cell source for children with congenital immunodeficiency when a HLA-matched related donor is not available.

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Mismatched human leukocyte antigen class II-restricted CD8$^+$ cytotoxic T cells may mediate selective graft-versus-leukemia effects following allogeneic hematopoietic cell transplantation

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Partial human leukocyte antigen (HLA)-mismatched hematopoietic stem cell transplantation (HSCT) is often performed when an HLA-matched donor is not available. In these cases, CD8$^+$ or CD4$^+$ T cell responses are induced depending on the mismatched HLA class I or II allele(s). Herein, we report on an HLA-DRB1*08:03-restricted CD8$^+$ CTL clone, named CTL-1H8, isolated from a patient following an HLA-DR-mismatched HSCT from his brother. Lysis of a patient Epstein–Barr virus-transformed B cell line (B-LCL) by CTL-1H8 was inhibited after the addition of blocking antibodies against HLA-DR and CD8, whereas antibodies against pan-HLA class I or CD4 had no effect. The 1H8-CTL clone did not lyse the recipient dermal fibroblasts whose HLA-DRB1*08:03 expression was upregulated after 1 week cytokine treatment. Engraftment of HLA-DRB1*08:03-positive primary leukemic stem cells in non-obese diabetic/severe combined immunodeficient/γc-null (NOG) mice was completely inhibited by the *in vitro* preincubation of cells with CTL-1H8, suggesting that HLA-DRB1*08:03 is expressed on leukemic stem cells. Finally, analysis of the precursor frequency of CD8$^+$ CTL specific for recipient antigens in post-HSCT peripheral blood T cells revealed a significant fraction of the total donor CTL responses towards the individual mismatched HLA-DR antigen in two patients. These findings underscore unexpectedly significant CD8 T cell responses in the context of HLA class II. (*Cancer Sci* 2011; 102: 1281–1286)

Allogeneic hematopoietic stem cell transplantation (HSCT) has been used successfully for the treatment of hematological malignancies. Although HSCT from human leukocyte antigen (HLA)-identical siblings or unrelated donors is feasible to minimize the risk of acute graft-versus-host disease (aGVHD), HSCT from HLA-mismatched donors can be performed when a patient has advanced disease and no HLA matched donor is available.[1] It has been shown that aGVHD and survival rates are comparable between patients receiving HLA-mismatched unrelated HSCT and those receiving fully HLA-matched HSCT when the mismatch combination is not non-permissive.[2] Because the mismatched HLA molecule(s) may serve as a target for donor T cells, the immune response to these HLA in patients receiving a zero non-permissible mismatch HSCT could give rise to a favorable graft-versus-leukemia (GVL) effect with minimal risk of aGVHD. Following HLA-mismatched HSCT, it is commonly believed that CD8$^+$ or CD4$^+$ T cell responses are induced, depending on the mismatched HLA class I or II allele(s), based on the binding of cognate coreceptors to MHC molecules stabilizing weak interactions between T cell receptors (TCR) and MHC.[3]

In the present study, we characterized an HLA class II-restricted CTL clone isolated from a patient with acute myeloid leukemia who received HLA-DR/DP loci-mismatched HSCT. The CTL clone, named CTL-1H8, was CD8$^+$ and its cytotoxicity was blocked by an anti-CD8 antibody as well as by an anti-HLA-DR antibody. The CTL-1H8 clone lysed primary leukemic cells possessing the mismatched HLA-DRB1*08:03, but not cytokine-treated recipient dermal fibroblasts. Engraftment of HLA-DRB1*08:03-positive primary leukemic stem cells in immunodeficient mice[4] was completely inhibited by *in vitro* preincubation with CTL-1H8. Furthermore, we demonstrated by CTL precursor (CTLp) frequency analysis that a significant fraction of the total donor CD8$^+$ CTL response in this patient was directed against the HLA-DRB1*08:03 molecule. These findings underscore the *in vivo* immunological relevance of a CD8$^+$ T cell response against mismatched HLA class II molecule(s).

