Expression of T-lineage–affiliated transcripts and TCR rearrangements in acute promyelocytic leukemia: implications for the cellular target of t(15;17)

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Acute promyelocytic leukemia (APL) is the most differentiated form of acute myeloid leukemia (AML) and has generally been considered to result from transformation of a committed myeloid progenitor. Paradoxically, APL has long been known to express the T-cell lymphoid marker, CD2. We searched for other parameters indicative of T-cell lymphoid specification in a cohort of 36 APL cases, revealing a frequent but asynchronous T-cell lymphoid program most marked in the hypogranular variant (M3v) subtype, with expression of PTGER3, sterile TCRA, and TCRG transcripts and TCRG rearrangement in association with sporicid cytoplasmic expression of CD3 or TdT proteins. Gene-expression profiling identified differentially expressed transcription factors that have been implicated in lymphopoiesis. These data carry implications for the hematopoietic progenitor targeted by the PML-RARA oncoprotein in APL and are suggestive of a different cellular origin for classic hypergranular (M3) and variant forms of the disease. They are also consistent with the existence and subsequent transformation of progenitor populations with lymphoid/myeloid potential. (Blood. 2006;108:3484-3493)

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Introduction

To gain further insights into mechanisms of leukemogenesis and provide opportunities for the development of novel therapeutic approaches, it is of critical importance to establish the nature of the hematopoietic progenitors subject to leukemic transformation, characterize how so-called “leukemic stem cells” differ from their normal counterparts, and determine the extent to which the level at which target progenitors reside within the hematopoietic hierarchy influences the biologic behavior and characteristics of the leukemic output. As far as acute myeloid leukemia (AML) is concerned, evidence based largely upon engraftment characteristics of primary leukemic blasts in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice suggests that the disease arises in multipotent CD34+ CD38− progenitors, which give rise to more differentiated progeny that correspond to the bulk of the leukemic clone.1 However, results from early studies suggested that acute promyelocytic leukemia (APL), which is characterized by the PML-RARA oncprotein generated by the t(15;17) translocation, may be distinct from other forms of AML with respect to its cellular outputs. As far as acute myeloid leukemia (AML) is concerned, influences the biologic behavior and characteristics of the leukemic transplant recipient. Paradoxically, APL has long been considered to result from transformation of a committed myeloid progenitor. Paradoxically, APL has long been known to express the T-cell lymphoid marker, CD2. We searched for other parameters indicative of T-cell lymphoid specification in a cohort of 36 APL cases, revealing a frequent but asynchronous T-cell lymphoid program most marked in the hypogranular variant (M3v) subtype, with expression of PTGER3, sterile TCRA, and TCRG transcripts and TCRG rearrangement in association with sporicid cytoplasmic expression of CD3 or TdT proteins. Gene-expression profiling identified differentially expressed transcription factors that have been implicated in lymphopoiesis. These data carry implications for the hematopoietic progenitor targeted by the PML-RARA oncoprotein in APL and are suggestive of a different cellular origin for classic hypergranular (M3) and variant forms of the disease. They are also consistent with the existence and subsequent transformation of progenitor populations with lymphoid/myeloid potential. (Blood. 2006;108:3484-3493)

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in an open chromatin configuration in primary M3v APL blasts in a similar way to the pattern observed in CD2+ T lymphocytes. This T-cell configuration was seen irrespective of CD2 cell-surface expression in M3v APL.6

Mechanisms underlying cross-lineage antigen expression have been the subject of debate for more than 2 decades, being considered to be the result of aberrant gene expression consequent to leukemogenesis (“lineage infidelity” model) or, alternatively, to reflect the immunophenotype of the progenitor subject to leukemic transformation, which is then perpetuated in the leukemic progeny (“lineage promiscuity”). One approach to address this issue is to undertake a systematic analysis of the extent to which the lymphoid program is activated in a clearly molecularly defined subset of AML. APL provides an excellent model, because the cell of origin is presumed to be the progenitor in which the initiating t(15;17) chromosomal translocation occurred. Any somatic rearrangement that has occurred in this leukemic progenitor will be clonally transmitted to its progeny. Clonal immunoglobulin (IG) and/or T-cell receptor (TCR) rearrangements therefore represent useful genetic fingerprints that can reflect cellular ancestry indicative of at least early lymphoid specification.

