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Augmentation of immune response by altered peptide ligands of the antigenic peptide in a human CD4$^+$ T-cell clone reacting to TEL/AML1 fusion protein

Key words:
HLA class II; human; pre-B acute lymphoblastic leukemia; T lymphocytes; TEL/AML1; tumor immunity

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Abstract: The 12;21 chromosomal translocation occurs in leukemic cells from 20–30% of patients with B-lineage childhood acute lymphoblastic leukemia, the result being the TEL/AML1 fusion gene carrying a sequence different from TEL or AML1. Because the protein newly formed by TEL/AML1 fusion is probably not tolerated by human immune system, the fusion region is a good candidate for tumor antigen expressed only in TEL/AML1-positive leukemic cells. We established two human CD4$^+$ αβ T-cell clones (T31.1 and Y41.2) reacting to the TEL/AML1 fusion region, from two unrelated healthy donors. In order to do this, we stimulated peripheral blood mononuclear cells with synthetic peptides corresponding to the TEL/AML1 fusion region. Both T31.1 and Y41.2 proliferated in response to TEL/AML1 fusion protein as well as to a peptide IGRIAECILGMNPSR, in the context of HLA-DP5 and DP17, respectively, and killed B lymphoblastoid cells pulsed with the peptide. Furthermore, these T-cell clones proliferated in response to several altered peptide ligands carrying a single residue substitution in the TEL/AML1 peptide, and some induced augmentation of proliferation and production of Th1-type cytokines. These superagonistic altered peptide ligands can be given consideration for anti-leukemic immunotherapy.

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The specific 12;21 chromosomal translocation identified in childhood acute lymphoblastic leukemic cells (1) results in a fusion gene between TEL (2) on chromosome 12p13 and AML1 (3) on chromosome 21q22 (4). Both TEL and AML1 are likely to be transcription factors. AML1 regulates the expression of several hematopoietic lineage-specific genes and plays a pivotal role in hematopoiesis (5). Both genes are fused with other genes to form fusion genes and these TEL- or AML1-associated fusion genes are thought to disrupt normal hematopoiesis, thus contributing to the pathogenesis of myeloid and lymphoid leukemia (6, 7). The 12;21 chromosomal translocation is difficult to detect by classical cytogenetics, but can be readily detected using reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence *in situ* hybridization (FISH).

TEL/AML1, observed in leukemic cells from 20–30% of patients with B-lineage childhood acute lymphoblastic leukemia, is thought to be one of the most frequent gene abnormalities associated with childhood malignancies (8).

In various tumors, tumor antigens and peptides recognized by T cells were identified and characterized (9). Tumor antigens are classified into mutated gene products (10), fusion proteins (11, 12) and normal proteins preferentially expressed or overexpressed in tumors (13). TEL and AML1 fuse in-frame but result in two kinds of fusion proteins with amino acid sequences that differ from TEL or AML1 (14). These fusion proteins are expressed only in TEL/AML1-positive leukemic cells, and not in normal tissues. These regions are candidates for tumor antigens because they would not be tolerated by the immune system.

Cytoplasmic or nuclear tumor antigens are processed into antigenic peptides in tumor cells to be presented on the cell surface, in the context of HLA class I molecules. Otherwise, apoptotic tumor cells are engulfed by APCs and processed into antigenic peptides to be presented on the surface of APCs, in the context of HLA class I and class II molecules (15). In anti-tumor immune responses, $CD8^+$ T cells recognize the major histocompatibility complex (MHC) class I-peptide complex on tumor cells and directly kill them (16). $CD4^+$ T cells recognize antigenic peptide, in the context of MHC class II molecules and exhibit anti-tumor activity by secreting cytokines, in addition to direct cell-killing activity mediated by Fas-ligand or tumor necrosis factors (TNF). $CD4^+$ T cells also amplify responses of CTLs, activate APCs, and maintain immunological memory (17). Thus, not only $CD8^+$ T cells but also $CD4^+$ T cells play important roles in tumor immunity.

Recognition of antigenic peptide by T cells is flexible and altered T-cell responses caused by altered peptide ligands (APLs) have been described (18). T cells can be activated by some APLs with single or multiple amino acid substitutions in agonistic wild-type peptides. Some of these APLs work as "superagonists"; they have a more potent activity than wild-type peptide to stimulate T-cell proliferation and cytokine production. We reported that immune response of a human $CD4^+$ αβ T-cell clone recognizing mutated p21 Ras proteins was augmented in recognition of a superagonistic APL (19). Enhancement of activation and proliferation of anti-tumor T cells using APLs may be one approach to evoke potent anti-tumor immunity.

