Gene Expression Analysis of Angioimmunoblastic Lymphoma Indicates Derivation from T Follicular Helper Cells and Vascular Endothelial Growth Factor Deregulation

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Abstract
Angioimmunoblastic lymphoma (AILT) is the second most common subtype of peripheral T-cell lymphoma (PTCL) and is characterized by dismal prognosis. Thus far, only a few studies have dealt with its molecular pathogenesis. We performed gene expression profile (GEP) analysis of six AILT, six anaplastic large cell lymphomas (ALCL), 28 PTCL-unspecified (PTCL/U), and 20 samples of normal T lymphocytes (including CD4+, CD8+, and activated and resting subpopulations), aiming to (a) assess the relationship of AILT with other PTCLs, (b) establish the relationship between AILT and normal T-cell subsets, and (c) recognize the cellular programs deregulated in AILT possibly looking for novel potential therapeutic targets. First, we found that AILT and other PTCLs have rather similar GEP possibly sharing common oncogenic pathways. Second, we found that AILTs are closer to activated CD4+, rather than to resting or CD8+ lymphocytes. Furthermore, we found that the molecular signature of follicular T helper cells was significantly overexpressed in AILT, reinforcing the idea that AILT may arise from such cellular counterpart. Finally, we identified several genes deregulated in AILT, including PDGFRA, REL, and VEGF. The expression of several of these molecules was then studied by immunohistochemistry on tissue microarrays containing 45 independent AILT cases. Notably, we found that the vascular endothelial growth factor (VEGF) was expressed not only by reactive cells, but also by neoplastic cells, and that nuclear factor-kB (NF-kB) activation is uncommon in AILT, as suggested by frequent exclusively cytoplasmic c-REL localization. Our study provides new relevant information on AILT biology and new candidates for possible therapeutic targets such as PDGFRA (platelet-derived growth factor α) and VEGF. [Cancer Res 2007;67(22):10703–10]

Introduction
Periipheral T-cell lymphomas (PTCL) represent 10% to 15% of all lymphoid neoplasms (1). They are a heterogeneous group of tumors that in the Revised European-American Lymphoma (REAL)/WHO classification are subdivided into specified and unspecified forms (1, 2). Angioimmunoblastic T-cell lymphoma (AILT) is a PTCL characterized by systemic disease, a polymorphic infiltrate primarily involving lymph nodes, with prominent proliferation of high endothelial venules and follicular dendritic cells (1). AILT is the second most common PTCL subtype, accounting for 15% to 20% of cases (1). It occurs in the middle age and elderly, the male/female ratio being 1/1. Patients usually present with generalized peripheral lymphadenopathy, hepatosplenomegaly, and frequent skin rash with pruritus. Bone marrow involvement, as well as B symptoms, is common (3). Other common clinical signs are edema, pleural effusion, arthritis, and ascites. Laboratory findings include polyclonal hyperγ-globulinemia, circulating immunocomplexes, cold agglutinins with hemolytic anemia, positive rheumatoid factor, and anti-smooth muscle antibodies. The clinical behavior is very aggressive, the response to therapy is scarce, and the long-term outcome is dismal (4).

The tumor morphology is constituted by a polymorphous population of small- to medium-sized lymphocytes, usually with clear cytoplasm (2, 5–7). The lymph node architecture is largely effaced, and regressed follicles may be seen. The lymphocytes show minimal cytologic atipia, and this form of lymphoma may be difficult to distinguish from atypical T-zone hyperplasia. Neoplastic cells are admixed with small reactive lymphocytes, eosinophils, plasma cells, histiocytes, and abundant follicular dendritic cells (1, 2, 7). High endothelial venules are numerous and show arborization. Cells positive for EBV are found in the majority of cases. However, the EBV cells are mainly B cells and rarely T cells (1). Immunohistochemistry generally shows T-cell–associated molecule expression, although the phenotypic profile is aberrant in the majority of cases, CD5 and CD7 being the most frequently defective antigens, whereas CD10 is frequently present (8). CD4 is most commonly expressed rather than CD8, but notably, the latter two antigens are coexpressed or even not expressed in more than 50% of cases (double-positive and double-negative cases, respectively; ref. 8). The differential diagnosis with other PTCLs and, in particular with PTCL unspecified (PTCL/U), is puzzling and mainly based on different features of reactive components.
(i.e., prominent vascular structures and abundant follicular dendritic cells) and phenotype (CD10 and BCL6 expression). Notably, no unique markers can reliably discriminate between these two entities. Recently, CXCL13 has been proposed as a possible candidate to distinguish the two diseases (7, 9–11).