Materials and Methods

Cells, HLA transfectants, and antibodies. Peripheral blood mononuclear cells (PBMC) were collected and cryopreserved before and after HSCT from a male patient who had received his brother's bone marrow (BM) for AML (M6; French American British subtype, M6). The HLA genotype of the recipient was A*24:02/*33:03, B*52:01/*44:03, C*12:02/*14:03, DRB1*08:03/*13:02, DQB1*06:01/*06:04, DPB1*02:02/*04:01, whereas that of the donor was mismatched by DRB1*15:02 instead of DRB1*08:03 and by DPB1*05:01 instead of DPB1*02:02. The patient developed grade II aGVHD limited to the skin and extensive chronic GVHD, but has been free from disease recurrence for over 2 years. B-Lymphoblastoid cell lines (B-LCL) were established from the donor and recipient, as well as from normal volunteers. All blood, BM, and tissue samples were collected after the subjects had provided written informed consent, and the study was approved by the Institutional Review Board of Aichi Cancer Center. The B-LCL, including the HLA class I negative B-LCL line 721.221, were

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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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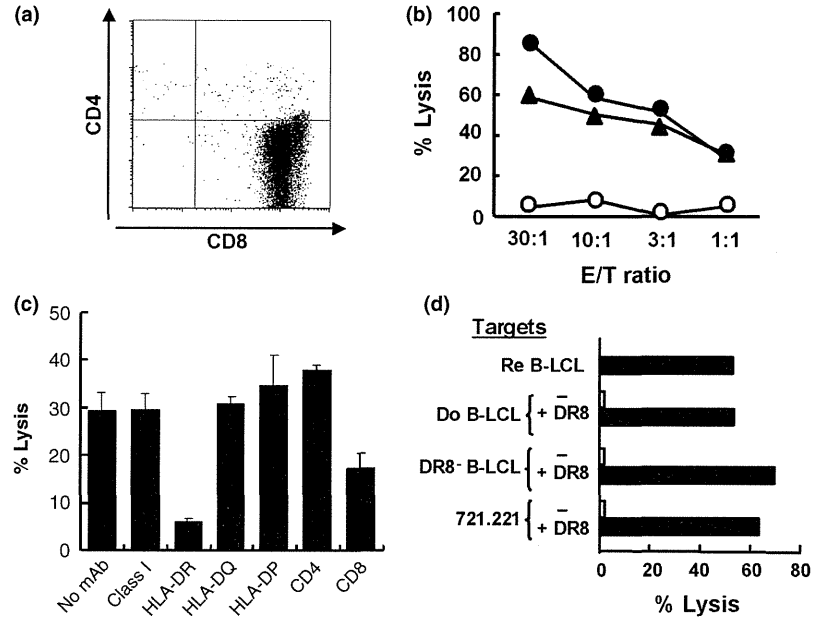

Fig. 1. Characteristics of the human leukocyte antigen (HLA)-DR8-restricted CTL clone, 1H8. (a) The cytolytic activity of CTL-1H8 was evaluated in a standard 4-h ^{51}Cr release assay. The recognition by CTL-1H8 of target cells was examined against B-lymphoblastoid cell lines (B-LCL) derived from the 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.

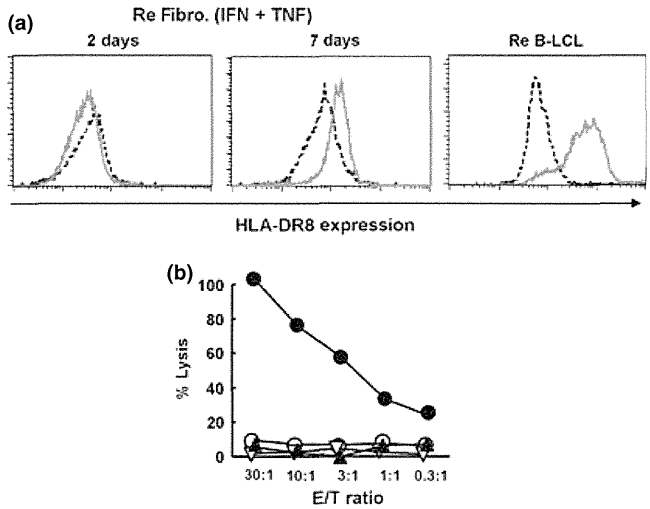

Fig. 2. Induction of human leukocyte antigen (HLA)-DR8 by cytokines and susceptibility of cytokine-treated dermal fibroblasts to CTL-1H8. (a) The recipient (Re) dermal fibroblasts (Fibro.) were incubated with 100 U/mL interferon (IFN)-γ and 10 ng/mL tumor necrosis factor (TNF)-α for 2 or 7 days and analyzed 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