To examine the possibility that peptides formed by TEL/AML1 fusions function as a tumor-antigen for possible use in anti-leukemic immunotherapy, we sought to determine whether $CD4^+$ T cells recognizing the peptides exist in healthy individuals. We found evidence for such T cells and we also identified numbers of superagonistic APLs derived from the epitope.

Material and methods

Peptides and recombinant proteins

Six kinds of the 21-mer peptides with a 15-residue-overlap, designed according to the two different TEL/AML1 junctional sequences reported elsewhere (2, 14, 20) (Table 1), were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corporation, Kyoto, Japan) based on the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy, and purified by C_{18} reverse-phase high-performance liquid chromatography (Millipore, Waters, Milford, MA, USA).

The TEL/AML1 cDNA clone (kindly provided by Dr. S.W. Hiebert) (21) covering codon 219–402, including a major type break point at the TEL residue 338 and myelin-oligodendrocyte glycoprotein (MOG) cDNA clone (kindly provided by Dr. A. Ben-Nun) (22) were inserted into pET28a expression vector (Novagen, Milwaukee, WI, USA). Recombinant proteins were expressed in a bacterial expression system and purified according to manufacturer's instructions. The buffer was replaced with culture medium by dialysis. To determine the purity and amount of proteins, soluble recombinant-human-TEL/AML1 (rh-TEL/AML1) and recombinant-human-MOG (rh-MOG) were analyzed by SDS-polyacrylamide gel electrophoresis.

Generation of T-cell clones recognizing the TEL/AML1 fusion region

Peripheral blood mononuclear cells (PBMCs) (1.5×10^5/well) from two healthy donors were incubated with a mixture of six peptides (1.0 μM each) covering two distinct TEL/AML1 fusion regions, in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented

Amino acid sequences of synthetic peptides covering TEL/AML1 fusion points

Peptide	Amino acid sequence	
	TEL exon 5	AML1 exon 2
TA1	**VSVSPPEEHAMPIGRIA/ECIL**	
TA2	**EEHAMPIGRIA/ECILGMNPSR**	
TA3	**IGRIA/ECILGMNPSRDVHDAS**	
	TEL exon 5	AML1 exon 3
TA4	**VSVSPPEEHAMPIGRIA/DAST**	
TA5	**PEEHAMPIGRIA/DASTSRRFT**	
TA6	**IGRIA/DASTSRRFTPPSTALS**	

Peptides from TA1 to TA3 were encoded by the gene carrying the major type of TEL/AML1 translocation, TEL exon 5 followed by AML1 exon 2, and peptides from TA4 to TA6 were encoded by the gene carrying the minor type of translocation, TEL exon 5 followed by AML1 exon 3. Fusion-points are indicated by slashes.

Table 1

with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μM 2-mercaptoethanol (2-ME) and 10% pooled, heat-inactivated normal human male plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). 2-ME was added to prevent formation of S-S bonds between Cys-residues of the peptides. After 7 days, irradiated (3,000 cGy) autologous PBMC (1.5×10^5/well) pulsed for 6 h with peptide mixture (5 μM each) were added to the culture wells carrying T-cell blasts and the cultures were maintained for 7 additional days. Subsequently, culture wells showing reactivity against the mixture of overlapping peptides were selected. Cloning was done in Terasaki plates (Nunc, Roskilde, Denmark) by limiting dilution at 0.3 cells per well in the presence of irradiated autologous PBMC (3×10^4/well) pulsed with peptide mixture, rhIL-2 (100 U/ml), and the same medium as described above. Growing microcultures were expanded at weekly intervals first in a 96-well plate, and then in a 24-well plate by feeding with irradiated autologous PBMC pulsed with the peptide mixture, in the presence of rhIL-2.

The surface markers of T-cell clones were analyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA), using anti-CD3, anti-CD4, anti-CD8, and anti-T cell receptor α/β monoclonal antibodies (mAbs) conjugated with either fluorescein isothiocyanate or phycoerythrin (PharMingen, San Diego, CA, USA).

Antigen-specific response of T-cell clones

Antigen-specific proliferation of the T-cell clones was assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottom culture plates in the presence of indicated concentrations of antigenic peptide or protein, and irradiated autologous PBMC (1.5×10^5/well), in multiplicate cultures. Cells were cultured for 72 h, in the presence of 1 μCi/well of 3H-TdR during the final 16-h period, and the incorporated radioactivity was measured by liquid scintillation counting. rh-TEL/AML1 and rh-MOG proteins were also tested for antigenicity.