The molecular pathogenesis of AILT, as in general of all peripheral T-cell neoplasms, is poorly understood. The karyotype is often characterized by complex abnormalities, but specific alterations have not been identified (12), and only few reports focused on gene expression profile (GEP) of nodal PTCLs (13–16). In one study, an AILT molecular signature has been identified together with a molecular link between AILT and T follicular helper (TFH) lymphocytes (16). Recently, we analyzed the GEP of a large panel of PTCLs and normal T lymphocytes by focusing on PTCL/U (17). We identified a molecular signature typical of PTCL/U and many tumor-associated pathways shared by both PTCL/U and AILT (17).

In the present study, we analyzed the GEP of AILT, PTCL/U, anaplastic large cell lymphoma (ALCL), and normal T-cell subpopulations aiming to (a) assess the relationship of AILT with other PTCLs, (b) establish the relationship between AILT and normal T-cell subsets, and (c) recognize the cellular programs that are altered as a consequence of malignant transformation in AILT and identify genes that might be suitable as novel potential therapeutic targets.

Materials and Methods

**Case selection.** Cryopreserved samples of 28 PTCL/U, 6 AILT, and 6 ALCL (4 ALK− and 2 ALK+) were collected on the basis of stringent criteria to guarantee the homogeneity of the series studied: (a) lymph node biopsy performed at disease presentation in the absence of any previous treatment, (b) excellent RNA preservation, and (c) presence of more than 70% neoplastic cells in the specimen. In addition, 20 samples of normal T lymphocytes (including CD4+, CD8+, activated, and resting T cells) were isolated from peripheral blood and tonsils of healthy donors.

The diagnosis of PTCL/U, AILT, and ALCL had previously been confirmed according to the REAL/WHO Classification (1, 2) by at least two experienced hematopathologists. In addition, AILT samples were studied for the expression of CXCL13, which was detected in all of them. All the samples had been obtained at the time of diagnosis, before any treatment had been given. Clinical details of these cases were previously reported (17).

In addition, the paraffin blocks of 193 PTCLs (148 PTCLs/U and 45 AILT) were collected. The phenotype was assessed by a large panel of reagents against CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD15, CD20, CD30, CD56, CD57, CD79a, TIA-1, Granzyme B, ALK protein, and EBER (8).

Informed consent had been obtained from all the enrolled patients, and the tissue collection was approved by each institutional ethical committee.

**Normal T-cell isolation.** T-cell subpopulations, CD4+ (n = 5), CD8+ (n = 5), HLA-DR+ (n = 5), and HLA-DR+ (n = 5), were purified from healthy donors as previously described (17).

**GEP analysis.** GEPs were generated and analyzed as previously reported (17) using the HG-U133 2.0 plus microarrays (Affymetrix, Inc.). For details on GEP generation and analyses, see Supplementary File 1.

Gene expression studies were conducted according to MIAMI guidelines.9

**Tissue microarray construction.** Tissue microarray (TMA) construction was performed as described in a previous publication referring to the same series (8).

**Immunohistochemistry.** After appropriate antigen retrieval (8), the sections obtained from each TMA were tested with specific antibodies against the following molecules: PDGFRα/CD140A, p-PDGFRα [representing the phosphorylated activated form of the platelet-derived growth factor receptor (PDGFR)], CYB61, LIFR-1, IGFBP7, BCL10, c-caldesmon, CXCL13, vascular endothelial growth factor (VEGF), VEGFR2, c-Rel, c-RelB, nuclear factor-κB (NF-κB) p65, selected because of their potential biological and clinical relevance. For additional details, see Supplementary File 1.