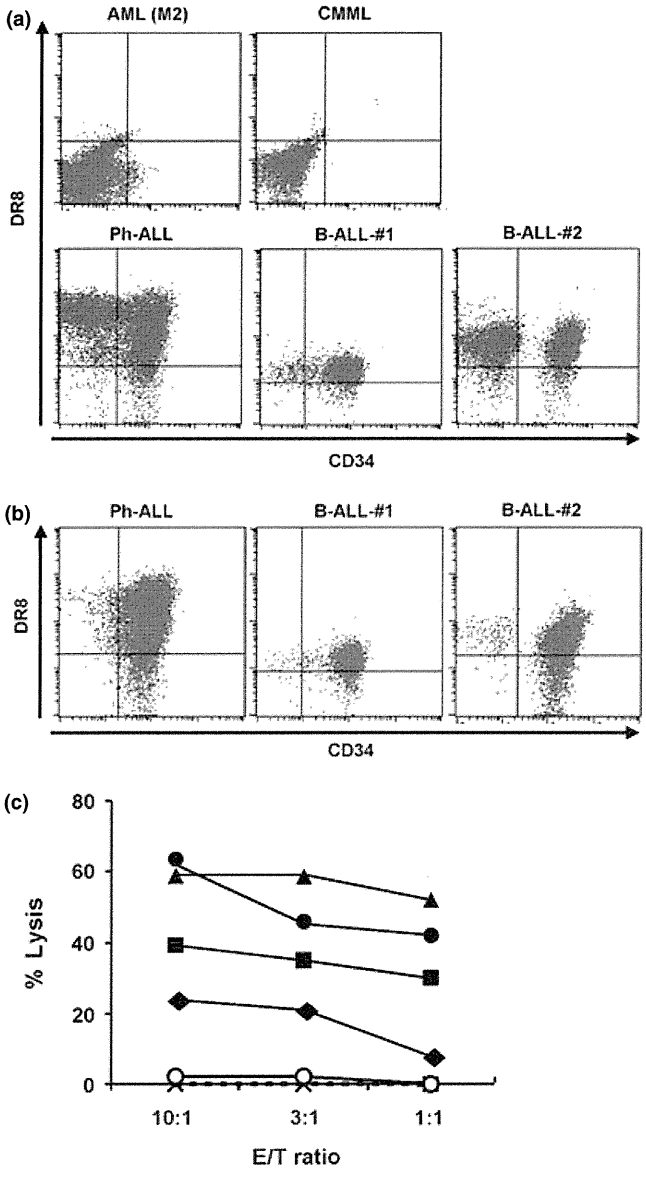

Fig. 3. Expression of human leukocyte antigen (HLA)-DR8 in primary leukemia cells and their susceptibility to CTL-1H8. (a) Expression of DR8 on five primary leukemia samples possessing the HLA-DRB1*08:02 or DRB1*08:03 genotype was examined in conjunction with CD34. (b) Purity and HLA-DR8 expression of 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.

