Brief report

Direct recognition and lysis of leukemia cells by WT1-specific CD4+ T lymphocytes in an HLA class II–restricted manner

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Wilms tumor gene 1 product (WT1) has been recognized as an attractive target antigen of immunotherapy for various malignancies including leukemia. Because tumor-associated antigen-specific CD4+ T lymphocytes undoubtedly play an important role in the induction of an antitumor immune response, we attempted to generate WT1-specific CD4+ T lymphocytes in vitro and examined their antileukemia functions. A CD4+ T-cell line, designated NIK-1, which proliferated and produced Th1 cytokines specifically in response to stimulation with the WT1-derived peptide, $WT1_{337-347}$ LSHLQMHSRKH, in an HLA-DP5–restriced manner was established. NIK-1 exhibited cytotoxicity against HLA-DP5–positive, WT1-expressing leukemia cells but did not lyse HLA-DP5–negative, WT1-expressing leukemia cells or HLA-DP5–positive, WT1–negative cells. NIK-1 did not inhibit colony formation by normal bone marrow cells of HLA-DP5–positive individuals. This is the first report to describe WT1-specific and HLA class II–restricted CD4+ T lymphocytes possessing direct cytotoxic activity against leukemia cells. (Blood. 2005;106: 1415-1418)

Introduction

Because Wilms tumor gene 1 product (WT1) is expressed in most cases of acute leukemia but not in normal tissues, it would be an attractive target antigen for immunotherapy against various malignancies including leukemia.[1-4] Recently, we and other investigators have succeeded in generating $CD8^+$ cytotoxic T lymphocytes (CTLs) that recognize WT1-derived peptides in vitro.[5-9] These WT1-specific CTLs efficiently lysed leukemia cells and solid tumor cells, but not normal cells, in an HLA class I–restricted manner. On the basis of these findings, clinical trials of cancer vaccine using WT1 peptides have been initiated.[10,11]

Increasing evidence from both murine and human studies indicates that tumor-associated antigen-specific $CD4^+$ T lymphocytes play a central role in orchestrating the host immune response against malignancies and infectious diseases.[12] Because identification of epitopes on WT1 recognized by $CD4^+$ T lymphocytes is essential for development of effective cellular immunotherapy for malignaicies targeting WT1, we attempted to generate WT1-derived peptide-specific $CD4^+$ T lymphocytes and examined their antileukemia functions.

Study design

Approval for the present study was obtained from the Institutional Review Board of Ehime University School of Medicine. Informed consent was obtained according to the Declaration of Helsinki.

On the basis of the amino acid sequence of WT1, a comprehensive panel of 43 20-mer peptides with 10 overlapping amino acids were synthesized. The WT1 peptide–specific $CD4^+$ T-cell lines were generated as reported previously.[13] Briefly, peripheral blood mononuclear cells (PBMCs) were stimulated 3 times with synthetic peptides at a concentration of 10 μg/mL. Cells showing a positive response to a WT1 peptide were cultured continuously in interleukin-2 (IL-2)–containing culture medium, and mitomycin C–treated autologous PBMCs and WT1 peptide were added to the wells every 1 to 2 weeks.

Chromium-51 release cytotoxicity assays were performed as described previously.[14] In some experiments, the target cells were incubated with an anti–HLA-DR monoclonal antibody (mAb) (L243), an anti–HLA-DQ mAb (HU-11), or anti–HLA-DP mAb (B7/21) at an optimal concentration for 30 minutes before adding the effector cells. Cold-target inhibition assays were performed as described previously.[15]

WT1 mRNA expression levels in cells were determined by quantitative reverse-transcription polymerase chain reaction (PCR) and calculated relative to that in the human leukemia cell line K562 as described previously.[16]

The effect of WT1-specific T lymphocytes on the growth of normal bone marrow cells was examined by performing the colony-forming assays as described previously.[5]

Results and discussion

A $CD4^+$ T-cell line, designated NIK-1, which proliferated specifically in response to stimulation with one of the 20-mer WT1

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Table 1. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 1

Target cells	% cytotoxicity, at E/T ratio		
	10:1 ratio	5:1 ratio	2.5:1 ratio
Auto LCL (HLA-DP5–positive)			
Without WT1 peptide	10.3	6.3	3.1
With WT1 peptide	70.2	63.2	49.1
Allo LCL no. 1 (HLA-DP5–positive)			
Without WT1 peptide	8.9	7.3	3.4
With WT1 peptide	73.9	62.1	50.1
Allo LCL no. 2 (HLA-DP5–positive)			
Without WT1 peptide	5.3	5.1	4.1
With WT1 peptide	63.2	56.1	47.3
Allo LCL no. 3 (HLA-DP5–negative)			
Without WT1 peptide	1.0	0.5	0.0
With WT1 peptide	2.1	0.8	0.1
Allo LCL no. 4 (HLA-DP5–negative)			
Without WT1 peptide	2.1	0.0	0.1
With WT1 peptide	4.1	0.8	0.0