Bound antibodies were visualized either by the alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes technique or the EnVision method (8). For negative controls, the primary antibodies were omitted.

Notably, each TMA was also tested with anti-CD20 and CD3 antibodies to define the amount of reactive B cells comprised within the neoplastic T-cell population.

The adopted equipment included an Olympus RX41 microscopy with UPlanFI objectives (20×/0.50 and 40×/0.75), an Olympus Camedia C-7070 digital camera and the Olympus Master 1.1 software for images acquisition and evaluation.

**Criteria for immunohistochemical marker evaluation.** Each section was independently evaluated by at least two experienced hematopathologists. Cases were considered positive if 30% or more of the tumor cells were stained with an antibody. The number of positive cells was estimated by each observer. The intensity of staining was also evaluated, but was not used to determine positivity because it can vary depending on tissue fixation (8, 17).

Results

**GEPs of AILT and PTCL/U are similar.** We performed a gene expression analysis on six angioimmunoblastic lymphomas (AILT) and, for comparison, on 28 PTCLs/NOS, 6 ALCLs, and 20 samples of purified normal T cells (including CD4+, CD8+, HLA-DR+, and HLA-DR+ cells). First, we applied unsupervised analysis to AILT, PTCL/U, and ALCL to assess whether they are clearly distinct entities. With this method, no complete separation was feasible among the three PTCL histologic subtypes. In particular, AILT and PTCL/U showed a quite similar GEP, whereas ALCL tended to cluster more closely (Supplementary Fig; S1; ref. 17).

**AILT are related to activated peripheral CD4 T cells with TFH functional differentiation.** To evaluate the relationship between AILT and different normal T-cell subsets, we first studied AILT for the expression of genes previously shown to be differentially expressed in purified resting (HLA-DR−) and activated (HLA-DR+) T cells (17). We found that AILT are definitely closer to activated T lymphocytes (Fig. 1A). An analogous investigation was performed using a profile of genes differentially expressed between purified CD4 and CD8 T-cell subsets (17). The analysis revealed that AILT are closer to CD4 cells (Fig. 1B). Furthermore, we looked for the expression of genes characteristic of different functional stages of T cells, such as T-central memory (TCM), T-effector memory (TEM), and TFH (18, 19), in our AILT. Interestingly, whereas TCM and TEM signatures were not significantly expressed in AILT in comparison with other PTCLs, the molecular signature typical of TFH was significantly overexpressed in AILT (P = 0.03; Fig. 1C).

Taken together, our results indicate that AILT are molecularly related to activated peripheral CD4 T cells with TFH functional differentiation and strongly support the hypothesis that they are derived from the transformation and clonal expansion of such cells.

**AILT differs from normal T cells for the expression of genes involved in matrix constitution, adhesion, immune response, response to stress, and homeostasis.** To identify the genes and cell programs deregulated in AILT, we used a supervised analysis directly comparing the tumor cases with the closer normal cellular counterparts (CD4+-activated cells). In addition, CD8+ cells were

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8 http://www.affymetrix.com/support/index.affx

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also included in the comparison to abrogate the possible puzzling effect of contaminating normal CD8⁺ lymphocytes in AILT samples. Four hundred seventy-four probe sets were differentially expressed, corresponding to 346 unique genes. Two hundred and four were up-regulated, and 142 were down-regulated in AILT (Fig. 3A, Supplementary Tables S1 and S2). Subsequently, these genes were classified according to functional categories defined by Gene Ontology. We found some categories to be significantly overrepresented also when considering the number of genes belonging to each category that were comprised within the GeneChip (P < 0.05; refs. 17, 20), such as matrix constituents, adhesion, immune response, response to stress, and homeostasis (Supplementary Table S3).