To determine restriction molecules for antigen presentation, the T-cell clones were cultured with irradiated autologous PBMC, with or without saturating amounts of either anti-HLA-class II mAbs HU-4 (anti-HLA-DRB1+DRB5 IgG2a, monomorphic) (23), L243 (anti-HLA-DRB1+DRB4 IgG2a, monomorphic) (24), or 1a3 (anti-HLA-DQ IgG2a, monomorphic) (Leinco Technologies, Inc., Manchester, UK), MAB1764 (anti-HLA-DQ IgG1 monomorphic) (Chemicon International Inc.), or B7/21 (anti-HLA-DP IgG1, monomorphic) (25). Allogeneic PBMC or Epstein-Barr virus-transformed B lymphoblastoid cell lines (BLCLs) were also used as APCs. BLCLs, LBUF and Wa were distributed by the 11th International Histocompatibility Workshop (26). Culture supernatants of the T-cell clones stimulated by APCs and soluble peptide (1.0 μM) were collected after 56 h of stimulation, and cytokine concentrations were determined. The human IL-4 ELISA kit (R&D systems), IFN-γ ELISA kit (Otsuka, Tokyo, Japan) and GM-CSF ELISA kit (BioSource, Camarillo, CA, USA) were used for quantitation of cytokines in culture supernatants, performed according to the manufacturer's instructions.

Cytotoxicity assay

The cytotoxic activity of CD4+ T-cell clones was tested by ^{51}Cr release assay, as described (27). BLCLs were labeled with ^{51}Cr (CJS11, Amersham, UK), subsequently pulsed with or without 25 μM TA3 peptide at 37°C for 1.5 h and washed with RPMI 1640 medium, to be used as target cells. To block the peptide presentation by BLCL, anti-DP mAb B7/21 was added to the culture, 30 min before the addition of effector cells. The target cells (2×10^3/well) were plated in triplicate in 96-well round-bottom tissue culture plates and mixed with the indicated numbers of effector cells. After 4 h of incubation, radioactivity in the supernatants of each well was measured. The result was expressed as a % specific lysis, calculated as follows: (experimental release – spontaneous release) / (maximum release – spontaneous release) ×100.

HLA typing

DRB (DRB1, DRB3, DRB4 and DRB5), DQA1, DQB1, DPA1 and DPB1 alleles of healthy donors and DPA1 and DPB1 alleles of B-cell lines were determined by hybridization of HLA-DR, DQ and DP genes amplified by polymerase chain reaction with sequence-specific oligonucleotide probes recommended at the 12th International Histocompatibility Workshop (28). The nomenclature of the HLA-DR, DQ, and DP alleles was according to the WHO Nomenclature Committee for factors of the HLA system (29).

Results

Establishment of T-cell clones recognizing the TEL/AML1 fusion point

Two T-cell clones, T31.1 and Y41.2, recognizing the mixtures of TEL/AML1 fusion peptides and established from two unrelated healthy donors were used for further experiments. These T-cell clones specifically proliferated in response to two of six TEL/AML1 fusion peptides, TA2 and TA3, which were encoded for by a major type of TEL/AML1 fusion gene, TEL exon 5 followed by AML1 exon 2 (Fig. 1). No T-cell clone reactive to minor type of TEL/AML1

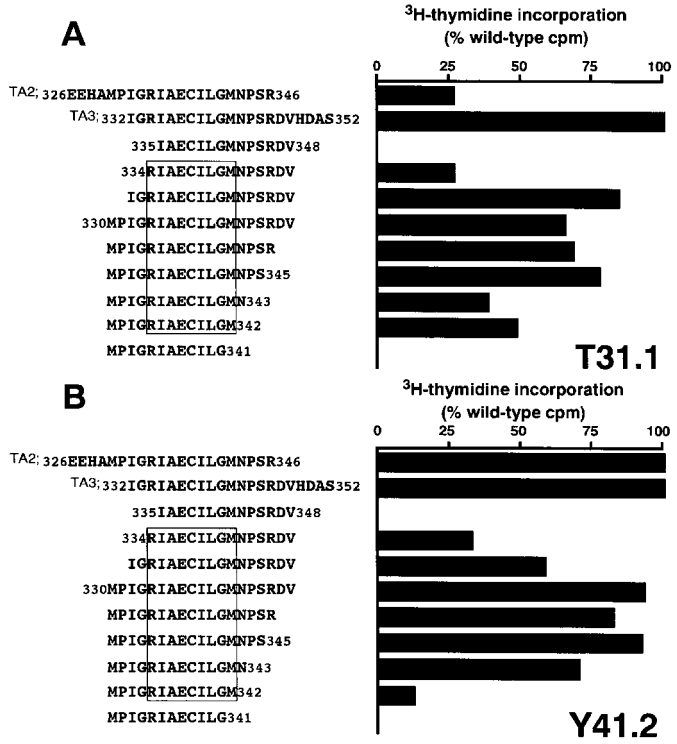

Fig. 1. **Proliferative responses of T-cell clones to TEL/AML1 fusion peptides truncated from N- or C-terminus.** Autologous PBMC was used as APC, and T cells were cultured for 72 h in the presence of 1.0 μM of each soluble peptide. The results from two independent experiments were combined for T-cell clones T31.1 (A) and Y41.2 (B). Responses of T cells to each truncated peptide were compared by calculating percentage to response to the wild-type peptide TA3 by using mean values of triplicate responses as follows: (value obtained with a truncated peptide – that with medium alone)/(value obtained with the TA3 peptide – that with medium alone) ×100. One hundred per cent wild-type response ranged from 20,000 to 30,000 cpm, and medium control responses without peptides were less than 100 cpm. Putative core epitope recognized by T cells are boxed.

