# *In vivo* T-cell immune response against anaplastic lymphoma kinase in patients with anaplastic large cell lymphomas

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Background and Objectives. Anaplastic lymphoma kinase (ALK) oncogenic fusion proteins, expressed in about 60% of anaplastic large cell lymphomas (ALCL), are tumor-specific molecular targets for such a malignancy. One of the promising ALK-targeted therapeutic options is cancer vaccination. In this study, we investigate whether ALK is a tumor-associated antigen suitable for immune interventions.

Design and Methods. The frequency and the functional phenotype of the anti-ALK CD8 precursor repertoire in freshly isolated peripheral blood mononuclear cells (PBMC) from healthy donors and ALK-positive patients were determined by major histocompatibility complex (MHC)/tetrameric analyses. The anti-ALK secondary immune responses were evaluated as PBMC-specific interferon (INF-γ) release by ELISPOT. In addition, the ability of the anti-ALK immune response to specifically lyse ALK-positive lymphoma cells was investigated by *in vitro* stimulation with ALK-derived peptide p280-89.

Results. Tetrameric MHC/peptide complexes revealed high frequencies of CD8/ALK-tetramer-positive cells both in patients and in healthy individuals. However, the functional phenotype of the CD8/ALK-tetramer-positive lymphocytes showed the presence of effector and memory T lymphocytes only in patients. The anti-ALK cytotoxic T lymphocytes (CTL) of patients, but not healthy donors, displayed thresholds of activation comparable to those of CTL precursors of a recall antigen (influenza virus). A polyclonal ALK-specific tumor-reactive T-cell line was isolated from patients' peripheral blood lymphocytes.

Interpretation and Conclusions. The presence of an anti-ALK effector/memory lymphocyte population in the peripheral blood of ALK-positive patients indicates an *in vivo* antigenic challenge. Thus, ALK is a lymphoma-associated antigen suitable for immune interventions. The high number of anti-ALK memory CD8 T cells present in patients' PBMC may represent a valid source of activated CTL suitable for cancer cell lysis.

Key words: ALK, tumor antigen, ALCL, immunotherapy, cancer vaccine.

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CD30+ anaplastic large cell lymphomas (ALCL), differentiated by the expression of the oncoprotein anaplastic lymphoma kinase (ALK), can be considered as a distinct clinicopathologic entity.[1-4] ALK is a receptor tyrosine kinase normally expressed within the developing brain and nervous system.[5,6] Hematopoietic tissues and normal lymphoid cells show no detectable expression of ALK.[7,8] In contrast ,intracellular ALK is aberrantly overexpressed in about 60-70% of ALCL.[2] The pathological overexpression of ALK in lymphoid cells is always associated with chromosomal translocations. These translocations lead to the synthesis of chimeric ALK proteins. The most frequent and best characterized ALK fusion protein is NPM/ALK accounting for ~75% of ALK-positive ALCL.[9] However, at least ten other ALK fusion partners have been identified in ALCL patients including TMP3, TFG, ATIC, CLTCL, MSN and ALO17.[10] In all ALK variant proteins, the fusion partner works as an oligomerization domain that leads to homodimerization and constitutive activation of the ALK tyrosine kinase domain.[11,12] It has been extensively demonstrated that ALK fusion proteins trigger the malignant transformation of normal lymphoid cells by promoting both mitogenic and anti-apoptotic signaling cascades.[12-16] Despite the fact that patients with ALK-positive lymphomas appear to benefit from highly intensive chemotherapeutic regimens, the risk of relapse and/or of remission failure is still high (approximately 20-30%).[17-19] No effective therapeutic regimens (conventional chemotherapy and/or alternative strategies) are available for relapsed or refractory patients.[1,10] Antigen-based immunotherapy is crucially dependent on the identification of immunogenic tumor-associated antigens (TAA) able to activate specific tumor-rejection responses. Mainly because of its selective overexpression in lymphoma cells and its direct requirement in ALCL pathogenesis,[20,21] ALK could potentially be an ideal target for immunotherapy. Following initial activation through the T-