Among others, the newly identified genes that were notably overexpressed in AILT, included (a) FNI (fibronectin 1), PCOLCE, COL1A1, COL3A1, COL4A1, COL6A1, COL12A1, COL13A1, COL4A2, and COL6A2 (encoding for collagen components), and MMP9, whose expression might be correlated with tissue invasiveness and angiogenesis (21–23); (b) ABCC3 and NVMT, which may contribute to the resistance to chemotherapy, a common feature of AILT (1); (c) PDGFRα (platelet-derived growth factor α), a tyrosine kinase possibly involved in neoplastic transformation, whose activity can be inhibited by imatinib mesylate (24, 25); (d) molecules involved in cell adhesion such as EMILIN1, LAMB1, PARVA, TNC, FLRT2, ENG, LAMC1, CDH5, CDH11, and RAB13; and finally, (e) chemokines including CCL2, CCL14, CCL19, CCL21, CXCL9, CXCL11, CXCL12, and CXCL13 (Supplementary Fig. S3), which may be responsible for reactive cell recruitment (26). Within the group of down-regulated genes, there were PPP1R15A, STK17B, and GADD45-A and GADD45-B (growth arrest and DNA damage-inducible α and β), which are involved in the apoptosis control. Notably, expression of the two latter is induced by histone deacetylase inhibitors (27–31). Moreover, we found REL to be down-regulated in AILT, suggesting that NF-κB pathway is not consistently active in these tumors.

**Supervised comparison among AILT, PTCL/U, and ALCL.**

The 474 gene signature identified in AILT at supervised analysis (Fig. 2A; Supplementary Tables S1 and S2) was largely shared by ALCL and PTCL/U (Fig. 2A). This finding might reflect either the influence of reactive components or the existence of common tumor-associated pathways (17). The latter hypothesis finds some support in the fact that among the deregulated genes, there were those shown to be tumor cell–specific by immunohistochemistry on TMAs (17). Moreover, they included many (142) down-regulated genes that are unlikely referable to nontumoral components. A further supervised analysis revealed 89 genes differently expressed between AILT on one hand and PTCL/U and ALCL on the other (Supplementary Table S4). Among the latter, there were FGF11, TP53, MMP11, LIFR, EDNRB, FOXP2, and CLK1. Finally, as differential diagnosis of AILT and PTCL/U is actually based on relatively subjective elements, we compared by supervised analysis these two PTCL subtypes. We found 80 genes consistently differentially expressed, including CCL19, CD81, IFNGR1, metallothionein 1X, 2A, 1G, 1F, and VEGF (Supplementary Table S5).

Because we found VEGF among genes overexpressed in AILT, we specifically looked at the expression of VEGFRs (VEGFR1, VEGFR2, and VEGFR3). We found VEGFR2/KDR to be significantly overexpressed in AILT versus normal T cells (P = 0.0008, Supplementary Fig. S2).

**Immunohistochemical validation on TMAs.** To investigate whether or not the up-regulated mRNA levels of PTCL/U-associated genes corresponded to elevated levels of the encoded proteins, we stained TMAs constructed from 45 AILT (including the cases subjected to gene expression analysis) using specific monoclonal and polyclonal antibodies raised against the selected
molecules. This method was previously shown to be very effective in identifying tumor-associated molecules and discriminate those related to reactive elements (17, 32).

The expression in AILT cells was confirmed for PDGFRα, VEGF, VEGFR2/KDR, LIFR, ILGFBP7, and BCL10, CALD1, and NF-κB components (namely, RELA/p65, RELB/p50, REL/c-rel; see examples in Figs. 3 and 4). The number of evaluable cases varied among the analyses. The main reasons for case exclusion were core loss, unrepresentative sample, or suboptimal antigen preservation. PDGFRα was expressed in 36/36 cases (100%); of note, it turned out to be phosphorylated in 21/22 evaluable cases (95%). ILGFBP7
was expressed in 17/27 cases (63%); LIFR in 12/28 (43%), and CYR61 in 36/37 cases (97%). Finally, AILT cells seemed to be VEGF positive in 18/18 cases (100%), whereas VEGFR2/KDR was present in 27/27 samples (100%). With regard to NF-κB pathway, RELA/p65 was expressed in 16/16; in six cases, the staining was detected at the cytoplasmic level and, in seven cases, at the cytoplasmic and nuclear levels. RELB/p50 was expressed in 18/23 cases, with cytoplasmic, cytoplasmic/nuclear, and nuclear staining in 14, 2, and 2 cases, respectively. REL/c-rel was expressed in 24/24 cases, with cytoplasmic or cytoplasmic + nuclear staining in 19 and 5 cases, respectively (Table 1).