The processes involved in normal T-lineage specification have been extensively studied. It is clearly established that TCR loci rearrange in a highly coordinated fashion: TCRD, TCRG, TCRB, and then TCRA,6 but the precise cellular stage at which recombinase occurs is not so clear. TCRD rearrangement was reported to start at the CD5+ CD1a− stage and TCRG and TCRB at the CD1a+ stage, just prior to the start of cTCRB expression at the CD4 intermediate single-positive/double-positive (ISP/DP) transition.9 However, a recent study demonstrated that TCR rearrangements occur earlier during T-cell development, with TCRD recombination starting at the CD34+ CD38− CD1a− stage, TCRG at the CD34+ CD38+ CD1a− stage, and complete TCRB rearrangement being detectable at the ISP stage.10 Rearrangement usually commences with the J3’ proximal of V or D segments and the V/D 5’ proximal of J segments and proceeds sequentially toward 5’ V and 3’ J segments.11 Expression of TCRoβ is preceded by expression of a pre-TCR composed of the preTo (PTCRA) invariant glycoprotein associated with the β TCR chain and the CD3 proteins. The pre-TCR is expressed at low levels at the DP thymocyte surface, where it mediates a proliferative and survival signal, a process known as β selection.12 PTCRA transcription precedes completion of TCRB rearrangement and is maximal at the CD4 ISP and DP stage.13 As such, it occurs in cells that have clearly undergone T-cell lymphoid restriction. Within T-cell acute lymphoblastic leukemias (T-ALLs), significant PTCRA transcription coincides with that of RAG1 in cells that have undergone TCRD, TCRG, and at least partial TCRB rearrangements.14 PTCRA is not expressed in mature T cells or B cells, natural killer (NK) cells, or myeloid cells and as such can be considered to be specific to the T-cell lymphoid lineage.15 TCR rearrangement is usually preceded by sterile transcripts within the locus, which reflect chromatin accessibility and may play a role in the initiation of rearrangement.16 One such sterile transcript that is indicative of opening of the TCR locus is the spliced product of the T-early alpha locus (TEA), a DNA sequence located at the 5’ limit of the Jα genes, to the TCR constant region.17 TEA–Ca is transcribed in immature thymocytes but is deleted, usually on both alleles, in TCRoβ-expressing lymphocytes and is not transcribed in TCRγδ mature lymphocytes.17 Although the presence of TEA–Ca is not absolutely required for TCRα Jα recombination,18 it modifies accessibility to the 5’ end of the Jα cluster on both alleles, thus targeting the first wave of Vα-Jα rearrangement.19 Cytoplasmic terminal deoxynucleotidyl transferase (TdT) expression precedes TCR rearrangement and is indicative of early lymphoid orientation, although this is not specific for the T-cell lineage.

In the present study, we have undertaken a comprehensive analysis of early T-lineage–affiliated markers in primary APL samples considered in relation to patterns observed in T-ALL and in the context of normal lymphopoiesis. Despite its strong “myeloid pedigree,” T-lineage–affiliated markers were detected in primary APL blasts, particularly associated with the hypogranular variant form of the disease. These findings not only provide insights into the progenitor populations to leukemic transformation in APL but also carry important implications for normal pathways of hematopoiesis.

Materials and methods

Samples

Diagnostic samples of peripheral blood or bone marrow were taken from 36 patients with APL diagnosed between 1993 and 2005 and treated within the APL93 and APL2000 French and United Kingdom Medical Research Council (UK MRC) AML 10 and AML 12 trials. These studies were approved by the French Lille University Hospital Comité Consultatif de Protection des Personnes dom la Recherche Biomédicale (CCPRB) and by the multicenter Research Ethics Committee (MREC) for Wales, for British patients. Informed consent was provided in accordance with the Declaration of Helsinki. These were compared for real-time quantitative PCR (RQ-PCR) analysis with normal bone marrow samples and 10% dilution of the same normal bone marrow into U937.14 Cells underwent Ficoll gradient centrifugation before immunophenotyping and DNA and RNA extraction, which was performed directly or after freezing in DMSO. RT-PCR was performed to confirm the presence of PML-RARA fusion genes and distinguish 5’ (bcr3) and 3’ (bcr1, bcr2) PML breakpoints.20 Patients were classified according to French-American-British (FAB) criteria21 as M3v or classic hypergranular (M3) APL.22 Thirty-six diagnostic AMLs and 106 previously published T-ALLs were used as controls.14

Immunophenotyping

Immunophenotyping was performed in the diagnostic center on fresh material and was completed at Necker-Enfants Malades from cryopreserved material as previously described.14 All APL cytometric analyses were performed on the ficolled fraction by triple staining with a PerCP CD45 gate for identification of the leukemic population within the morphologically gated blast population (Figure 1A). Triple labeling was performed to detect myeloid (CD13, CD33, CD117, MPO), T (CD2, cCD3, TdT, CD7), B (CD79a, CD19), and CD34 cell markers. Cytoplasmic CD3 (cCD3) and nuclear TdT were analyzed by triple staining with CD45 PerCP, cCD3 PE, and TdT FITC after permeabilization with Leucoperm (Serotec, Cergy Saint-Christophe, France). Antibodies used for triple staining were titrated to minimize nontargeted staining. Samples with more than 20% labeled leukemic cells and/or relative fluorescent intensity (RFI) greater than 2 within the CD45 leukemic gate were considered positive.