**Table 1.** Patients' characteristics.

| Patient | HLA-A2.1 | Date of diagnosis | Age at diagnosis | Treatment* analysis | Time of T cell | Disease status# |
|---|---|---|---|---|---|---|
| PT 1 | + | April 1996 | 14 | CH | December 2000 | CR |
| PT 2 | + | April 1999 | 52 | CH, RT February 2003 | June 2002 CR | CR |
| PT 3 | + | October 1997 | 27 | CH, RT | July 2001 | CR |
| PT 4 | + | March 1996 | 19 | CH, RT April 2002 | June 2001 CR | CR |
| PT 5 | + | June 2001 | 52 | - | June 2001 diagnosis | at |
| PT 6 | + | April 1992 | 3 | CH January 2003 July 2003 | February 2002 CR CR | relapse |
| PT 7 | - | August 2001 | 30 | - | August 2001 diagnosis | at |

*CH: chemotherapy, RT: radiotherapy; #CR: clinical remission.*

cell receptor (TCR), naïve T cells begin to differentiate into effector and subsequently memory cells. This differentiation process is complex and many fundamental aspects are still not understood.[22,23] T cells can be subdivided into four distinct populations with different functional properties on the basis of the surface expression of the leukocyte common antigen CD45RA and the chemokine receptor CCR7. CD45RA$^+$ naïve cells uniformly display high levels of CD62L and CCR7. Effector cells down-regulate CCR7 expression (CD45RA$^+$ CCR7$^-$). In contrast, CD45RA$^-$ memory T cells can be divided into CD62L$^+$ CCR7$^+$ non-polarized *central memory* and CD62L$^-$ CCR7$^-$ polarized *effector memory* cell populations.[24–27]

In this study, we investigated whether a natural anti-ALK immune response occurs in ALK-positive ALCL patients, by assessing the frequency of circulating anti-ALK cytotoxic T lymphocyte (CTL) precursors and analyzing their functional phenotype. We also discuss the implications for a potential anti-ALK immunotherapy strategy.

## Design and Methods

### Patients' characteristics

Seven patients previously diagnosed as having ALK-positive ALCL were studied (Table 1). All patients, except patient 7 (HLA-A*01030), were typed for HLA class I molecules including HLA-A*0201. Peripheral blood samples were drawn at diagnosis before induction chemotherapy (patients 5 and 7), at relapse before treatment (patient 6, February 2002) or at least 6 months after the end of treatments and in clinical remission. Blood samples from patients and healthy volunteers were collected after informed consent and processed according to the guidelines approved by the ethical Committee of the *Istituto Nazionale Tumori.*

### Peptides

The following HLA-A*0201-restricted peptides were used in this study: ALK-derived peptides p280-89 (SLAMLDLLHV) and p376-85 (GVLLWEIFSL), FLU peptide derived from the influenza matrix protein amino acid position 58-66 (GILGFVFTL) and CEA peptide derived from the carcino-embryonic antigen amino acid position 571-579 (YLSGANLNL). Peptides purified by high-performance liquid chromatography were synthesized and purchased from Sigma-Genosys (Cambridge, UK) at a minimum purity of 90%.

### Tetramer staining and flow cytometry

Streptavidin-phycoerythrin conjugated HLA-A2.1/p280-89 tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility (NIAID, Emory University Vaccine Center, Atlanta, GA, USA). The stock solution contained 0.7 mg monomer/mL. Staining conditions and tetramer dilution were determined using the anti-ALK-derived epitopes p280-89 and p376-85 specific CTL lines previously described.[28] Tetramer staining was carried out on fresh PBMC at 4°C for 45 minutes in FACS staining buffer (phosphate-buffered saline pH 7.5 containing 0.5 % bovine serum albumin) at a final dilution of 1/400 relative to the stock reagent. Cells were concurrently stained for surface markers using the following Becton Dickinson (Mountain View, CA, USA) anti-human antibodies: anti-CD8$^{APC}$, anti-CD45RA$^{FITC}$ and anti-CCR7$^{BIOTIN}$ detected by further incubation with SA$^{PerCP}$ (PharMingen, San Diego, CA, USA). Cells were analyzed on a FACScalibur instrument (Becton Dickinson) using Cell Quest software. For the analysis, CD8$^{high+}$ lymphocytes were gated according to their side scatter profile. Minimal tetramer binding was observed on HLA-A2.1- CD8$^+$ peripheral blood lymphocytes. The maximum background level has been determined to be 0.03 % of CD8$^+$ cells, thus samples containing less than 0.03 % of CD8/tetramer-positive cells were considered negative. Tetramer-positive cells were quantified by flow cytometry and expressed as % of CD8$^+$ cells. The difference between tetramer-positive cells in patients and healthy donors was assessed using a two-sided non-parametric hypothesis test based on the Wilcoxon rank sum test statistics. Graphics were created using GraphPad Prism, Version 4 (GraphPad Softwer, San Diego, CA, USA). In order to determine the functional phenotype frequencies of ALK-tetramer-positive, cells at least $5\times10^2$ CD8$^+$/tetramer$^+$ cells were analyzed for each staining. Frequencies were averaged from between two and four replicate staining analyses. Given the limited number of CD8/A2ALKp280 tetramer-positive cells, to minimize the effect of unspecific background in the evaluation of the CD45RA and CCR7 subset staining, a significant effector and/or memory subset was arbitrary defined as ≥20% of CD8/A2ALKp280 tetramer-positive gated cells.

