CD4 T-Helper Responses to the Anaplastic Lymphoma Kinase (ALK) Protein in Patients with ALK-Positive Anaplastic Large-Cell Lymphoma

Kamel Ait-Tahar,[1] Martin C.N. Barnardo,[2] and Karen Pulford[1]

[1]Nuffield Department of Clinical Laboratory Sciences, Leukemia Research Fund Immunodiagnostics Unit, John Radcliffe Hospital, and [2]Transplant Immunology, Oxford Transplant Center, Churchill Hospital, Oxford, United Kingdom

Abstract

We have previously shown both humoral and CTL responses to anaplastic lymphoma kinase (ALK) in patients with ALK-positive anaplastic large-cell lymphoma (ALCL). However, because $CD4^+$ T-helper (Th) cells also play a vital role in developing and maintaining tumor immunity, we investigated the presence of a $CD4^+$ Th response in ALK-positive ALCL. Using an IFN-γ ELISPOT assay, we identified two ALK-derived DRB1-restricted 24-mer promiscuous peptides, $ALK1_{278–301}$ and $ALK2_{233–256}$, as being immunogenic in six ALK-positive ALCL patients but not in two ALK-negative ALCL patients or five normal subjects. A significant interleukin-4 response to the ALK peptides was detected in only one ALK-positive patient. $CD4^+$ Th cell lines lysed ALK-positive ALCL cell lines in a MHC class II–restricted manner. This first report of a $CD4^+$ Th response to ALK provides valuable information for developing future immunotherapeutic options for ALK-positive ALCL patients who fail to respond well to conventional therapies. [Cancer Res 2007;67(5):1898–901]

Introduction

Anaplastic large-cell lymphoma (ALCL) comprises 30% to 40% of pediatric large-cell lymphomas. The majority of ALCL are associated with the aberrant expression of anaplastic lymphoma kinase (ALK) fusion proteins and constitute the tumor entity ALK-positive ALCL (1). ALK expression is considered to be a primary oncogenic event in ALCL, playing a pivotal role in the survival of the tumor cells (2). This factor, combined with a lack of peripheral tolerance (due to the restricted normal distribution of ALK), identifies ALK as an ideal immunotherapeutic target (3).

Evidence confirming ALK as a tumor-associated antigen has been obtained from studies reporting the presence and persistence not only of an antibody response to ALK (4–6) but also of ALK-specific CTLs in patients with ALK-positive ALCL (6, 7). However, increasing evidence that $CD4^+$ T-helper (Th) cells play a significant role in the regulation and maintenance of the CTL response to tumors (8–10) means it is important to identify $CD4^+$ Th cell peptide-binding epitope(s) within the ALK protein to maximize the efficacy of future ALK-based immunotherapeutic strategies. We provide here the first description of a $CD4^+$ Th response to ALK in ALK-positive ALCL patients.

Requests for reprints: Kamel Ait-Tahar, Nuffield Department of Clinical Laboratory Sciences, Leukemia Research Fund Lymphoma Antigens Group, Room 5501, Level 5, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom. Phone: 44-1865-220536-222913; Fax: 44-1865-222912; E-mail: kamel.ait-tahar@ndcls.ox.ac.uk.

doi:10.1158/0008-5472.CAN-06-4427

Materials and Methods

Subjects. Six ALK-positive ALCL (patients 1–6) and two ALK-negative ALCL (patients 7 and 8), diagnosed according to the WHO classification (2), and five normal control subjects (normals 1–5) were studied. Following informed consent, blood was obtained from patients who were attending the Hematology Department, John Radcliffe Hospital, Oxford (patients 1, 2, 3, 7, and 8); Manchester General Hospital, Manchester (patient 4); Royal Marsden Hospital, London (patient 5); and Liverpool General Hospital, Liverpool (patient 6). Patients 4 and 5 were included in the Biological Studies project BS001 run by the United Kingdom Children's Cancer Study Group. Details of patients and samples are shown in Table 1.

HLA typing. HLA typing was done by PCR with sequence-specific primers using a modification of the phototyping method. PCR conditions were as previously described (12).