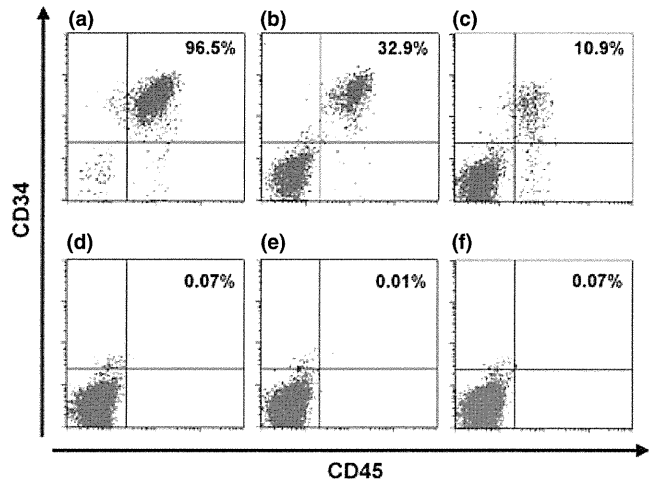

Fig. 4. Inhibition of human Philadelphia chromosome acute lymphoblastic leukemia (Ph-ALL) stem cell engraftment in non-obese diabetic/severe combined immunodeficient/γc-null (NOG) mice by CTL-1H8. The $CD34^+$ fraction (3.0×10^6) of a Ph-positive primary ALL sample was preincubated for 16 h either alone 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.

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 $CD8^+$ 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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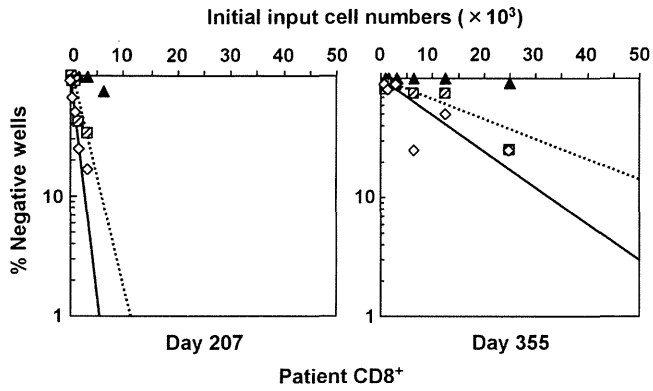

Fig. 5. Human leukocyte antigen (HLA)-DR8-specific $CD8^+$ T cells were detectable in recipient (Re) post-transplant peripheral blood mononuclear cells (PBMC) following HLA-DR-mismatched hematopoietic stem cell transplantation (HSCT). The proportion of $CD8^+$ CTL precursors specific for HLA-DRB1*08:03 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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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,