Cytotoxicity of NIK-1 against autologous (auto) and various allogeneic (allo) LCL loaded or unloaded with WT1 peptide. E/T ratio indicates effector-target ratio.

peptides ($WT1_{336\text{-}355}$ KLSHLQMHSRKHTGEKPYQC) was established. More than 99% of NIK-1 cells were $CD3^+CD4^+CD8^-$. NIK-1 appeared to produce large amounts of T helper-1 (Th1) cytokines, such as interferon-γ, IL-2, and IL-12, upon stimulation with WT1 peptide in the presence of autologous PBMCs (data not shown). In addition to the proliferative response, NIK-1 showed strong cytotoxicity against a WT1 peptide–loaded autologous B-lymphoblastoid cell line (LCL) (Table 1). The restriction element of NIK-1 appeared to be HLA-DP, because the cytotoxicity and proliferative response of NIK-1 against WT1 peptide were significantly inhibited by adding anti–HLA-DP mAb. Because the donor was an HLA-DP5 homozygote, the cytotoxicity of NIK-1 against WT1 peptide–loaded *HLA-DP5* gene–transfected L cells was examined. As expected, NIK-1 exerted cytotoxicity against WT1 peptide–loaded HLA-DP5–positive L cells but not against $HLA\text{-}DP9^+$ L cells (Table 2). In addition, NIK-1 showed cytotoxicity against WT1 peptide–loaded HLA-DP5–positive but not HLA-DP5–negative allogeneic cells (Table 1), demonstrating that NIK-1 is the HLA-DP5–restricted $CD4^+$ T-cell line.

Table 2. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
Auto LCL (HLA-DP5–positive)	
Without WT1 peptide	10.5
With WT1 peptide	63.6
With WT1 peptide and anti–HLA-DR mAb	59.9
With WT1 peptide and anti–HLA-DQ mAb	62.5
With WT1 peptide and anti–HLA-DP mAb	11.6
L-DP5 (HLA-DP5–positive)	
Without WT1 peptide	5.7
With WT1 peptide	68.3
With WT1 peptide and anti–HLA-DR mAb	62.6
With WT1 peptide and anti–HLA-DQ mAb	64.7
With WT1 peptide and anti–HLA-DP mAb	8.9
L-DP9 (HLA-DP5–positive)	
Without WT1 peptide	2.5
With WT1 peptide	4.2

Cytotoxicity of NIK-1 against autologous LCL, *HLA-DP5* gene–transduced L cells, and *HLA-DP9* gene–transduced L cells loaded or unloaded with WT1 peptide in the presence or absence of anti-HLA-DR, anti-HLA-DQ, or anti-HLA-DP mAb. E/T ratio indicates effector-target ratio.

Table 3. WT1-specific and HLA-DP5–restricted cytotoxicity by NIK-1: experiment 3

WT1 peptide loaded	% cytotoxicity, at 10:1 E/T ratio
None	3.5
KLSHLQMHSRKHTGEKPYQC	73.5
KLSHLQMHSRKHTGEKPYQ	68.5
KLSHLQMHSRKHTGEKPY	73.1
KLSHLQMHSRKHTGEKP	58.6
KLSHLQMHSRKHTGEK	70.2
KLSHLQMHSRKHTGE	71.5
KLSHLQMHSRKHTG	71.2
KLSHLQMHSRKHT	73.1
KLSHLQMHSRKH	72.7
KLSHLQMHSRK	32.5
KLSHLQMHSR	7.3
KLSHLQMHS	7.5
LSHLQMHSRKHTGEKPYQC	63.9
SHLQMHSRKHTGEKPYQC	32.4
HLQMHSRKHTGEKPYQC	6.6
LQMHSRKHTGEKPYQC	6.9
QMHSRKHTGEKPYQC	4.9
MHSRKHTGEKPYQC	6.4
HSRKHTGEKPYQC	5.9
SRKHTGEKPYQC	7.2
RKHTGEKPYQC	3.5
KHTGEKPYQC	7.8
HTGEKPYQC	7.7
LSHLQMHSRKH	72.1

Cytotoxicity of NIK-1 against autologous LCL loaded and unloaded with various WT1 peptides. E/T ratio indicates effector-target ratio.

We next examined the fine epitope on WT1 recognized by NIK-1. Experiments using deletion peptides clearly demonstrated that the minimal amino acid sequence recognized by NIK-1 is $WT1_{337\text{-}347}$ LSHLQMHSRKH (Table 3).