Peptides. The TEPITOPE prediction algorithm[3] was used to select ALK peptides predicted to bind with high affinity to DRB1 *0101, *0301, *0401, *0701, *1101, and *1501 (the most prevalent alleles among the Caucasian population; ref. 13). Three highly promiscuous peptides, $ALK1_{278–301}$ (PSSLAMLDLLHVARDIACGCQYLE), $ALK2_{233–256}$ (KFNHQNIVRCIGVSLQSLPRFILL), and $ALK3_{411–434}$ (PKNCPGPVYRIMTQCWQHQPEDRP), were identified lying within the kinase domain. $ALK1_{278–301}$ encompasses the highly immunogenic $ALK_{280–289}$ CTL epitope (6, 14). An irrelevant control peptide was $HIV_{121–140}$ (DESFRKYTAFTIPSMNNETP).

Cell lines. The t(2;5)-positive ALCL-derived cell lines SUDHL-1 (DRB1_0403) and KARPAS-299 (DRB1_0101) and the t(2;5)-negative ALCL cell line FEPD (DRB1_0301) were obtained and cultured as previously described (15).

Preparation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) in RPMI 1640 containing 10% FCS (RPMI 1640/FCS) were prepared as previously described (6). PBMCs (2×10^5) in 200 μL were added to each well of a 96-well flat-bottomed plate and incubated for 10 days with 10 μmol/L of $ALK1_{278–301}$, $ALK2_{233–256}$, $ALK3_{411–434}$, or the irrelevant $HIV_{121–140}$ peptide, or with 10 μg/mL of phytohemagglutinin (PHA; Sigma-Aldrich Co. Ltd., Dorset, United Kingdom). Recombinant interleukin-2 (IL-2; 20 IU/mL; Roche Diagnostics, Indianapolis, IN) and recombinant human IL-7 (25 ng/mL; R&D Systems, Minneapolis, MN) were added on days 2, 5, and 7.

ELISPOT assay. After 10 days of culture, cells were incubated for 18 h in complete RPMI 1640/FCS at 37°C in 5% CO_2 with $ALK1_{278–301}$, $ALK2_{233–256}$, $ALK3_{411–434}$, $HIV_{121–140}$, or PHA, or medium only. Peptides were used at 10 μmol/L and all cultures were carried out in triplicate. IFN-γ/IL-4 release ELISPOT assays were done (6) according to the manufacturer's instructions (Mabtech, Stockholm, Sweden). Spots were counted using an automated ELISPOT reader (Autoimmun-Diagnostika, Strassberg, Germany). The SD was calculated using standard techniques and Student's *t* test was used for statistical analysis.

CD4-enriched PBMC population. PBMCs cultured at a density of 2×10^6/mL were stimulated with 10 μmol/L of $ALK1_{278–301}$, $ALK2_{233–256}$, or $HIV_{121–140}$ peptide, or with medium only. After 72 h, an equal volume of

[3] http://www.vaccinome.com

Table 1. Clinical details of patients with ALK-positive ALCL

Patient	Age (y)	Sex	Stage	Treatment	Time of blood sample after diagnosis	Follow-up
1	46	M	II	CHOP	At diagnosis and 47 mo	CR
2	34	F	II	CHOP	At diagnosis and 12 mo	CR
3	17	M	IV	ALCL'98[1]	47 mo	CR
4	14	M	III	ALCL'99	At diagnosis	CR
5	5	M	II	ALCL'99	At diagnosis	PD
6	16	F	I	ALCL'99	At diagnosis	PD

NOTE: ALCL'98 and ALCL'99 use a modification of the NHL-BFM-B 90 treatment (11).
Abbreviations: CR, complete remission; PD, progressive disease; CHOP, cyclophosphamide, adriamycin, vincristine, prednisolone.

RPMI 1640/FCS containing 50 IU/mL recombinant IL-2 was added to each culture well; every 3 days thereafter, half of the medium was removed and replaced with fresh medium. In some experiments, a $CD4^+$ T-cell purification step was done (Dynabeads, Dynal, Oslo, Norway) following the manufacturer's instructions before the cells were used in an overnight ELISPOT assay for IFN-γ release. In other experiments, the HLA-DR–specific WR18 antibody (Abcam, Cambridge, United Kingdom) was added at 2 μg/mL to the $CD4^+$ Th cell population for 1 h before assay.

^{51}Cr cytolytic assay. SUDHL-1, KARPAS-299, and FEPD cells were radiolabeled with 100 μCi ^{51}Cr. The target cells were then incubated for 18 h with the ALK peptide–stimulated lines and cytolysis was analyzed as previously described (6).