fusion peptides was established. Flow cytometric analyses of these T-cell clones revealed $CD3^+$, αβ TCR^+, $CD4^+$ and $CD8^-$ phenotype (data not shown).

To determine whether T-cell clones recognize an epitope containing TEL/AML1 fusion point, we synthesized several peptides that contain overlapping region of TA2 and TA3, as well as their shorter derivatives truncated from either N- or C-terminus. As shown in Fig. 1, peptides deleted of Arg334 at N-terminus or Met342 at C-terminus did not induce proliferation in either T-cell clone even at 100 μM peptide concentration but both T-cell clones did proliferate in response to peptides that contain the region from Arg334 to Met342, RIAECILGM, which covered the TEL/AML1-fusion point. Peptides with one or two flanking residues were effective in inducing proliferation of T cells as strong as wild peptide. These data indicate that both T-cell clones recognize the epitope containing the major type of TEL/AML1 fusion point.

Restriction molecules of TEL/AML1-reactive T-cell clones

To identify HLA molecules that present TEL/AML1 peptides to established T-cell clones, blocking experiments using anti-HLA class II mAbs were done. The proliferative responses of both clones were completely blocked by B7/21 specific to DPB1 gene products, but L243, HU4, 1a3 and MAB1764 were not capable of blocking the response (Fig. 2). Thus, both T-cell clones were likely to be restricted by DP molecules.

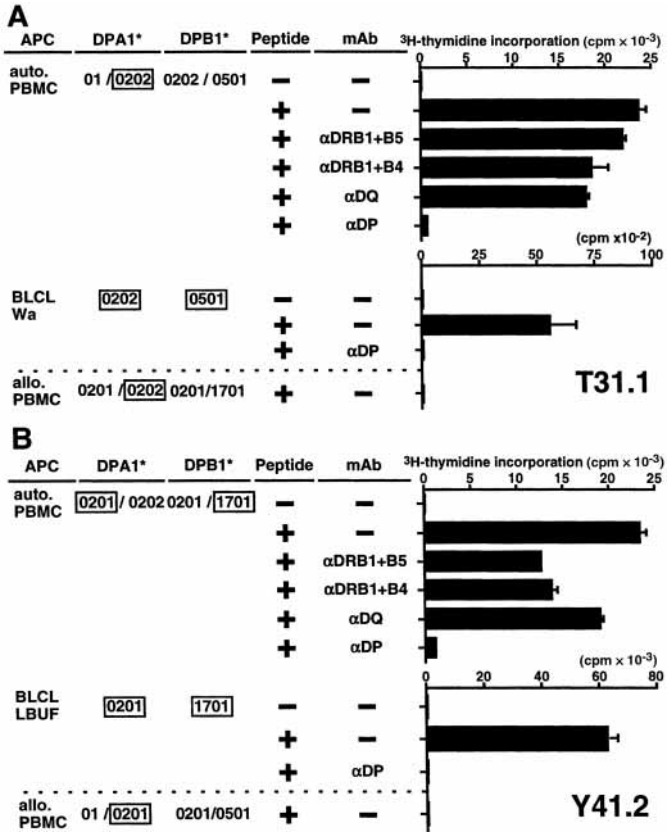

Fig. 2. **Identification of restriction HLA class II molecules of TEL/AML1-reactive T-cell clones.** Proliferative responses of T-cell clones were investigated in the presence of various APCs with or without anti-HLA mAbs. When autologous PBMCs were used as APC, soluble TA3 peptide (1.0 μM) was utilized for antigenic stimulus. When BLCL Wa (A) homozygous for DPA1*0202-DPB1*0501, BLCL LBUF (B) homozygous for DPA1*0201-DPB1*1701 and allogeneic PBMC were used as APC, they were pulsed with TA3 peptide (5.0 μM) for 2 h. To block the peptide presentation by APCs, saturating amounts of anti-HLA class II mAbs were added to APCs, 30 min before addition of soluble peptide or addition of T cells to peptide-pulsed APC. Boxed HLA alleles indicate those shared between T-cell donors and APC-donors. Values shown are the mean cpm of triplicate responses+SD.