Quantification of PTCRA, RAG1, TEA–Ca, and TCRG transcripts

RQ-PCR was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) in a total volume of 25 µL in the presence of 300 nM primers, 100 ng cDNA, and 200 nM probe using Europe Against Cancer standards.23 Quality of cDNA was assessed by quantification of the ABL housekeeping gene, and samples with Ct values greater than 32 (fluorescence threshold, 0.1) were considered degraded. Amplification efficiency was assessed using the logarithmic dilutions slope obtained from positive cell lines (HPB-ALL for ABL, PTCRA, and RAG1; NB4 for TEA–Ca). Primers and probes were as described for ABL, PTCRA, and RAG1,14 or as follows for TEA–Ca (TEA forward: 5’TGG AGC AGC CTG
TAG CAA GA3'; TEA reverse: 5'AGC TGG TAC AGC GCA GGG T3'; TEA probe: 5'FAM-AGT CTT GGT CAC AGA TAT CCA GAA CCC T3'MAR3-3'; or JyP1/Cy TCRG (TCRG JP1/2 forward: 5'ATT TCG TGA AGG GAC TAA GCT CAT AG3'; TCRG C reverse: 5'CTC AAG AAG ACA AAG GTA TGT TCC AG3'; TCRG C probe: 5'FAM TCC TTT AAT TCG TGA AAC AAA GCT CCA GAT T3'MAR3-3'). Results were normalized for RNA quality relative to the ABL gene. Quantification was based on the ΔCt method where ΔCt = CtABL - CtAGL. Percentage of expression (RAG1 or PTCRA or TEA-CaABL) is given by (1 + efficiency)ΔCt.

Additional molecular analyses

TCRD, TCRG, and TCRB rearrangements were analyzed as previously described using Biomed-2 BMH4-CT98-3936 Concerted Action protocols. Detection of FLRT3-ITD by PCR was performed from cDNA as described.23 PCR products were analyzed by Genescan analysis on an ABI PRISM 310.

Microarray analysis

Microarray analysis was performed using Affymetrix HG-U133A arrays (Affymetrix, Santa Clara, CA) and GeneChip Operating software (GCOS), as described.26 Microarray absolute signal expression intensities were normalized by scaling the raw data intensities to a target intensity value of 5000 given the recommended HG-U133A mask file.27 Following this strategy, the microarray background signal value usually ranged between 50 to 90, and genes could be reliably detected by the software as present with intensities above this background. Many significant transcripts in APL vary to 90, and genes could be reliably detected by the software as present with intensities above this background. Many significant transcripts in APL vary within the range cited for the T-cell–associated transcripts described here.

Statistical analysis

A χ2 test on a 2 × 2 table was performed for comparison of disease characteristics (PML-RARA breakpoint; FLRT3-ITD status; CD2, CD34, cCD3, TdT expression) within different leukemic subcategories. Expression analysis was performed using a Mann-Whitney U test. A P value lower than .05 was considered statistically significant.

Results

Characterization of APL samples

Thirty-six cases of PML-RARA–positive APL were evaluated, including 15 with the M3v form. They included 1 child aged less than 15 years with classic APL and 35 adults. APL samples were of bone marrow origin in 50% of cases. The proportion of APL blast cells after Ficoll ranged from 75% to 95% (median, 90%). Residual T lymphocytes, as assessed by CD45 and CD3 staining (Figures 1A and S1A; the latter is available on the Blood website by clicking on the Supplemental Materials link at the top of the online article), ranged from 1% to 15% in the 13 classic APLs tested (median, 5%) and from 1% to 8% in 13 of 15 M3v's tested (median, 3%). There was no correlation between the presence of T-cell lymphoid transcripts and the proportion of contaminating T lymphocytes (data not shown). Immunophenotypic analysis of the CD45 gated blasts showed that all cases expressed CD13 and CD33. As expected, M3v morphology was associated with significantly more frequent CD2 and CD34 expression compared with classic M3 AML (Table 1). CD117 tended to be expressed more frequently in M3v compared with M3. CD56 was expressed by 1 of 13 M3v’s and 2 of 11 classic APLs. None of the 11 APLs tested expressed CD5, but these included only 3 M3v's. CD19 was expressed by 2 of 15 M3v cases tested (13%) but CD79a by none of the 10 M3v’s tested. All 12 APLs tested expressed CD45RA, but the intensity of expression was higher in M3v compared with M3 AML (data not shown). FLRT3-ITD was, as expected, more frequent in M3v, with a trend for more frequent PML-RARA bcr3 breakpoints.

