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

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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T cells can be divided 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+ naive 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.

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 (SLAMLDDLHV) and p376-85 (GVILWEIFSL), 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-phycocerythrin 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. 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, anti-CD45RA, and anti-CCR7 detected by further incubation with SA Streptavidin phycoerythrin conjugated HLA-A2.1 and FITC-anti-CD8 (Becton Dickinson, Mountain View, CA, USA). The following HLA-A*0201-restricted peptides were synthesized and purchased from Sigma-Genosys (Cambridge, UK) at a minimum purity of 90%.

**Table 1. Patients’ characteristics.**

<table>
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<tr>
<th>Patient</th>
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<th>Time of T cell</th>
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<td>August 2001</td>
<td>30</td>
<td>CH, RT</td>
<td>August 2001</td>
<td>CR</td>
</tr>
</tbody>
</table>

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

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Institute of Allergy and Infectious Diseases Tetramer Facility, 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. 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, anti-CD45RA, and anti-CCR7 detected by further incubation with SA Streptavidin phycoerythrin conjugated HLA-A2.1 and FITC-anti-CD8 (Becton Dickinson, Mountain View, CA, USA). Cells were analyzed using Cell Quest software. For the analysis, CD8+ 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 Software, San Diego, CA, USA). In order to determine the functional phenotype frequencies of ALK-tetramer-positive, cells at least 5x10⁶ 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 0.5×10^6 PBMC were plated in triplicate and co-cultured overnight in the presence of 16×10^4/well T2 cells or T2 cells pulsed with 10 µM of the cognate peptide in plates pre-coated with 10 µg/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.


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, Uppsala, Sweden) gradients. The PBMC (10^6) 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, 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, Minneapolis, 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 (80 Gy) autologous PBMC as antigen-presenting cells.

CTL assays

For the cytokine release assays, 10^6 target cells (TAP-/T2 cells) were incubated overnight with the same amount of lymphocytes. Interferon-γ and granulocyte-macrophage colony-stimulating factor concentrations were assessed in culture supernatants using commercial ELISA kits (Human IFN-γ ELISA Assay, Mabtech, Stockholm, Sweden; Human GM-CF ELISA, Endogen, Woburn, MA, USA) following the manufacturers’ instructions. Cytolytic activity was measured in a standard 4-h 51chromium 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 0.08 %, 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 25th-75th 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.

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-
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+) 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 ALC. In contrast, healthy donors had predominantly naïve cells.

**Functional potential of ALK-specific memory T cells from ALC 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...
performed to determine the influenza matrix protein (FLU) and the carcinomaembryonic 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).
Both cytokine secretion and lytic activity were inhibited by the presence of the anti-HLA-A2 monoclonal antibody CR11.551, 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 extension of the anti-p280-89 precursor CTL population present within the T-cell repertoire of ALK-positive ALCL patients. It 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 with 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 (CD45RA−/CCR7−) and/or a memory (CD45RA−/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. Moreover, it has been recently demonstrated that only the reactivation of the memory T-cell subsets (effector memory and central memory cells), but not naive cells, can mediate in vivo tumor infiltration, recognition and rejection. For this reason, memory T cells, rather than naive/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. 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), 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. 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, and it is required for the neoplastic transformation of lymphoid cells so that it seems unlikely to assist tumor immunoevasion 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, 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, working in a combined strategy, following conventional chemotherapy to reduce tumor burden to a mini-
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 ALCCL and about 30% of adult patients, its expression is not restricted to a single individual greatly facilitating its hypothetical use in vaccination protocols.

To our knowledge, there are no widely accepted tumor-associated antigens for lymphomas except idiotypes, the tumor-specific immunoglobulin variable regions. 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 ALCCL and about 30% of adult patients, its expression is not restricted to a single individual greatly facilitating its hypothetical use in vaccination protocols.

**References**