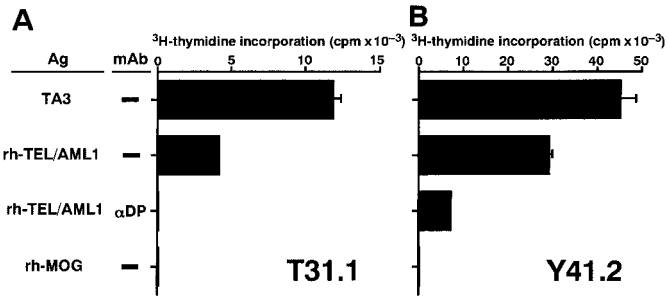

Fig. 3. **Proliferative responses of T-cell clones to TA3 peptide and rh-TEL/AML1 protein.** Autologous PBMC was used as APC, and T cell clones T31.1 (A) and Y41.2 (B) were cultured for 72 h in the presence of 1.0 μM of each peptide or recombinant proteins with or without anti-DP mAb. Values shown are the mean cpm of triplicate cultures+SD.

As shown in Fig. 2, T31.1 proliferated in response to the wild-type peptide presented by allogeneic PBMC and BLCL Wa sharing DPA1*0202 and DPB1*0501 with the donor of the T-cell clone. It was concluded that T31.1 was restricted by HLA-DP5 (DPA1*0202-DPB1*0501). The same analysis revealed that Y41.2 was restricted by DP17 (DPA1*0201-DPB1*1701). Thus, both T-cell clones recognized the same epitope but differed in restriction molecules.

Proliferative responses of T-cell clones to recombinant TEL/AML1 protein

To determine if APCs process TEL/AML1 fusion protein derived from leukemic cells into antigenic peptides, rh-TEL/AML1 protein corresponding to amino acid residues 219–402 was tested for the capacity to induce proliferative responses. As shown in Fig. 3, both T-cell clones proliferated in response to rh-TEL/AML1 protein but not to irrelevant rh-MOG protein. These proliferative responses were blocked by anti-DP mAb B7/21. We therefore concluded that the epitope recognized by T-cell clones was naturally processed from rh-TEL/AML1 and presented by APCs in the context of HLA-DP molecules to induce proliferation of the T-cell clones.

Cytotoxic activity of T31.1 and Y41.2

Cytotoxic activity of T31.1 and Y41.2 was then tested in a ^{51}Cr release assay, using BLCL pulsed with or without TEL/AML1 fusion peptide. As shown in Fig. 4, T31.1 exerted cytotoxic potential on peptide-pulsed Wa, a BLCL bearing DPA1*0202-DPB1*0501, but not on peptide-unpulsed Wa. Likewise, Y41.2 exerted cytotoxic potential only on peptide-pulsed LBUF, a BLCL bearing DPA1*0201-DPB1*1701, but not on peptide-unpulsed LBUF. Specific lysis of both T-cell clones increased in accordance with the increase in E:T ratio. Moreover, cytotoxic activities were abolished by anti-DP mAb B7/21. These observations indicate that both T-cell clones have cytotoxic potential, in a DP-restricted and peptide specific manner.

Augmentation of proliferative response and cytokine production in response to APLs

Some APLs carrying single or multiple amino acid residue substitutions in an antigenic peptide can augment proliferation and/or cytokine production of a human CD4+ T-cell clone restricted by HLA-DR (19) or HLA-DQ (30) molecules. To examine the effects on responses of TEL/AML1 reactive T-cell clones of minimal alternation in peptide sequence, we synthesized a series of APLs with conservative single amino acid substitutions.

As shown in Fig. 5B, four APLs, R1K (standing for APL carrying Arg to Lys substitution at N-terminus of the putative core epitope, RIAECILGM, recognized by T-cell clones), G8A, G8V and M9L, induced stronger proliferation of Y41.2 than wild-type peptide did. G8V in particular induced proliferation of Y41.2, even at 10 nM, a dose with which wild-type peptide did not induce proliferation. G8V at 1 μM induced about a 2-fold stronger proliferation in Y41.2. Seven APLs had 70–80% of the activity of wild-type peptide in T31.1. However, so far as we tested, no APLs induced stronger proliferation of T31.1 than did wild-type peptide (Fig. 5A).