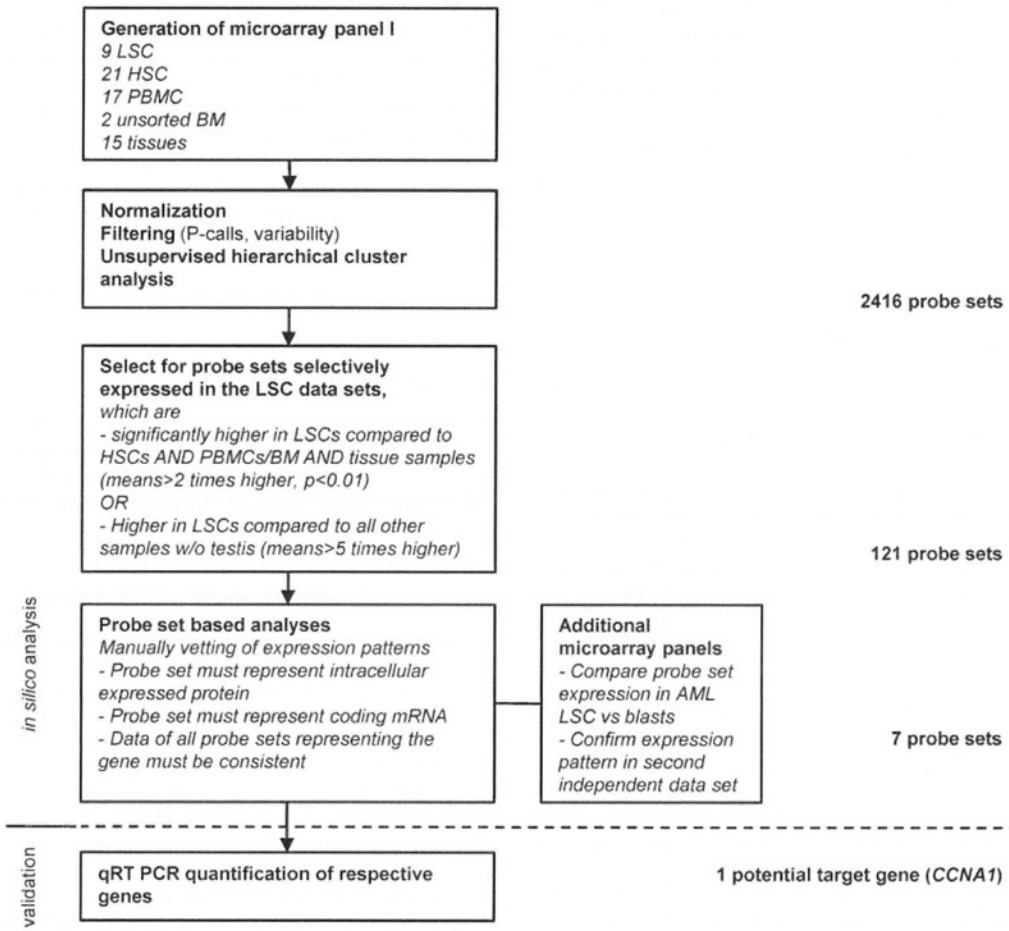

Figure 1. Systematic approach to identify potential target genes in AML LSCs based on 3′IVT expression microarray data. The microarray panel was created combining data files from 5 independent studies. Datasets were filtered for present calls in more than 10% of the samples and overall variability (reject 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