### ELISPOT assay

A total of $0.5\times10^6$ PBMC were plated in triplicate and co-cultured overnight in the presence of $16\times10^3$/well T2 cells or T2 cells pulsed with 10 μM of the cognate peptide in plates pre-coated with 10 mg/mL of primary anti-interferon-γ monoclonal antibody (1-D1K, Mabtech). After two washes, the biotinylated detection antibody (7-B6-1-Biotin, Mabtech) was added. Specific binding was visualized using an alkaline phosphatase-avidin system (Life Technologies, Gaithersburg, MD, USA). Spots were analyzed and counted by computer-assisted video image analysis with the AID Elispot- Reader (Bioline, Turin, Italy). Antigen-specific spots were calculated after subtracting the background obtained with unpulsed T2 cells.

### Statistical analysis

Statistical analyses were aimed at evaluating the difference in the average number of spots for the CEA, FLU, p280 and p376 peptides. Since the response variables were counts, underlying Poisson distributions were expected; therefore, the original response data were transformed by square root after adding 0.5 to improve the strength of the standard assumptions for the application of analysis of variance (ANOVA), namely: homogeneity of variances and Gaussian distribution of residuals. Such assumptions were then graphically evaluated. The determinations were replicated for each patient to account for different sources of experimental variability, according to a nested subsampling design. ANOVA for a mixed effects model, accounting for the unbalanced experimental designs, was adopted. In particular, the fixed effects for the difference of mean spot counts were assayed against the variance between subjects (random effect). However, the latter included nested random components, namely the variance between different experimental runs within subjects and that due to the replications within each experiment.

Statistical analyses and graphics were performed with SAS ver. 8.02 software (*SAS/STAT® User's Guide, Version 6, Fourth Edition, Volume 2*, 1989 SAS Institute Inc., Cary, NC, Proc MIXED, USA) and S-Plus v3.3 software (MathSoft, Inc.).

### Generation of CTL

PBMC were separated from the peripheral blood of an HLA-A*0201$^+$ patient with ALCL (patient 6) by centrifugation on Ficoll/Hypaque (Pharmacia, Upsala, Sweden) gradients. The PBMC ($10^7$) were cultured for 7 days in culture medium (50% RPMI 1640 from Gibco BRL, Rockville, MD, USA and 50% X-VIVO from BioWhittaker, Walkersvill, MA, USA) supplemented with 5% autologous serum containing 5 μM of synthetic ALK-p280 peptide. The reactive lymphoblasts were isolated on a Percoll (Amersham, Uppsala, Sweden) gradient,[29] further expanded for 7 days in the presence of 20 IU/mL recombinant human interleukin-2 (Chiron, Emeryville, CA USA), 10 ng/mL recombinant human interleukin-7 (R&D System, Mineapolis, MN, USA) and 10 ng/mL recombinant human interleukin-15 (PeproTech, London, UK), and restimulated at weekly intervals with the same amount of antigen plus irradiated (30 Gy) autologous PBMC as antigen-presenting cells.

### CTL assays

For the cytokine release assays, $10^5$ target cells (TAP -/- T2 cells) were incubated overnight with the same amount of lymphocytes. Interferon-γ and granulocyte-monocyte colony-stimulating factor concentrations were assessed in culture supernatants using commercial ELISA kits (Human IFN-γ ELISA Assay, Mabtech, Stockholm, Sweden; Human GMC-SF ELISA, Endogen, Woburn, MA, USA) following the manufacturers' instructions. Cytolytic activity was measured in a standard 4-h $^{51}$chromium release assay. A 10-fold excess of unlabeled K562 cells was added to offset natural killer cell activity. Target cells and effector-to-target (E:T) ratios are indicated in Figure 1. Specific lysis was determined according to the following formula: % specific lysis = cpm (sample-spontaneous)/ cpm (total-spontaneous) x 100. HLA-A2 blocking of T-cell activity was performed by pre-incubating target cells with the anti-HLA-A2 monoclonal antibody CR11.351.

