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

Nuffield Department of Clinical Laboratory Sciences, Leukemia Research Fund Immunodiagnostics Unit, John Radcliffe Hospital, and 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, ALK1278–301 and ALK2233–256, 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 MIIC 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.

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 (13) 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, ALK1278–301 (PSSLAMLDDLHVDIAGCGYQLE), ALK2233–256 (KFNHQNNLVRCIGVSLQSLDFELLL), and ALK3411–434 (PKNCGPVYRIMTCWQHQQDPEDR), were identified lying within the kinase domain. ALK1278–301 encompasses the highly immunogenic ALK280–289 CTL epitope (6, 14). An irrelevant control peptide was HIV121–140 (DESFRKTYATFTPSMNNETP).

Cell lines. The t(2;5)-positive ALK-derived cell lines SUDHL-1 (DRB1*0403) and KARPAS-299 (DRB1*0101) and the t(2;5)-negative ALCL cell line FPD (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 × 10^6) 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 ALK1278–301, ALK2233–256, or the irrelevant HIV121-140 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% CO2 with ALK1278–301, ALK2233–256, or HIV121–140, 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 ALK1278–301, ALK2233–256, or HIV121–140 peptide, or with medium only. After 72 h, an equal volume of
CD4⁺ T-Helper Response to ALK

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Stage</th>
<th>Treatment</th>
<th>Time of blood sample after diagnosis</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>M</td>
<td>II</td>
<td>CHOP</td>
<td>At diagnosis and 47 mo</td>
<td>CR</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>F</td>
<td>II</td>
<td>CHOP</td>
<td>At diagnosis and 12 mo</td>
<td>CR</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>M</td>
<td>IV</td>
<td>ALCL'98³</td>
<td>47 mo</td>
<td>CR</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>M</td>
<td>III</td>
<td>ALCL'99</td>
<td>At diagnosis</td>
<td>CR</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>M</td>
<td>II</td>
<td>ALCL'99</td>
<td>At diagnosis</td>
<td>PD</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>F</td>
<td>I</td>
<td>ALCL'99</td>
<td>At diagnosis</td>
<td>PD</td>
</tr>
</tbody>
</table>

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.

BPMI 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.

³¹Cr cytolytic assay. SUDHL-1, KARPAS-299, and FEPD cells were radiolabeled with 100 µCi ³¹Cr. The target cells were then incubated for 18 h with the ALK peptide–stimulated lines and cytosis 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).

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

<table>
<thead>
<tr>
<th>Patients</th>
<th>MHC class II</th>
<th>Titres circulating anti-ALK*</th>
<th>ELISPOT response to peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALK1278–301 (IFN-γ/IL-4)</td>
<td>ALK2233–256 (IFN-γ/IL-4)</td>
</tr>
<tr>
<td>ALK-positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>DRB1_0401</td>
<td>16,750</td>
<td>56 ± 8/10/4</td>
</tr>
<tr>
<td>Patient 2</td>
<td>DRB1_0403</td>
<td>16,750</td>
<td>126 ± 14/48 ± 8</td>
</tr>
<tr>
<td>Patient 3</td>
<td>DRB1_0401</td>
<td>12,250</td>
<td>38 ± 6/12 ± 2</td>
</tr>
<tr>
<td>Patient 4</td>
<td>DRB1_0701</td>
<td>1,750</td>
<td>64 ± 12/8 ± 2</td>
</tr>
<tr>
<td>Patient 5</td>
<td>DRB1_1303</td>
<td>1,675</td>
<td>36 ± 6/13 ± 4</td>
</tr>
<tr>
<td>Patient 6</td>
<td>DRB1_0401</td>
<td>1,675</td>
<td>74 ± 10/6/10</td>
</tr>
<tr>
<td>ALK-negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>DRB1_0403</td>
<td>None</td>
<td>12 ± 2/10/4</td>
</tr>
<tr>
<td>Patient 8</td>
<td>DRB1_0701</td>
<td>None</td>
<td>14 ± 4/10 ± 6</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 1</td>
<td>DRB1_0401</td>
<td>None</td>
<td>18 ± 2/14/4</td>
</tr>
<tr>
<td>Normal 2</td>
<td>Not available</td>
<td>None</td>
<td>2 ± 2/4 ± 4</td>
</tr>
<tr>
<td>Normal 3</td>
<td>DRB1_0701</td>
<td>None</td>
<td>12 ± 6/10/4</td>
</tr>
<tr>
<td>Normal 4</td>
<td>DRB1_0803</td>
<td>None</td>
<td>9 ± 2/6/2/2</td>
</tr>
<tr>
<td>Normal 5</td>
<td>DRB1_0401</td>
<td>None</td>
<td>10 ± 2/2 ± 2</td>
</tr>
</tbody>
</table>

NOTE: Significant IFN-γ responses to the ALK1278–301 and ALK2233–256 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.
ALK1278–301 and ALK2233–256 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 ALK1278–301 (>80%) and ALK2233–256 (>100%) were detected following repeated peptide stimulation of PBMCs (Fig. 1A). Depletion of the CD4+ cell population or the addition of anti–HLA-DR antibody abrogated the IFN-γ response to both 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 ALK1278–301 and ALK2233–256 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 (HIV121-140). The results are the mean ± SD and were from triplicate cultures.

CD4+ Th cell lines specific for ALK1278–301 and ALK2233–256 raised from patient 2 PBMCs lysed ALK-positive SUDHL-1 and KARPAS-299 cells (Fig. 1D; 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. 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 ALK1278–301 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.

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Figure 1. PBMC and CD4+ Th responses of ALK-positive ALCL patient 2 in IFN-γ release ELISPOT and 51Cr release cytolytic assays. A to C, PBMCs were maintained in culture and stimulated with ALK1278–301 and ALK2233–256 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 ALK1278–301 and ALK2233–256 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 ALK1278–301 and ALK2233–256 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 (HIV121-140). The results are the mean ± SD and were from triplicate cultures.
References


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