Because NIK-1 showed WT1 peptide–specific cytotoxicity, we addressed the question of whether NIK-1 can lyse leukemia cells. Because most leukemia cell lines are HLA class II negative, only one HLA-DP5–positive leukemia cell line expressing WT1 was available. As shown in Table 4, NIK-1 exerted strong cytotoxicity against HLA-DP5–positive WT1-expressing leukemia cell lines but not against HLA-DP5–negative leukemia cell lines or HLA-DP5–positive or HLA-DP5–negative lymphoma cell lines that are negative for WT1 expression. Similarly to the cytotoxicity against cell lines, HLA-DP5–positive but not HLA-DP5–negative freshly isolated leukemia cells were lysed efficiently by NIK-1. Cytotoxicity of leukemia cells mediated by NIK-1 appeared to be restricted by HLA-DP5, because only HLA-DP5–positive leukemia cells were lysed by NIK-1 and the cytotoxicity of leukemia cells mediated by NIK-1 was inhibited by adding anti–HLA-DP mAb (Table 5).

To further confirm that the cytotoxicity of NIK-1 against leukemia cells was mediated by the specific recognition of endogenously processed WT1, we performed cold-target inhibition experiments. As shown in Table 6, the addition of WT1 peptide–loaded autologous LCL decreased the cytotoxicity of NIK-1 against leukemia cells, whereas the addition of peptide-unloaded autologous LCL had no effect on the cytotoxicity. These data strongly suggest that WT1 is naturally processed in leukemia cells and recognized by WT1-specific $CD4^+$ CTLs in the context of HLA-DP5.

We then addressed the issue of whether NIK-1 recognizes WT1 peptide expressed on normal bone marrow progenitor cells and

Table 4. Direct recognition and lysis of leukemia cells by NIK-1: experiment 1

Target cells	WT1 expression level	HLA-DP5 expression	% cytotoxicity, at E/T ratio		
			20:1 ratio	10:1 ratio	5:1 ratio
Cell lines					
C2F8; leukemia	2.3×10^{0}	+	38.8	34.4	31.2
MEG01; leukemia	8.4×10^{-1}	−	3.6	1.8	1.2
KAZZ; leukemia	1.1×10^{0}	−	2.1	2.7	0.9
IZU; lymphoma	1.1×10^{-5}	+	1.5	0.5	1.0
IKE; lymphoma	8.6×10^{-5}	+	0.7	1.8	0.5
Daudi; lymphoma	3.8×10^{-5}	−	2.0	0.3	0.4
Freshly isolated leukemia cells					
From donor no. 1 (AML M1)	3.5×10^{-1}	+	45.1	34.2	21.0
From donor no. 2 (AML M2)	2.7×10^{-1}	+	33.6	24.3	18.9
From donor no. 3 (AML M2)	8.6×10^{-2}	+	31.2	18.9	13.1
From donor no. 4 (AML M1)	6.5×10^{-1}	−	2.7	2.1	0.6
From donor no. 5 (AML M2)	7.2×10^{-1}	−	1.4	1.8	1.0
From donor no. 6 (ALL L2)	5.8×10^{-1}	−	0.0	0.3	0.0

Cytotoxicity of NIK-1 against various cell lines and freshly isolated leukemia cells. E/T ratio indicates effector-target ratio.

suppresses their growth. As shown in Table 7, after coculture with NIK-1 in the absence of WT1 peptide, the numbers of granulocyte-macrophage colony-forming units (CFU-GMs) and erythroid burst-forming units (BFU-Es) generated from bone marrow cells of 2 HLA-DP5–positive individuals were almost the same as those generated from bone marrow cells cultured alone. However, the numbers of CFU-GMs and BFU-Es decreased significantly when HLA-DP5–positive bone marrow cells were pretreated with WT1 peptide and then cocultured with NIK-1. As expected, NIK-1 had no effect on colony formation by HLA-DP5–negative bone marrow cells that had been pretreated with WT1 peptide or left untreated.

In the present study, we demonstrated for the first time the generation of WT1-specific $CD4^+$ T lymphocytes that can recognize directly leukemia cells in an HLA class II–restricted manner. It is well known that induction of the $CD8^+$ CTL response requires cognate $CD4^+$ T-lymphocyte help.[17] $CD4^+$ T lymphocytes recognize major histocompatibility complex (MHC) class II–binding peptides on antigen-presenting cells, such as dendritic cells (DCs), and their interaction may result not only in activation and priming of $CD4^+$ T lymphocytes but also in activation of the DCs themselves.[18] Consequent to this mutual activation, DCs prime and activate $CD8^+$ CTLs specific for tumor-associated antigens. On the basis of this scenario, it is expected that WT1-specific $CD4^+$ T lymphocytes may be effective for efficient induction of WT1-specific $CD8^+$ CTLs in vivo.