Overall, NF-κB units turned out to be mostly localized in the cytoplasm (39/60 evaluable cores) and, in a minority of cases, at the cytoplasmic and nuclear (14/60 cores) or nuclear (5/60 cores) level. These findings were actually consistent with the gene expression results, not suggesting a constitutive/significant activation of this pathway in the majority of cases (Figs. 4 and 5 and Table 1).

The immunohistochemical analysis results are summarized in Table 2.

Taken together, these results indicate that there is a rather good correspondence between mRNA and protein expression. In
addition, the immunohistochemical determination allows an unambiguous definition of the cellular compartment carrying the phenotypic attribute (Table 1).

**Discussion**

This paper has addressed critical questions regarding the cell of origin and pathogenesis of AILT using the gene expression profiling technology. Overall, the panel of cases herein described was sufficient to show the distinctive tracts of the molecular signature of this disease, although the overall molecular profile of AILT is relatively similar to that of PTCL/U and ALCL (Supplementary Fig. S1; ref. 17). This fact may reflect either the influence of non-neoplastic elements or the existence of common tumor-associated pathways (17). To definitely solve this problem, gene expression analysis of purified PTCL cells is necessary. In addition, we could identify the closer cellular counterparts and discover alterations in cellular programs that might be of biological and clinical relevance. On the other hand, at present, we could not determine the prognostic impact of the novel identified molecules because the available panel was not designed, and, thus, sufficient, for such analysis.

The cellular derivation of AILT. Morphology and general phenotype of AILT suggests their derivation from mature T lymphocytes. Interestingly, immunohistochemical studies suggested the derivation of AILT from germinal center regulatory T cells (33) and, in particular, T follicular helper cells (TFH) according to the expression of CXCL13 (7, 9–11), a typical TFH marker (18). Furthermore, a recent GEP analysis showed that AILT are linked to TFH at the molecular level (16). In our study, we provided further evidence of the close relationship between AILT and TFH cells. In particular, we show that the global molecular profile of AILT is intimately related to that of activated CD4+ T lymphocytes. Furthermore, we could show that AILT do express the TFH molecular signature at significantly higher levels rather than PTCL/U and ALCL, whether T-central memory and T-effector memory signatures (19) were not significantly expressed. Together, these data strongly reinforce the hypothesis that AILT are actually derived from TFH cells. Nevertheless, at present, it is not possible to conclude that CXCL13 immunohistochemical expression (confirmed also in our series) represent a definitive marker for discriminating AILT and PTCL/U cases, but rather a useful additional information together with morphology, complete phenotype (including CD21, CD10, and BCL6), and clinical presentation.