Production by T-cell clones of cytokines related to anti-cancer immunity, such as IFN-γ and GM-CSF, was determined. G8V induced production of both cytokines by Y41.2, even at 10 nM, and

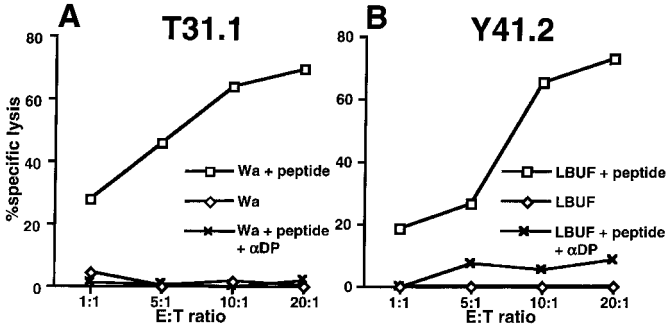

Fig. 4. **Cytotoxic activity of T-cell clones against peptide-pulsed BLCL.** ^{51}Cr-labeled BLCLs, Wa (A) and LBUF (B), were subsequently pulsed with or without peptide and be used as target cells. The T-cell clones, T31.1 (A) and Y41.2 (B), were co-incubated with target cells at the indicated E/T ratio, and ^{51}Cr release was measured after 4 h incubation. To block the peptide presentation by BLCL, anti-DP mAb B7/21 was added to the culture, 30 min before the addition of effector cells. Percent specific lysis was calculated by using mean cpm of triplicate cultures and SD of triplicate cpm did not exceed 10% of mean cpm. The spontaneous release from each target cell was usually less than 20–25% of the maximum release induced by treatment with 0.1% TritonX-100 and SD of triplicate cpm did not exceed 10% of mean cpm.

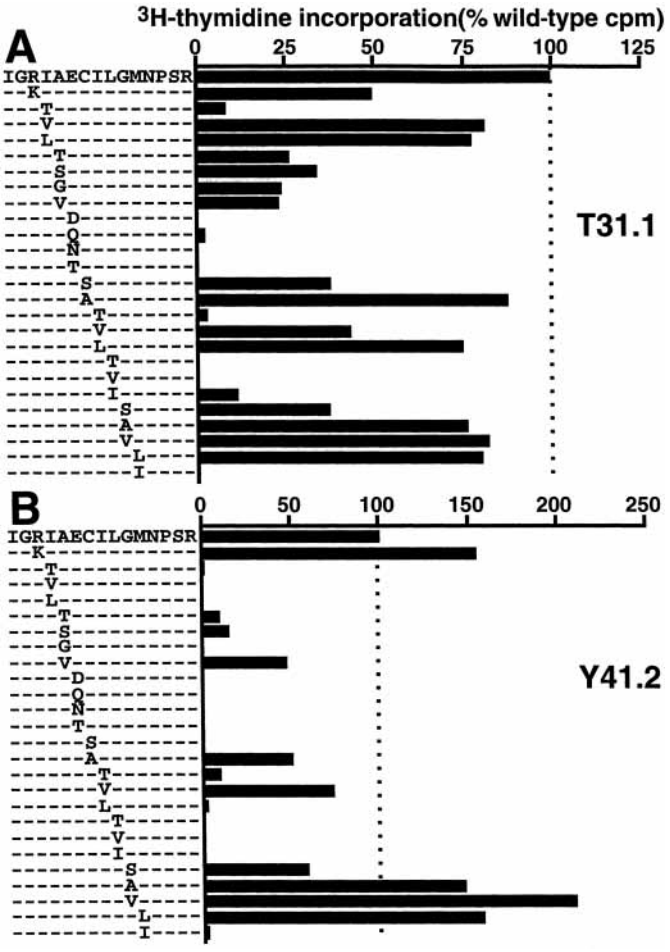

Fig. 5. **Proliferative response of T-cell clones to APLs.** T cells were cultured for 72 h in the presence of 1.0 μM of soluble APLs and irradiated autologous PBMC. Per cent wild-type response were calculated by using the mean values of triplicate cultures. One hundred per cent wild-type response ranged from 10,000 to 20,000 cpm, and medium control responses without peptides were less than 100 cpm.