Detection of the antibody response to ALK. Cytocentrifuge preparations of COS cells, which had been transiently transfected with cDNA encoding for ALK, NPM-ALK, or vector only, were prepared and stained using patient's plasma in an indirect immunoperoxidase technique as previously described (4).

Results and Discussion

A significant IFN-γ response to the $ALK1_{278–301}$ and $ALK2_{233–256}$ peptides was detected in all six ALK-positive ALCL patients ($P < 0.05$). Peptide $ALK3_{411–434}$ was weakly immunogenic to PBMCs from patient 4 only (Table 2). All six patients exhibited an antibody response to ALK (Table 2). The absence of an IFN-γ response in patients with ALK-negative ALCL and normal controls shows the specificity of the anti-ALK responses observed here. The ability of

Table 2. Summary of the $CD4^+$ Th and B-cell immune responses to ALK in ALCL patients and normal donors

Patients	MHC class II	Titres circulating anti-ALK*	ELISPOT response to peptides					
			$ALK1_{278–301}$ (IFN-γ/IL-4)	$ALK2_{233–256}$ (IFN-γ/IL-4)	$ALK3_{411–434}$ (IFN-γ/IL-4)	None (IFN-γ/IL-4)	$HIV_{121–140}$ (IFN-γ/IL-4)	PHA (IFN-γ/IL-4)
ALK-positive								
Patient 1	DRB1_0401	1:6,750	56 ± 8/10 ± 4	44 ± 6/18 ± 4	22 ± 6/6 ± 2	12 ± 2/4 ± 2	10 ± 2/6 ± 1	188 ± 22/144 ± 12
Patient 2	DRB1_0403	1:6,750	126 ± 14/48 ± 8	78 ± 8/52 ± 6	40 ± 6/26 ± 4	20 ± 4/18 ± 4	14 ± 4/16 ± 4	240 ± 36/170 ± 16
Patient 3	DRB1_0401	1:2,250	38 ± 8/12 ± 4	34 ± 6/16 ± 2	12 ± 2/18 ± 4	14 ± 6/12 ± 2	12 ± 2/10 ± 2	48 ± 12/38 ± 8
Patient 4	DRB1_0701	1:750	64 ± 12/8 ± 2	72 ± 12/8 ± 4	22 ± 6/6 ± 2	6 ± 4/4 ± 1	10 ± 2/8 ± 2	108 ± 22/94 ± 12
Patient 5	DRB1_1303	1:6,750	36 ± 6/13 ± 4	26 ± 6/18 ± 4	12 ± 2/12 ± 4	10 ± 2/8 ± 3	12 ± 2/10 ± 2	48 ± 8/58 ± 16
Patient 6	DRB1_0401	1:6,750	74 ± 10/ND	58 ± 6/ND	28 ± 10/ND	15 ± 4/ND	16 ± 4/ND	132 ± 34/ND
ALK-negative								
Patient 7	DRB1_0403	None	12 ± 2/10 ± 4	10 ± 4/6 ± 2	12 ± 4/14 ± 6	14 ± 6/10 ± 2	14 ± 2/12 ± 3	122 ± 22/108 ± 14
Patient 8	DRB1_0701	None	14 ± 4/10 ± 6	12 ± 2/8 ± 2	10 ± 4/8 ± 2	16 ± 6/6 ± 2	10 ± 2/8 ± 2	82 ± 18/68 ± 10
Normal								
Normal 1	DRB1_0404	None	18 ± 4/14 ± 4	22 ± 6/24 ± 8	26 ± 6/12 ± 2	22 ± 4/14 ± 4	18 ± 6/16 ± 4	148 ± 44/96 ± 10
Normal 2	Not available	None	2 ± 2/4 ± 2	6 ± 2/8 ± 4	12 ± 4/2 ± 1	12 ± 2/4 ± 2	8 ± 2/6 ± 2	18 ± 12/22 ± 6
Normal 3	DRB1_0701	None	12 ± 6/10 ± 4	12 ± 4/16 ± 8	9 ± 2/4 ± 4	10 ± 2/10 ± 3	10 ± 4/6 ± 2	38 ± 12/42 ± 8
Normal 4	DRB1_0803	None	9 ± 2/ND	10 ± 4/ND	6 ± 2/ND	9 ± 3/ND	6 ± 1/ND	172 ± 38/ND
Normal 5	DRB1_0401	None	10 ± 2/2 ± 2	8 ± 2/12 ± 2	2 ± 1/1 ± 1	4 ± 2/2 ± 1	12 ± 3/3 ± 2	108 ± 24/88 ± 18

NOTE: Significant IFN-γ responses to the $ALK1_{278–301}$ and $ALK2_{233–256}$ peptides with respect to the irrelevant peptide and medium only controls were found only in the six ALK-positive ALCL patients but not in ALK-negative ALCL patients or normal subjects. The results ± SD are from triplicate ELISPOT cultures.
Abbreviation: ND, not done.
*These figures refer to the highest dilution that the sera could be used before labeling of ALK-positive transfectants disappeared.