## Results

### The ALK tetramer$^+$ CD8$^+$ repertoire in the peripheral blood of healthy donors and patients

In order to directly assess the frequency of anti-ALK circulating CD8$^+$ T cells *ex vivo*, uncultured PBMC were analyzed by tetrameric MHC/p280-89 ALK-derived peptide. The epitope-restricted recognition of the A2/ALKp280 tetramer was confirmed by selective staining of the anti-ALK p280-89 epitope-restricted, but not p376-85 epitope-restricted, CTL line (Figure 1A). Moreover, A2/ALKp280 tetramer-positive cell detection was dependent on the expression of HLA-A*0201 molecules (Figure 1B). No samples scored 0.03 %, corresponding to the background level determined in HLA-A*0201-positive individuals (*data not shown*). High frequencies of A2/ALKp280-tetramer-positive cells were observed in the peripheral CD8 repertoire of all HLA-A*0201 healthy donors and ALK-positive patients tested and no major differences in frequencies were observed (median frequency and 25$^{th}$-75$^{th}$ percentiles: 0.22% 0.17-0.29, and 0.19% 0.15-0.3 of CD8 cells in normal individuals and ALCL patients, respectively) (Figure 1C). A2/ALKp280 tetramer-positive CD8 T cells showed a range of fluorescence intensity suggesting that the anti-ALK CTL repertoire is heterogeneous comprising CD8 T cells with wide ranges of T-cell receptor affinity and levels of expression.[30-32]

### Functional phenotype of ALK-specific CD8$^+$ T-cell populations

In order to understand whether the presence of anti-ALK specific lymphocytes in patients correlates with the generation of a T-cell effector/memory phenotype as the consequence of *in vivo* immune recognition of ALK, we analyzed CCR7 and CD45RA isoform expres-

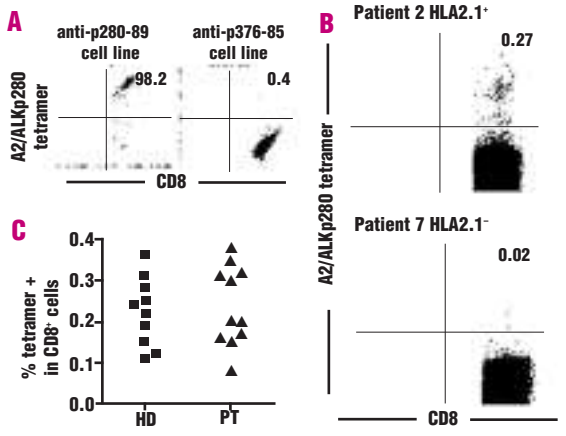


**Figure 1.** A2/ALKp280 tetramer binding is epitope-specific and HLA-restricted. (A) CTL lines elicited from peripheral blood of healthy individuals and specific for ALK-derived p280-89 and p376-85 28 peptides were stained with phycoerythrin-labeled A2/ALKp280 tretramer and anti-CD8APC as described in the Methods section. (B) PBMC from HLA-A*0201+ and HLA-A*0201- individuals were stained with the A2/ALKp280 tretramer and anti-CD8 antibody to determine the HLA-restricted pattern of staining. Demonstrative examples of an HLA-A*0201 positive individual (patient 2) and negative individual (patient 7) are presented. (C) Circulating CD8+A2/ALKp280 tetramer+ T cells are detectable *ex vivo* in both lymphoma patients and healthy individuals. Summary plot of CD8+A2/ALKp280 tetramer+ T-cell frequencies detected in the PBMC of a healthy donor (HD) and an ALK-positive patient (PT). No statistically by significant differences were observed (Wilcoxon's rank-sum test $p$=0.89).

sion on CD8/A2ALKp280 tetramer-positive cells. PBMC from healthy donors and patients were co-labeled with anti-CD8, anti-CD45RA and anti-CCR7 antibodies and the A2/ALKp280 tetramer and analyzed by four-color flow cytometry. Some patients (patients 2, 3, 4 and 6) were analyzed at different time points. The results are presented in Table 2.