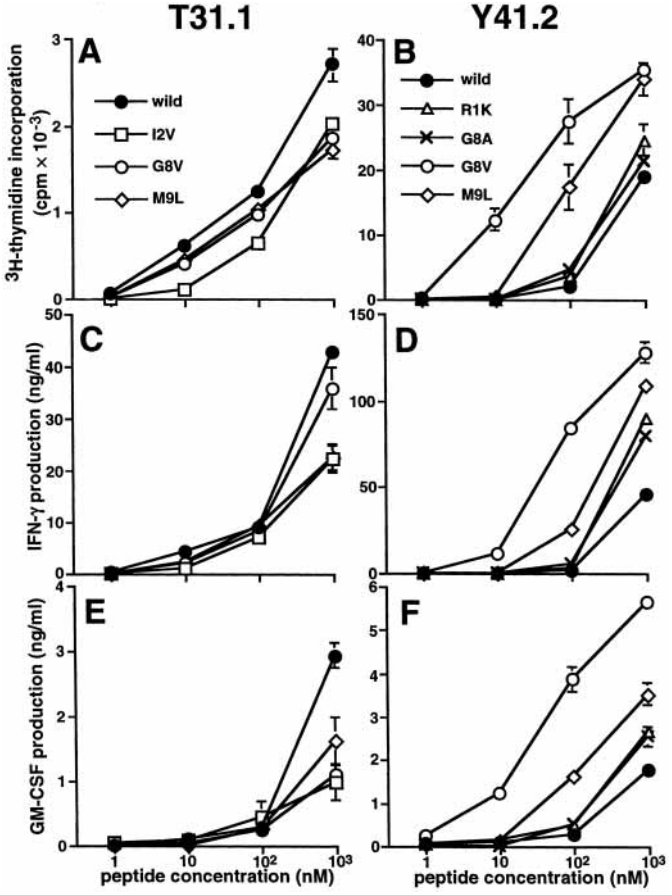

Fig. 6. **Proliferation of and cytokine production by T-cell clones in response to various doses of APLs.** T-cell clones T31.1 (A, C, E) and Y41.2 (B, D, F) were cultured with irradiated autologous PBMC in the presence of APL, at the indicated concentrations. After 56 h incubation, 70 μl from a total 150 μl of culture supernatants were collected and examined for IFN-γ (C, D) and GM-CSF (E, F) production by ELISA. To determine the proliferative responses, T cells were cultured for an additional 16 h (A, B). Results are expressed as mean values of duplicate cultures±SD.

significantly augmented cytokine production at the range of 0.1–1 μM (Fig. 6D,F). Three other APLs that augmented proliferative responses of Y41.2 also augmented the production of both cytokines. These APLs stimulated a similar amount of cytokines production in T31.1, as compared with findings in case of the wild-type peptide (Fig. 6C,E). IL-4 production was also determined (data not shown), but T-cell clones secreted very scant amounts of IL-4.

Discussion

In this study, we established from two healthy donors CD4+ T-cell clones recognizing for TEL/AML1 fusion region. It is conceivable that human CD4+ T cells do not acquire tolerance to the TEL/AML1 fusion region, and that MHC class II-restricted T cells recognizing this region do exist in healthy donors. Leukemogenesis-associated fusion proteins, such as BCR/ABL or PML/RARα, were reported to have antigenicity and to act as a tumor antigen specifically expressed in leukemic cells (11, 12). We show here that TEL/AML1 fusion protein is another possible candidate for the target of anti-leukemic immune response mediated by human CD4+ T cells. Because our T-cell clones responded to rh-TEL/AML1 protein, APCs could incorporate TEL/AML1 protein derived from leukemic cells and process it to present the T-cell epitope on their cell surface, in the context of HLA-DP molecules. Using lysates of pre-B leukemic cell lines or dendritic cells which engulfed UV-induced apoptotic leukemic cells, we tested leukemic cells for their capacity to induce

proliferative responses of T cells, but so far we find no T-cell response (data not shown). It still remains to be investigated whether T-cell clones described in this study are capable of directly or indirectly eradicating leukemic cells not only *in vivo* but also *in vitro*.

The putative core epitope RIAECILGM recognized by our T-cell clones was a 9 mer peptide carrying basic amino acid arginine at the N-terminus. In case of HLA-DR- or -DQ-restricted T cells, the most N-terminal and the first DR or DQ anchor residues are often hydrophobic amino acids (31). On the contrary, it was reported that hydrophilic and basic amino acids are suitable for the first anchor residue of DP-binding peptides (32, 33). Binding peptide motif of DP9 was reported by Dong et al. (32) and they described that DP9 specific binding motif was composed of R or K at position 1, A, G, or L at position 6, and L or V at position 9. Hori et al. (33) also reported that minimal T-cell epitope presented by DP5 was "KVTVAFNQF" and its N-terminal residue was a hydrophilic and basic amino acid. To our knowledge, there have been no previous reports of detailed binding motifs for HLA-DP5 or DP17 and except for position 1, the putative core epitope described in our current study does not fit with these reports. Our present observation provides further evidence of characteristics of DP-restricted T-cell epitopes.

MHC class II molecules usually bind peptides derived from membrane proteins or from extra-cellular proteins (34), and they exceptionally present peptides derived from cytoplasmic proteins (35). However, Bosch et al. reported that BCR/ABL-positive leukemic blasts presented endogenous BCR/ABL derived peptides, in the context of HLA-DR molecules, and that BCR/ABL-specific T cells could proliferate in response directly to these leukemic cells (36). Moreover, Th1 cells mediate cytotoxicity (37) and our T-cell clones also exerted cytotoxic activity to peptide-pulsed BLCL. If TEL/AML1-positive leukemic cells could present peptides derived from endogenous TEL/AML1 proteins, in the context of HLA class II molecules (all five TEL/AML1-positive leukemic cell lines tested, Reh, Sup-B26, Sup-B28, UoC-B4 and UoC-B6 (38), were positive for HLA-DP expression, our unpublished observation), it is possible that $CD4^+$ T cells possessing cytotoxic activity directly recognize these complexes and specifically exert cytotoxic activity to leukemic cells.