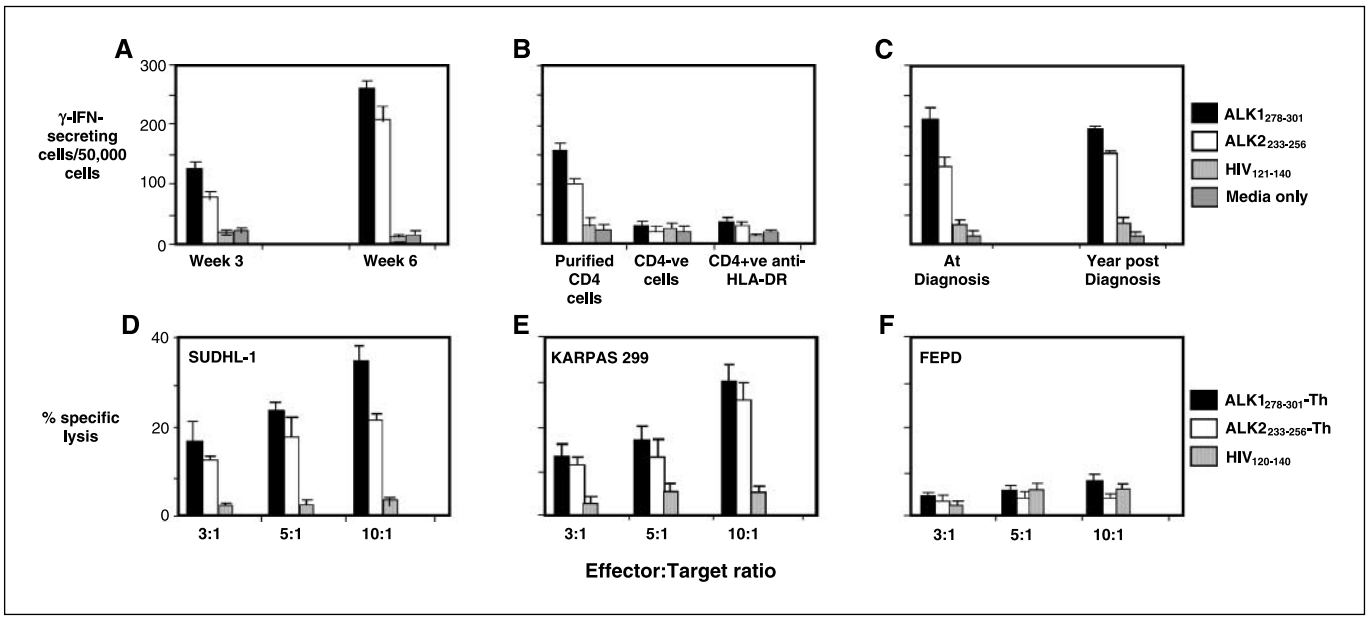

Figure 1. PBMC and CD4$^+$ Th responses of ALK-positive ALCL patient 2 in IFN-γ release ELISPOT and ^{51}Cr release cytolytic assays. *A* to *C,* PBMCs were maintained in culture and stimulated with $ALK1_{278–301}$ and $ALK2_{233–256}$ peptides for 10 d. CD4$^+$ Th cell lines were raised after 6 wk of repeated stimulation with ALK peptides. *A,* the CD4$^+$ Th–enriched cells showed a significantly increased IFN-γ response to both ALK peptides when compared with PBMC cultures ($P < 0.05$). In contrast, no significant response to the control HIV peptide or medium only was observed. *B,* CD4$^+$ cells were prepared using a magnetic purification step. After overnight incubation with the $ALK1_{278–301}$ and $ALK2_{233–256}$ peptides in an ELISPOT assay, the CD4$^+$ cells released IFN-γ in response to both ALK peptides. No significant response was detected to the irrelevant control peptide. The use of a CD4$^+$ depletion step or the addition of anti–HLA-DR antibody abrogated the IFN-γ response to the ALK peptides. *C,* monitoring the CD4$^+$ Th responses of this patient at time of diagnosis and during remission shows that responses to both peptides remained significantly high even after 1 y ($P < 0.05$). *D* to *F,* the cytolytic activity of CD4$^+$ Th cell lines raised from patient 2 against $ALK1_{278–301}$ and $ALK2_{233–256}$ peptides was tested in an 18-h Cr release assay on the ALK-positive SUDHL-1 (DRB1_0403) and KARPAS-299 (DRB1_0101) and the ALK-negative FEPD (DRB1_0301) ALCL-derived cell lines. Significant lysis, even at an effector/target ratio of 3:1, was observed in cultures containing the ALK-positive SUDHL-1 (*D*) or KARPAS-299 (*E*) cells. This was in contrast to the lack of lysis observed of the ALK-negative FEPD cell line (*F*). No significant lysis was observed with CD4$^+$ Th cells raised against the irrelevant peptide ($HIV_{121–140}$). The results are the mean +/− SD and were from triplicate cultures.

$ALK1_{278–301}$ and $ALK2_{233–256}$ peptides to stimulate an IFN-γ response from patients displaying different HLA-DRB1 alleles possibly reflects the potential promiscuity of these peptides enabling them to recognize and interact with various HLA-DRB1 alleles (13).

The detection of a significant IL-4 response in patient 2 only (Table 2) suggests the presence of an IFN-γ–driven Th1 type response to the ALK peptides. This is of interest because the majority of the ALK-positive patients studied are currently in remission. Previous reports have described that melanoma patients who remained disease-free exhibited highly polarized Th1 immune responses (16). In contrast, progressive disease was skewed towards Th2 or Th1/Th2 responses in a murine leukemia/lymphoma model (17).