When the functional phenotype of p280-tetramer+/CD8 lymphocytes was studied, the majority of healthy donor lymphocytes were CD45+CCR7+, consistent with a naïve phenotype, and significant amounts (≥20% of total CD8/tetramer+cells) of effector and memory T cell were not detected. In contrast, in ALK-positive patients a considerable proportion of p280-tetramer+/CD8 lymphocytes were effector cells, effector memory cells and central memory cells. In four out of six cases an effector CD45+CCR7- phenotype was dominant (from 20.3% up to 54.2% of the tetramer-positive cells). The CD45-CCR7- effector memory subset was less represented, being detected in two patients (patients 5 and 6). Central memory CD45RA-CCR7+ cells were observed in three patients, accounting from 24.2% up to 31.9% of the p280-tetramer+/CD8 lymphocytes. In patient 1 only naïve cells were observed, indicating that no anti-ALK immune response occurred. Remarkably, in patient 2 the only subsets observed were central memory together with naïve cells, even in samples collected after 20 months, suggesting the establishment of a long-lasting immunological memory. It is noteworthy that the sample taken in February 2002 from patient 6 contained the highest percentage of effector cells (54.2%) and no memory cells. In a sample taken in 2003 (one year after the relapse) an effector memory subset was present and six months later (July 2003) only a central memory subset was evident, as in patient 2, indicating the developmental course of the immune response in this patient over time.

Taken together these results indicate that there was *in vivo* immune recognition of ALK in five out of six patients with ALK-positive ALCL. In contrast, healthy donors had predominantly naïve cells.

**Table 2.** *Ex vivo* functional phenotype of circulating CD8/A2ALKp280 tetramer-positive lymphocytes.

| | Phenotype of CD8/ tetramer+ T cells% | | | |
|---|---|---|---|---|
| *sample* | ***NAIVE*** *CD45RA+ CCR7+* | ***EC*** *CD45RA+ CCR7-* | ***EM*** *CD45RA- CCR7-* | ***CM*** *CD45RA- CCR7+* |
| HD 1 | 81.8 | 4.6 | 7 | 4.6 |
| HD 2 | 93.7 | | | 6.3 |
| HD 3 | 71.4 | 11.9 | 2.4 | 14.3 |
| HD 4 | 75 | 8.3 | | 16.7 |
| HD 5 | 61.2 | 11.8 | 12 | 4.8 |
| HD 6 | 68.8 | 18.2 | 5.5 | 7.5 |
| HD 7 | 83.4 | 7.5 | 4.5 | 4.5 |
| HD 8 | 91.4 | 2.9 | 2.6 | 3 |
| HD 9 | 85.9 | 3.5 | 3.2 | 7.4 |
| HD 10 | 77.9 | 9.6 | 6.1 | 9.6 |
| HD 11 | 69.5 | 13.9 | 3.9 | 12.6 |
| (#) mean±SEM | 78.2±3 | 8.4±1.6 | 4.3±1 | 8.3±1.3 |
| Patient 1 | | | | |
| December 2000 | 86.7 | 5.1 | 0.8 | 7.5 |
| Patient 2 | | | | |
| June 2001 | 64.7 | 5.9 | | **29.4** |
| February 2003 | 66.4 | 1.7 | | **31.9** |
| Patient 3 | | | | |
| June 2001 | 45 | **20.8** | 10 | **24.2** |
| April 2002 | 66.7 | **30.3** | 3.3 | 0 |
| Patient 4 | | | | |
| July 2001 | 56.4 | **22.5** | 6.9 | 14.2 |
| August 2002 | 62.1 | **34.5** | 3.4 | |
| Patient 5 | 6-'01 | | | |
| June 2001 | 40.2 | **20.3** | **23.2** | 16.3 |
| Patient 6 | | | | |
| February 2002 | 37.5 | **54.2** | 8.3 | |
| January 2003 | **53.1** | **21.9** | **25** | |
| July 2003 | 70.8 | 0.5 | 3.4 | **25.3** |
| (#)mean±SEM | 59±4.4** | 19.8±4.9* | 7.7±2.6 | 13.5±3.8 |

*Relevant immune responses defined as ≥20% of CD8/tetramer+ T cells in the effector cell (EC); effector memory (EM) and central memory (CM) subsets are highlighted in bold characters. (#) Frequencies were averaged from two to four replicate stainings. Overall mean values (± standard deviation) of all independent stainings are reported. *p≤0.05; **p≤0.01.*