The other interesting finding of this study is that WT1 peptide–specific $CD4^+$ T lymphocytes exerted strong cytotoxicity against WT1-expressing leukemia cells in an HLA class II–restricted manner. In general, endogenous antigens are degraded in the cytoplasm to oligopeptides and bind to newly synthesized MHC class I molecules. On the other hand, exogenous antigens are processed into peptides capable of binding to MHC class II molecules. However, it has recently been shown that the MHC class II pathway can process and present endogenous antigens as well as exogenous antigens. For example, virus-infected cells are recognized by $CD4^+$ T lymphocytes in a viral antigen–specific and MHC class II–restricted manner in vitro and in vivo.[19,20] It has also been reported that tumor cells transfected with syngeneic MHC class II genes could present endogenously synthesized tumor-associated protein-derived peptides in the context of MHC class II molecules to $CD4^+$ T lymphocytes.[21] Taken together with previous data, our present findings strongly suggest that leukemia cells can process and present endogenously synthesized WT1 protein to $CD4^+$ T lymphocytes in the context of HLA class II molecules.

Table 5. Direct recognition and lysis of leukemia cells by NIK-1: experiment 2

Target cells	% cytotoxicity, at 10:1 E/T ratio
C2F8 cells	
No anti-HLA mAb	27.6
With anti-HLA-DR mAb	28.0
With anti-HLA-DQ mAb	30.3
With anti-HLA-DP mAb	3.1
Freshly isolated leukemia cells	
From donor no. 1 (AML M1)	
No anti-HLA mAb	45.1
With anti-HLA-DR mAb	43.2
With anti-HLA-DQ mAb	41.6
With anti-HLA-DP mAb	5.6
From donor no. 2 (AML M2)	
No anti-HLA mAb	33.6
With anti-HLA-DR mAb	31.7
With anti-HLA-DQ mAb	35.1
With anti-HLA-DP mAb	5.6

Cytotoxicity of NIK-1 against leukemia cell line and freshly isolated leukemia cells in the presence or absence of anti-HLA- DR, anti-HLA-DQ, or anti-HLA-DP mAb.

E/T ratio indicates effector-target ratio.

Table 6. Direct recognition and lysis of leukemia cells by NIK-1: experiment 3

Hot target cells and cold target cells	% cytotoxicity, at cold-hot target cell ratio			
	0	5:1	10:1	20:1
C2F8 cells				
WT1 peptide–loaded autologous LCL	31.3	22.5	14.3	9.6
Peptide-unloaded autologous LCL	31.3	30.5	29.7	28.1
Freshly isolated leukemia cells from donor no. 1 (AML M1)				
WT1 peptide–loaded autologous LCL	42.1	26.4	15.2	8.8
Peptide-unloaded autologous LCL	42.1	41.3	39.3	40.2

Cytotoxicity of NIK-1 against leukemia cells in the presence or absence of WT1 peptide–loaded autologous LCL or peptide-unloaded autologous LCL at an effector–hot target cell ratio of 10:1.

Table 7. Direct recognition and lysis of leukemia cells by NIK-1: experiment 4

Donor	Colony formation	
	CFU-GM	BFU-E
HLA-DP5–positive donor no. 1		
Without NIK-1	56 ± 5	86 ± 8
With NIK-1	59 ± 7	93 ± 10
With NIK-1 and WT1 peptide	29 ± 3	37 ± 7
HLA-DP5–positive donor no. 2		
Without NIK-1	136 ± 14	167 ± 24
With NIK-1	138 ± 8	170 ± 23
With NIK-1 and WT1 peptide	66 ± 14	62 ± 13
HLA-DP5–negative donor no. 3		
Without NIK-1	86 ± 15	96 ± 18
With NIK-1	88 ± 7	100 ± 10
With NIK-1 and WT1 peptide	85 ± 15	103 ± 8

The colony formation by normal bone marrow cells cocultured with or without NIK-1 cells in the presence or absence of WT1 peptide.

In summary, we have demonstrated WT1-specific $CD4^+$ T lymphocytes capable of producing Th1 cytokines and exerting direct cytotoxicity against leukemia cells in an HLA class II–restricted manner. Because most types of leukemic cells are positive for HLA class II expression,[22] WT1-specific $CD4^+$ CTLs may play an important role in the antileukemia response through cytotoxic activity as well as helper function for $CD8^+$ CTL induction. On the basis of this concept, we are planning a clinical trial of WT1 peptide vaccination using a combination of peptides derived from epitopes recognized by $CD4^+$ T lymphocytes as well as $CD8^+$ T lymphocytes.

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