Increased IFN-γ responses to ALK peptides $ALK1_{278–301}$ (>80%) and $ALK2_{233–256}$ (>100%) were detected following repeated peptide stimulation of PBMCs (Fig. 1*A*). Depletion of the CD4$^+$ cell population or the addition of anti–HLA-DR abrogated the IFN-γ response to both peptides (Fig. 1*B*), supporting the CD4$^+$-mediated and DRB1-restricted nature of this response.

The presence of a significant IFN-γ response in patient 2 at time of diagnosis and after 1 year in remission (Fig. 1*C*; $P < 0.05$) suggests the presence of a pool of memory CD4$^+$ Th cell subsets. Such cells could play an important role not only in protective tumor immunity (18) but also in the maintenance of the CD8$^+$ memory CTLs previously reported in ALK-positive ALCL (6, 7).

CD4$^+$ Th cell lines specific for $ALK1_{278–301}$ and $ALK2_{233–256}$ raised from patient 2 PBMCs lysed ALK-positive SUDHL-1 and KARPAS-299 cells (Fig. 1*D*; $P < 0.001$) despite the fact that the ALK-positive cell lines express different HLA-DRB1 alleles. A possible explanation is the high degree of homology between the DRB1_0403 and DRB1_0101 molecules.[4] No significant lysis was detected of the ALK-negative FEPD cell line. Thus, the CD4$^+$ Th cells were capable of recognizing endogenous ALK peptides on the ALK-positive tumor cells.

Studies have shown the potential of using epitopes predicted to bind to both MHC Class I and Class II to obtain more effective immune responses (19, 20). The $ALK1_{278–301}$ peptide encompassing a CTL epitope previously shown to be immunogenic in ALK-positive patients (7) could, therefore, represent an attractive peptide for inclusion in a vaccine formulation.

We show, for the first time, ALK peptides capable of eliciting strong CD4$^+$ Th responses in patients with ALK-positive ALCL. These peptides constitute potential vaccine candidates for ALK-positive patients who respond poorly to conventional treatment.

Acknowledgments

Received 12/4/2006; revised 12/22/2006; accepted 1/11/2007.

Grant support: Leukemia Research Fund and Cancer Research United Kingdom.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Chris Hatton (John Radcliffe Hospital), Dr. Denise Williams (Addenbrookes Hospital), the clinicians and nurses in the United Kingdom Children's Cancer Study Group centers who enrolled patients in our study, Dr. Rachel Hobson (United Kingdom Children's Cancer Study Group Data Centre), and Prof. Vincenzo Cerundolo for his helpful comments.