### Functional potential of ALK-specific memory T cells from ALCL patients

To evaluate the functional activity of effector /memory T cells, ELISPOT analysis was performed. This detects interferon-γ-producing T cells in freshly isolated PBMC without any prior *in vitro* sensitization in order to elicit secondary but not primary immune responses. In addition, simultaneous analyses were

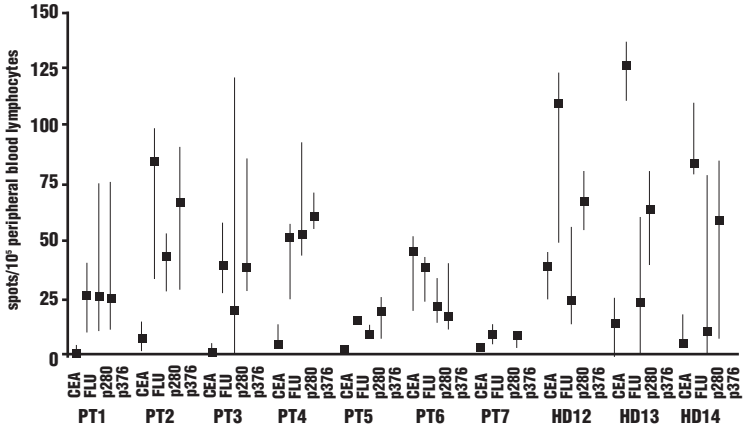


Figure 2. ELISPOT assay measuring interferon-γ secretion by peripheral blood CD8⁺ T cells in response to ALK-, FLU- and CEA-derived peptides in ALK-positive ALCL patients (PT) and healthy donors (HD). Results are represented as median values (small squares) of spot/$10^5$ peripheral blood lymphocytes of at least three independent experiments each performed in triplicate. Lower and upper boundaries represent the 25th and 75th percentile, respectively. Mean values for spot numbers (including 95% confidence intervals) are reported in the graph below; patient 7 was excluded from the calculation because HLA-A*0201 negative.

*Overall mean values of spots/$10^5$ peripheral blood lymphocytes in ALCL patients*

| CEA | FLU | p280 | p376 |
|---|---|---|---|
| 7.25 | 34.84 | 31.65 | 33.70 |
| 2.28-14.73 | 16.25-60.29 | 16.06-52.36 | 17.66-54.77 |

performed to determine the influenza matrix protein (FLU) and the carcinoembryonic antigen (CEA), as paradigms of recall and self-antigen, respectively. Our results show that the overall frequency of patient p280-89 and p376-85 specific T cells was comparable to the frequency of anti-FLU precursors and always higher than the low frequency of anti-CEA self-antigen. In contrast, the frequency of healthy donor p280-89 and p376-85 specific T cells was lower than the frequency anti-FLU (Figure 2). These findings indicate a different functional status of anti-p280 and anti-p376 precursors in healthy donors and lymphoma patients, suggesting that patient anti-ALK specific lymphocytes possess a lower activation threshold, similar to that of recall antigens, and consistent with a T-cell memory-like functional status.

### *Patients' circulating CTL include ALK-specific tumor-reactive T cells*

To test the ability of anti-p280-89 CTL precursors to recognize tumor cells, patients' PBMC were cultured for one week in the presence of the ALK-derived peptides p280-89 (5 μM) and subsequently restimulated with irradiated autologous PBMC pulsed with related peptide. After three rounds of *in vitro* stimulation, a significant enrichment of p280-specific cells was observed in the resulting CTL cell line (Figure 3A). The specificity of p280-reactive cells was demonstrated by interferon-γ and granulocyte-monocyte colony-stimulating factor release upon exposure to T2 cells pulsed with cognate peptide (p280-89). No release was detected in the presence of T2 alone or T2 pulsed with an irrelevant (FLU) peptide (Figure 3B). Anti-p280 CTL also lysed the HLA-A2.1⁺ NPM/ALK⁺ ALCL-derived cell lines SU-DHL1 and SUP-M2. No lytic activity above the background level was observed in the HLA-A*0201⁻ no/NPM/ALK⁺ ALCL-derived cell line Karpas-299 or in the HLA-A*0201⁺ colon carcinoma cell line HCT-116 expressing irrelevant antigens (Figure 3C and D).