On the other hand, there is evidence that tumor cells by themselves downregulate T-cell responses, thus, they interfere with T-cell responses by releasing inhibitory cytokines (39) and induce anergy or apoptosis of T cells. Pre-B leukemic cells, with or without TEL/AML1 transcription, lack expression of costimulatory molecules required for optimal T-cell activation, and induce anergy in T cells recognizing alloantigen (40). Because we did not obtain HLA-DP5- or -DP17-positive and TEL/AML1-positive leukemic cells, we could not investigate whether T-cell clones respond to TEL/AML1-positive leukemic cells. In our preliminary observation, although T31.1 cells proliferated in response to peptide-pulsed BLCL, they did not proliferate in response to a peptide-pulsed pre-B type leukemic cell line expressing HLA-DP5 but not TEL/AML1 (data not shown). As reported by Cardoso et al. (40, 41), this may be due to differences in expression levels of CD80/86 costimulatory molecules between BLCL and pre-B leukemic cell line. We analyzed the expressions of CD80 and CD86 in BLCLs and pre-B leukemic cell lines used in this study by flow cytometry. All five pre-B leukemic cell lines described above expressed no CD80 and only a small amount of CD86, whereas BLCLs expressed much higher levels of both CD80 and CD86 than did leukemic cells.

Many studies have shown that the recognition of antigenic peptides by T cells is flexible, and qualitative and/or quantitative changes in T-cell responses can be induced in recognition of APLs. In this present study, we identified superagonistic APLs that augmented proliferation and cytokine production of the TEL/AML1-reactive T cell clone, Y41.2. Using such APLs, efficient *ex vivo* activation of $CD4^+$ T-cell clones reactive to tumor antigens might be achieved. It was reported that $CD4^+$ helper T cell, particularly Th1 cells, can assist the activation of CTL (42) and have important roles in anti-cancer immune responses (17). Indeed, the superagonistic APLs reported herein can induce and enhance production of Th1-type anti-tumor cytokines, indicating that APLs may be putative candidates for anti-leukemic-immunotherapy. However, whether TEL/AML1-reactive T cells are more efficiently propagated from PBMC by stimulation with superagonistic APLs than with wild-type peptide, and whether T cells propagated by stimulation of PBMC with superagonistic APLs can respond to wild-type TEL/AML1 protein remain to be determined.

A single amino acid substitution in the peptide affects interactions between MHC, peptide and TCR (43). One of the mechanisms for augmented proliferation and/or cytokine production of T-cell clones stimulated by APLs is that a single amino acid substitution in the peptide potentiates affinity of the peptide for MHC molecules and/or affinity of the peptide-MHC complex for TCR. We reported another mechanism for augmentation of T-cell response by APLs, APLs augmented IL-12 production by APCs to induce enhancement of IFN-γ production by a T-cell clone (30). Recently, Hemmer et al. reported that superagonistic APLs induce stronger TCR down-modulation and ZAP-70 phosphorylation than did wild-type peptide, at the same peptide concentration (44). It was also reported that an APL that binds to MHC with an affinity similar to that of the wild-type peptide, functioned as a superagonistic ligand (45). Detailed mechanisms for the APL-induced superagonism which we observed remain unclear.

T-cell clones, T31.1 and Y41.2, recognized the same epitope but differed in restriction molecules and responses to APLs. APLs sub-

stituted at position 2 induced proliferation of T31.1 but not Y41.2. APLs substituted at position 1, 8 and 9 functioned as superagonistic ligands for Y41.2 but were weak agonistic ligands for T31.1. Distinct responses to APLs of both T-cell clones may be due to differences in structures of restriction molecules and TCRs.

Yotnda et al. reported that HLA class I-restricted CD8$^+$ anti-ALL CTLs reactive to TEL/AML1(ETV6/AML1) fusion region exist in healthy donors and in leukemic patients, suggesting participation in host anti-leukemic defense (46). However, it has remained unclear whether this region is recognized by CD4$^+$ T cells. Our present study is the first to demonstrate that the TEL/AML1 fusion region is not tolerized and is indeed recognized by CD4$^+$ T cells in human, and that APLs derived from the TEL/AML1 fusion peptide augment proliferation and cytokine production by a T-cell clone. The function of our T-cell clones and APLs *in vivo* remains unclear. Anti-leukemic CTLs were seen to have too low a proliferative potential to the specific antigen to lead to leukemic cell eradication (46), however, TEL/AML1-specific CD4$^+$ T cells may possibly assist activation and proliferation of TEL/AML1-specific CTLs and promote anti-leukemic immune responses.

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