**Functional alterations in AILT.** This study provides new information concerning the functional alterations in AILT. The direct comparison of AILT with the closest cellular counterparts showed a consistent deregulation of genes that sustain relevant cellular functions, such as matrix remodeling, adhesion, immune response, response to stress, and homeostasis. In this regard, it should be considered that the up-regulation of some genes (especially those involved in the extracellular matrix and adhesion) may reflect the presence of contaminating stromal elements present in the lymph node tumor tissues. Our results are overall consistent with those reported by de Leval et al. (16). However, few differences could be recorded. First, our signature did not include some molecules known to be expressed in AILT, such as CD10. It was not really surprising as we previously showed that CD10 is not always expressed by AILT (8). Second, de Leval et al. (16) suggested that VEGF expression was mainly due to reactive nonneoplastic components surrounding AILT cells. On the contrary, using immunohistochemistry as further investigation, we could show that AILT neoplastic cells also do ectopically express VEGF, which might be a relevant therapeutic target, in line with a previous observation (34). Notably, we could also show the expression of VEGFR2 in AILT cells, suggesting the existence of an autocrine loop. On the other hand, other groups investigated PTCLs by GEP (14, 15), but did not specifically focus on AILT and did not find an AILT-associated signature. Interestingly, it was proposed that some AILT might present constitutive NF-κB activity as some PTCL/U (35). We found REL to be down-regulated in AILT in comparison with normal T cells, suggesting that this pathway is not constitutively active in this tumor. In addition, we confirmed our result by immunohistochemical analysis because we found cytoplasmic localization (i.e., not activation) of NF-κB components (p50, p65, and c-rel) in the majority of cases in an independent panel of AILT. In our opinion, it is likely that previous findings might be related at least in part to non-neoplastic components more than to intrinsic characteristics of the neoplastic cells, although rarer AILT cases that do actually express the functional NF-κB pathway might not be excluded. Certainly, further studies on larger series and especially on purified neoplastic cells are anyway warranted.

**Table 1.** NF-κB pathway evaluation by immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Evaluable cores</th>
<th>Positive: cytoplasm</th>
<th>Positive: nucleus and cytoplasm</th>
<th>Positive: nucleus</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>RELB/p50</td>
<td>23</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>REL/c-rel</td>
<td>24</td>
<td>19</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RELA/p65</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>39</td>
<td>14</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 2.** Immunohistochemical analysis on TMA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Evaluable cases</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>18</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>27</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>36</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>p-PDGFRα</td>
<td>22</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>IGF1B</td>
<td>27</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>LIFR-1</td>
<td>28</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>CYB61</td>
<td>37</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>BCL10</td>
<td>25</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>43</td>
<td>43*</td>
<td>0</td>
</tr>
</tbody>
</table>

*Stromal reactivity.*
Biological and clinical implications. The present study confirmed our previous observation that different PTCLs can share molecular abnormalities (17). On the other hand, different PTCL subtypes seemed to present significant differences to what their relationship with different cellular counterparts is concerned. We and others showed that AILT is closely related to FHC (16), whereas PTCL/U and ALC1 are more frequently related to TCM and TEM, respectively (30). These observations may suggest the intriguing hypothesis that different PTCLs may arise from different T cells, but through largely common oncogenic events.

In addition, our gene expression analysis identified several genes deregulated in AILT, which may be interesting for the clinical practice. For instance, AILT are characterized by really dismal prognosis (4). We found several chemokines to be deregulated in this tumor, which might represent possible therapeutic targets (26). Furthermore, we identified molecules involved in drug resistance, such as ABC3 and NNM1, which might sustain the typical chemoresistance of this tumor. In addition, our gene expression study showed the down-regulation in AILT of genes that positively regulate apoptosis, such as MOAP1, PPP1R15A, STK17B, and GADD45-A and GADD45-B. In particular, GADD45-A and GADD45-B regulate apoptosis through p38 activation and c-Jun-NH2 kinase inhibition (27–29). Intriguingly, histone deacetylase inhibitors can induce the expression of GADD45-B/A in a p33-independent manner (30) and were shown as effective treatment in a PTCL/U both ex vivo and in vivo (17, 31).

Finally, we documented the aberrant expression of PDGFRα in AILT as previously recorded for PTCL/U (17, 32), and we found VEGF to be aberrantly expressed by AILT cells. Of note, recently, a few articles reported on the clinical efficacy of AILT in thalidomide (37–39) and especially of bevacizumab (40), two compounds with antiangiogenic properties. Surely, the practical impact of inhibiting VEGF and PDGFRα may be the goal of future pilot clinical studies (25).

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10 P.P. Piccaluga and S.A. Pileri, unpublished data.

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