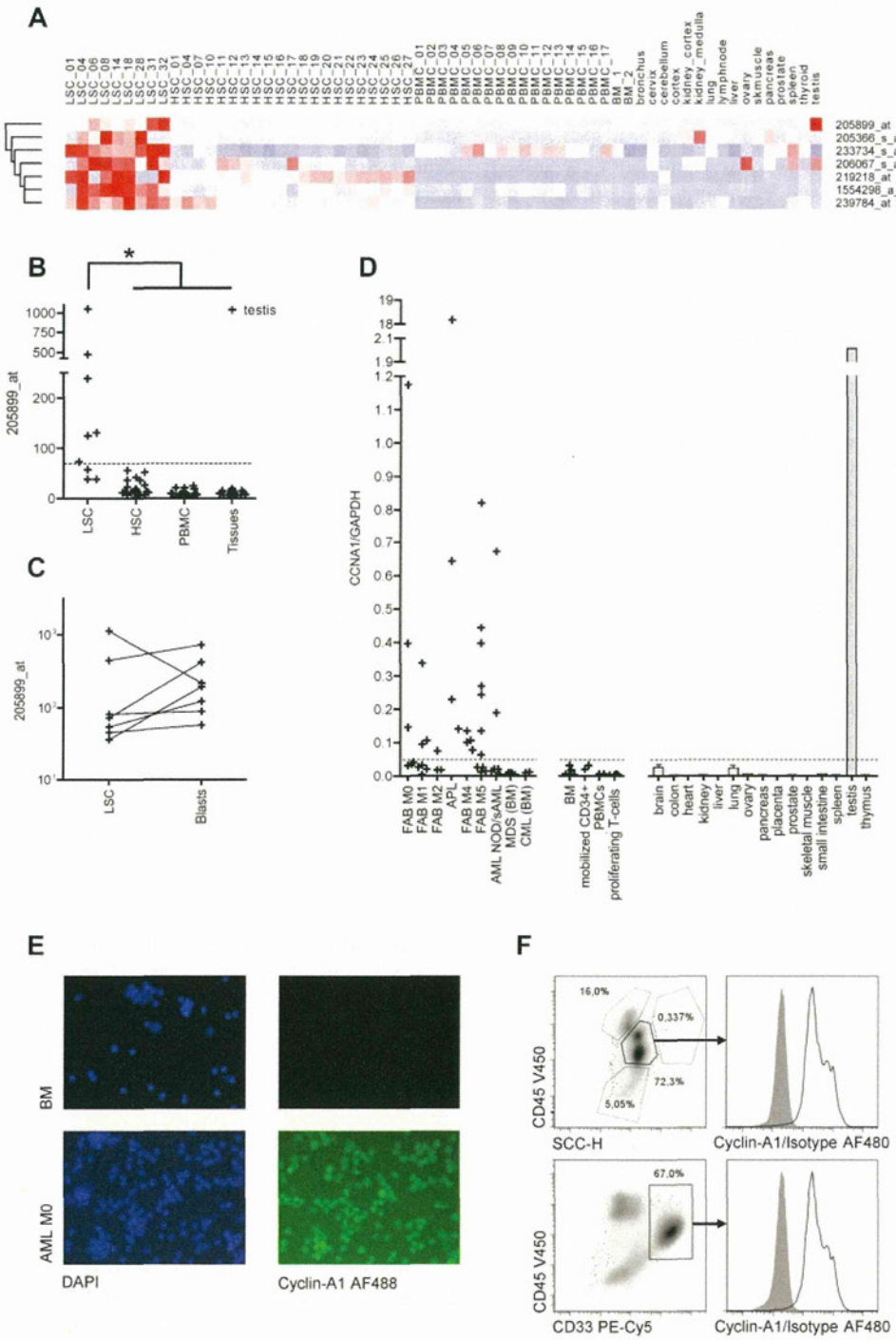

Figure 2. Identification of cyclin-A1 as target candidate by in silico expression analysis and RT-PCR quantification. (A) Seven candidate target genes predicted in silico: Heatmap of normalized probe sets. Each row represents one probe set, and each column represents one sample dataset. Red represents 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.

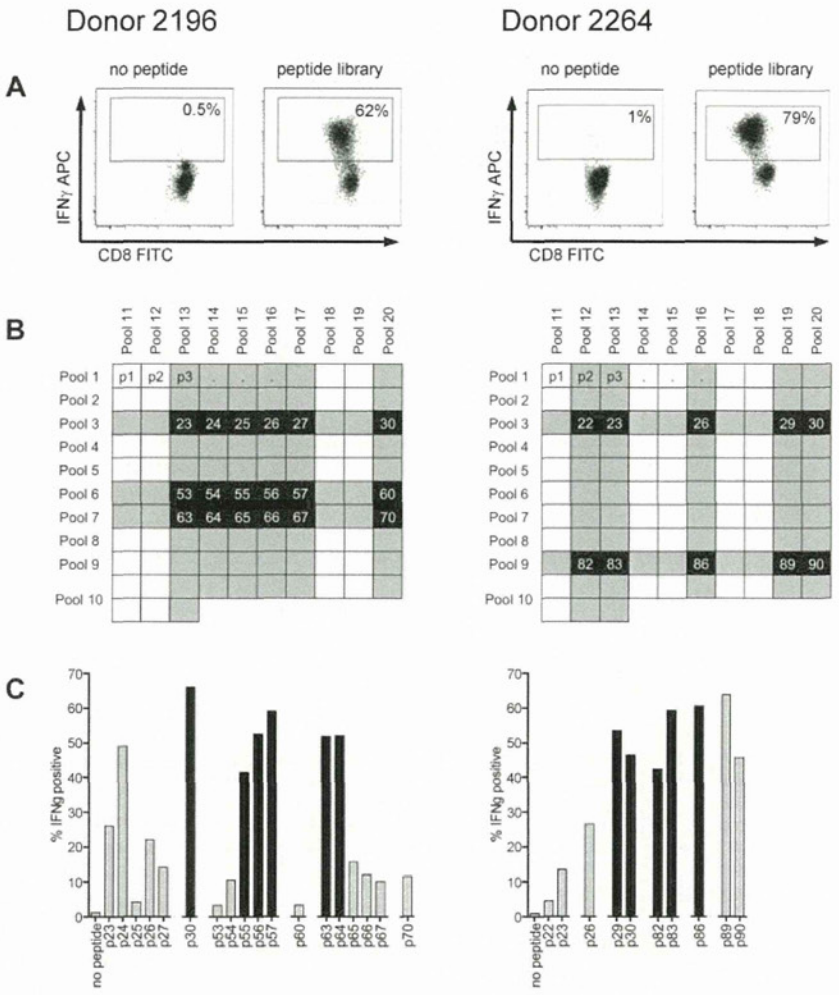

Figure 3. Reverse immunology strategy for epitope mapping in cyclin-A1 using ICS for IFN-γ. (A) After 4 stimulations with the peptide library, T-cell lines from both donors consisted of more than 60% specific cells (gated on $CD8^+$ cells). (B) The cell lines were subsequently tested against 20 peptide 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)