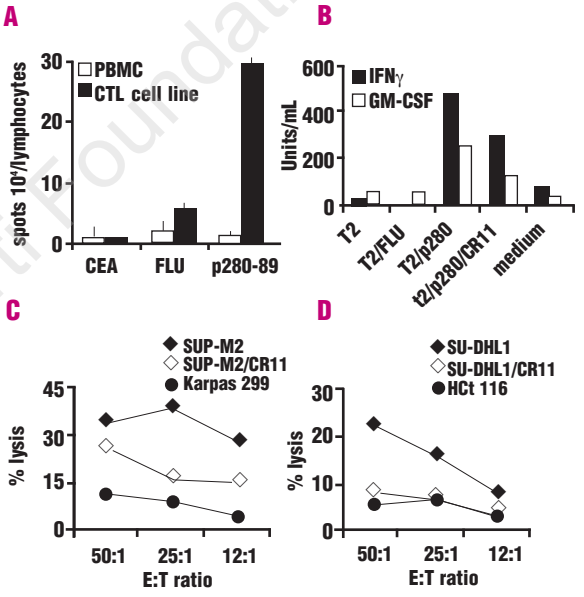


Figure 3. CTL generated from ALK-positive patients have *in vitro* anti-tumor antigen-specific activity. Peripheral lymphocytes were stimulated *in vitro* weekly with p280-pulsed autologous PBMC. (A) The enrichment in p280-reactive T cells after three rounds of *in vitro* stimulation was tested in an ELISPOT assay. Freshly isolated PBMC (open bars) and *in vitro* p280-89-sensitized cells (solid bars) were cultured overnight with T2 target cells pulsed with 5 μM of p280-89, FLU and CEA peptides. Each bar represents the average of spots in triplicate wells. (B) Specific cytokine release by *in vitro*-induced anti-p280 CTL lines was assessed after overnight culture with T2 cells alone or T2 cells loaded with 5 μM of FLU or p280-89m single peptide. Supernatants were checked for the presence of interferon-γ (IFN-γ) (■) and granulocyte-monocyte colony-stimulating factor (GM-CSF) (□) by ELISA. Inhibition of cytokine release was achieved by pre-incubating target cells with anti-HLA-A2 monoclonal antibody CR11.351. Data from one representative experiment of two performed are shown. Tumor cytotoxicity was measured by a conventional $51_{Cr}$-release assay at indicated effector to target (E:T) ratios. The following human tumor cell lines were used as target cells: (C) the NPM/ALK⁺ lymphoma cell line SUP-M2 (HLA-A2.2⁺) and Karpas 299 (HLA-A2.1⁻); (D) the HLA-A2.1⁺ tumor cell lines SU-DHL1 (NPM/ALK⁺ lymphoma) and HCT 116 (colon carcinoma). Lysis was inhibited by the presence of the anti-HLA-A2 monoclonal antibody CR11.351. The results of one representative experiment of two performed are shown.

Both cytokine secretion and lytic activity were inhibited by the presence of the anti-HLA-A2 monoclonal antibody CR11.351, consistent with a class I-restricted mechanism of recognition. No p280-89 reactive cell line could be generated from donor PBMC (*data not shown*).

These results represent a further demonstration that the anti-p280-89 precursor CTL population present within the T-cell repertoire of ALK-positive ALCL patients is functionally active and can be, at least *in vitro*, efficiently activated to react against ALK$^+$ tumor cells.

## Discussion

This study addresses the occurrence of a spontaneous *in vivo* immune response against ALK tyrosine kinase in ALCL patients confirmed by the presence of a long-lasting, ALK-specific memory T-cell population. Despite healthy donors and patients have comparable numbers of CD8/A2ALKp280-tetramer-positive cells, major functional differences in the anti-ALK T-cell repertoire were observed. ALK-specific CD8 lymphocytes from healthy individuals predominantly showed a naive phenotype (CD45RA$^+$/CCR7$^+$) revealing that they had never encountered their cognate antigen. In contrast, the anti-ALK CD8 repertoire of patients showed a substantial proportion of T cells expressing an effector (CD45$^+$CCR7$^-$) and/or a memory (CD45$^-$CCR7$^+$ and CD45$^-$/CCR7$^-$) phenotype providing evidence for an *in vivo* ALK antigenic challenge.

ELISPOT analysis, aimed at verifying the functional capacity of anti-ALK precursors, showed detectable levels of circulating CD8$^+$ T cells specifically producing interferon-γ in response to the ALK-derived p280-89 and p376-85 epitopes. Concurrently, as expected, a high number of FLU-peptide-reactive T cells was observed, correlating with a recall immune response against the viral FLU antigen, to which all individuals are supposed to have been exposed. Also as expected, a low number of CEA-peptide-reactive T cells was observed, in this case correlating with the existence of T-cell peripheral tolerance towards the CEA antigen widely expressed by normal epithelial cells. It is noteworthy that anti-ALK frequencies were closer to the frequency of FLU than to the frequency of CEA, indicating not only the presence of a high number of ALK precursors but also their prompt reactivity to antigenic stimulation suggesting an activation threshold for ALK similar to that of a recall antigen such as FLU.