[4] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

References

1. Delsol G, Ralfkaier E, Stein H, Wright D, Jaffe ES. Anaplastic large cell lymphoma. Pathology and genetics. Tumors of haematopoietic and lymphoid tissues. International classification of tumors. Lyon: IARC Press; 2001. p. 230–5.
2. Wan W, Albom MS, Lu L, et al. Anaplastic lymphoma kinase is essential for the proliferation and survival of anaplastic large-cell lymphoma. Blood 2006;107:1617–23.
3. Pulford K, Morris SW, Turturo F. Anaplastic lymphoma kinase proteins in growth control and cancer. J Cell Physiol 2004;199:330–58.
4. Pulford K, Falini B, Banham AH, et al. Immune response to the ALK oncogene tyrosine kinase in patients with anaplastic large cell lymphoma. Blood 2000;96:1605–7.
5. Borisch B, Yerly S, Cerato Ch, et al. ALK-positive anaplastic large-cell lymphoma: strong T and B antitumor responses may cause hypocellular aspects of lymph nodes mimicking inflammatory lesions. Eur J Haematol 2003;71:243–9.
6. Ait-Tahar K, Cerundolo V, Banham AH, et al. B and CTL responses to the ALK protein in patients with ALK-positive ALCL. Int J Cancer 2006;118:688–95.
7. Passoni L, Gallo B, Biganzoli E, et al. *In vivo* T-cell immune response against anaplastic lymphoma kinase in patients with anaplastic large cell lymphomas. Haematologica 2006;91:48–55.
8. Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T cells and their role in antitumor immune responses. J Exp Med 1999;189:753–6.
9. Knutson KL, Schiffman K, Disis ML. Immunization with Her-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. J Clin Invest 2001;107:477–84.
10. Ostrand-Rosenberg S. $CD4^+$ T lymphocytes: a critical component of antitumor immunity. Cancer Invest 2005; 23:413–9.
11. Seidemann K, Tiemann M, Schrappe M, et al. Short pulse B-non-Hodgkin lymphoma-type chemotherapy is efficacious treatment for pediatric anaplastic large cell lymphoma: a report of the Berlin Frankfurt-Munster Group Trial NHL-BFM 90. Blood 2001;96:3699–706.
12. Bunce M, O'Neill CM, Barnardo MC, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB5 and DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). Tissue Antigens 1995;46:355–67.
13. Southwood S, Sidney J, Kondo A, et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. J Immunol 1998;160: 3363–73.
14. Passoni L, Scardino A, Bertazzoli C, et al. ALK as a novel lymphoma-associated tumor antigen: identification of 2 HLA-A2.1-restricted $CD8^+$ T-cell epitopes. Blood 2002;99:100–6.
15. Pulford K, Lamant L, Morris SW, et al. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood 1997;89:1394–404.
16. Tatsumi T, Kierstead L, Ranieri E, et al. Disease-associated bias in T helper type 1 (Th1)/Th2 $CD4^+$ T cell responses against MAGE-6 in HLA-DRB1*0401^+ patients with renal cell carcinoma or melanoma. J Exp Med 2002; 196:619–28.
17. Lee PP, Zeng D, MaCaulay AE, et al. T helper 2-dominant anti-lymphoma immune response is associated with fatal outcome. Blood 1997;90:1611–7.
18. Broderick L, Yokota SJ, Reineke J, et al. Human $CD4^+$ effector memory T cells persisting in the microenvironment of lung cancer xenografts are activated by local delivery of IL-12 to proliferate, produce IFN-γ, and eradicate tumor cells. J Immunol 2005;174:898–906.
19. Bocchia M, Korontsvit T, Xu Q, et al. Specific human cellular immunity to bcr-abl oncogene-derived peptides. Blood 1996;87:3587–92.
20. Zeng G, Li Y, El-Gamil M, et al. Generation of NY-ESO-1 generation of NY-ESO-1 specific $CD4^+$ and $CD8^+$ T cells by a single peptide with dual MHC class I and class II specificities: A new strategy for vaccine design. Cancer Res 2002;62:3620–5.

CD4 T-Helper Responses to the Anaplastic Lymphoma Kinase (ALK) Protein in Patients with ALK-Positive Anaplastic Large-Cell Lymphoma

Kamel Ait-Tahar, Martin C.N. Barnardo and Karen Pulford

Cancer Res 2007;67:1898-1901.