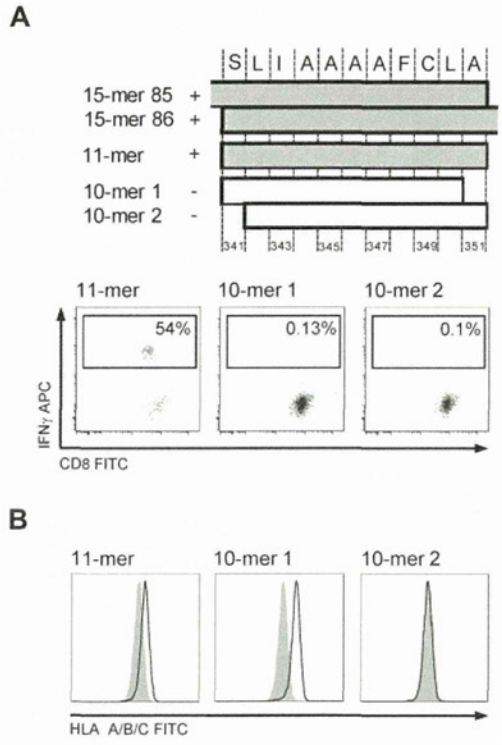

Figure 4. HLA A*0201-restricted epitope 341-351. Mapping the minimal immunogenic AA sequence and HLA stabilization. (A) The position of the different peptides in cyclin-A1. IFN-γ ICS of T-cell line (donor 2264) stimulated by autologous LCLs pulsed with the 10-mer 1, 10-mer 2, and 11-mer. Only pulsing 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

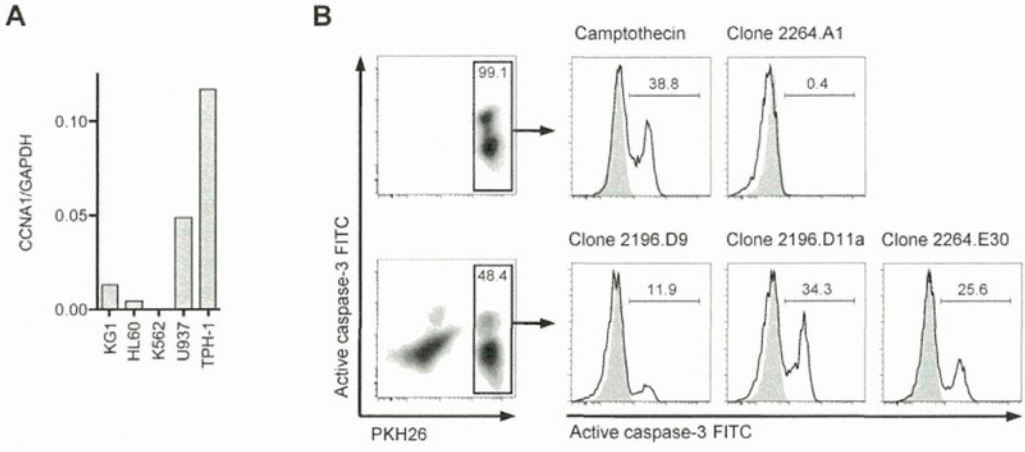

Figure 5. T-cell clones recognize endogenous processed epitopes 227-235 and 341-351. (A) Expression of cyclin-A1 in several myeloid cell lines quantified by quantitative RT-PCR. (B) Clones 2196.D9 and D11$_a$ (both specific for epitope 227-235) and clone 2264.E30 (specific for epitope 341-351) were tested 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.