Secondary immune responses by antigen-restimulated memory T cells are faster and stronger than primary responses. They require lower antigen concentrations and depend on co-stimulation for activation.[33] Moreover, it has been recently demonstrated that only the reactivation of the memory T-cell subsets (effector memory and central memory cells), but not naïve cells, can mediate *in vivo* tumor infiltration, recognition and rejection.[34] For this reason, memory T cells, rather than naïve/effector cells, might be a better target for vaccination protocols as an ideal source for generating effector cells. Thus, the detection of anti-ALK memory T cells among lymphoma patients' cells is therapeutically relevant for the design of an anti-ALK immune strategy.

Clinical trials performed up to date in solid tumors, mostly melanoma, have demonstrated that therapeutic cancer vaccines are easily administered to outpatients and generally do not cause systemic toxicity and are well tolerated. Unfortunately, overall clinical response rates have been below the expectations and characterized by the common paradox that immunization can elicit tumor antigen-specific T cells easily identifiable among circulating lymphocytes, but their presence does not seem sufficient to induce cancer rejection. Overall objective tumor regression rates of 2.6-3.3% have been reported among 1,205 patients with metastatic cancer treated in different types of active specific immunotherapy trials.[35] Complete clinical responses are, however, significantly more frequent in the treatment of lymphoid tumors than in solid tumors. The overall clinical response rate was ~ 40% among 60 patients with follicular lymphoma treated with anti-idiotypic vaccination (anti-Id),[36-40] suggesting that hematopoietic tumors may be more susceptible to active immunotherapy. The reasons for the different responses of solid and lymphoid tumors to active immunotherapy are not clear. A higher grade of vascularization and the expression of co-stimulatory molecules have been suggested.[35] Therefore, cancer vaccination could still represent a relevant, alternative strategy for tumors of hematopoietic cell origin.

We believe that ALK is a relevant tumor antigen, mostly because it is not expressed by normal somatic cells except in low amounts and in specific regions of the nervous system,[7] and it is required for the neoplastic transformation of lymphoid cells so that it seems unlikely to assist tumor immunoescape by ALK loss variants. Furthermore, activated anti-ALK specific CTL are unlikely to react against normal cells both because the nervous system is an immunologically privileged site and because of the limited normal level of ALK expression. Finally, translocated ALK is present in about 50-60% of all cases of CD30$^+$ ALCL but in almost the totality (84-95%) of pediatric ALCL[7,11,41] indicating that ALK is a stable lymphoma-specific hallmark and that its hypothetical use in vaccination protocols would be widely applicable. Patients' CTL precursors stimulated with p280-89 ALK-derived CTL epitope induced an anti-tumor ALK-specific immune response. Therefore, it is reasonable to consider an immune intervention such as a vaccination protocol based on the ALK target. Reinforcing the immunological memory by vaccination might help to prevent relapses, which occur in about 20-30 % of treated ALCL patients,[18,19,42,43] working in a combined strategy, following conventional chemotherapy to reduce tumor burden to a mini-

mum level.

To our knowledge, there are no widely accepted tumor-associated antigens for lymphomas except idiotypes, the tumor-specific immunoglobulin variable regions.[44,45] Although, idiotype is a truly tumor-specific antigen, wide use of idiotypic vaccines is hampered by the fact that autologous idiotype is not only a weakly immunogenic, self-antigen, but is also patient-specific so that the vaccine must be individually prepared for each patient. Since translocated ALK is expressed in the majority of pediatric ALCL and about 30% of adult patients, its expression is not restricted to a single individual greatly facilitating its hypothetical use in vaccination protocols.

*LP guided the experiments, supervised the analysis and the interpretation of data, prepared the manuscript; BG contributed substantially to data acquisition and analysis; EB and RS conducted all the statistical analyses; MM, MDN and AMG supervised the clinical documentation of the patients; CGP participated in drafting the manuscript. All authors contributed to the interpretation of the data, revised the manuscript critically for intellectual content, and approved its final version. The authors declare that they have no potential conflict of interest.*

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