Primary APL blasts express early T-cell lymphoid transcripts

APLs express PTCRA but not RAG1 transcripts. We investigated expression of T-cell- and lymphoid-specific markers, because CD2 is frequently expressed in APL. Quantification of PTCRA and RAG1 transcripts by RQ-PCR was performed in all APL samples and in 36 other cases of AML. PTCRA expression was significantly higher in APL compared with non-APL AML (P < .001), with the highest levels being observed in hypogranular M3v cases, where they were similar to those seen in T-ALL (Figure 2A). Although PTCRA transcripts were seen in peripheral blood, and to a lesser extent bone marrow, the levels seen in APL were much higher than those observed in 10% dilutions of these normal samples. This is higher than the median level of T-cell infiltration in APL (3% and 5% in M3v and M3, respectively), making a mature T-cell lymphoid origin for PTCRA unlikely. The levels observed in the control group of non-APL AML were heterogeneous and were significantly higher in M1 and M2 cases compared with M4 and M5 AML (P < .001). We therefore divided the control group into 21 “myeloid lineage” M1/M2 AML (10 M1 and 11 M2) and 15 “monocytic lineage” M4/M5 AML (3 M4 and 12 M5). PTCRA levels in M3v were higher than in classic APL (albeit not significant, P = .062; Table 1), M1/M2 AML (P < .001) and M4/M5 AML (P < .001). PTCRA levels observed in classic APL were also higher than those observed in M1/M2 AML (P = .031) and in M4/M5 AML (P < .001). Univariate analysis of the entire

Figure 1. Residual T lymphocytes as assessed by CD45 and CD3 staining. (A) Flow cytometric identification of weak cytoplasmic CD3 (cCD3) PE expression in APL blasts, identified by weak CD45 expression relative to the minor mature T-lymphocyte population (brighter CD45 and cCD3 expression). Cytoplasmic CD3 expression by APL was also weaker than (shaded) MPO expression (which uses a different isotype control to cCD3). Columns from left to right show the morphologic and CD45 gates and expression of cCD3 and MPO relative to isotype controls. (B) Absence of TdT expression is shown for both cCD3+ APLs.

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Table 1. Summary of genotypic, immunophenotypic, and TCR-rearrangement and transcriptional results in classic M3 and hypogranular M3v APL and in M1/M2 and M4/M5 AML

<table>
<thead>
<tr>
<th></th>
<th>M3</th>
<th>M3v</th>
<th>M1 + M2</th>
<th>M4 + M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>21</td>
<td>15</td>
<td>21 (10 + 11)</td>
<td>15 (3 + 12)</td>
</tr>
<tr>
<td>PML-RARA bcr3 (%)</td>
<td>8/21 (38)</td>
<td>9/15 (60)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FLT3-ITD (%)</td>
<td>4/21 (19)†</td>
<td>11/15 (73)</td>
<td>4/20 (20)†</td>
<td>2/15 (13)†</td>
</tr>
<tr>
<td>CD34+ (%)</td>
<td>0/9 (0)‡</td>
<td>8/15 (53)</td>
<td>13/20 (65)</td>
<td>3/20 (20)</td>
</tr>
<tr>
<td>CD117+ (%)</td>
<td>3/8 (38)</td>
<td>9/15 (60)</td>
<td>15/15 (100)</td>
<td>6/12 (50)</td>
</tr>
<tr>
<td>cTdT+ (%)</td>
<td>0/13 (0)</td>
<td>3/13 (23)</td>
<td>3/18 (17)</td>
<td>0/15 (0)‡</td>
</tr>
<tr>
<td>CD2+ (%)</td>
<td>1/14 (7)†</td>
<td>8/15 (53)</td>
<td>2/20 (10)‡</td>
<td>1/15 (7)†</td>
</tr>
<tr>
<td>cCD3+ (%)</td>
<td>0/12 (0)</td>
<td>2/13 (15)</td>
<td>3/17 (18)</td>
<td>0/15 (0)</td>
</tr>
<tr>
<td>CD7+ (%)</td>
<td>2/13 (15)</td>
<td>4/13 (31)</td>
<td>2/19 (10)</td>
<td>2/15 (13)</td>
</tr>
<tr>
<td>Median PTCRA/ABL, %</td>
<td>5.8</td>
<td>20.3</td>
<td>1.1†</td>
<td>&lt; 0.1†</td>
</tr>
<tr>
<td>Median TEA-Cα/ABL, %</td>
<td>1†</td>
<td>10.2</td>
<td>2.3‡</td>
<td>0.7†</td>
</tr>
<tr>
<td>TCR rearrangement (%)</td>
<td>1/17 (6)</td>
<td>4/15 (27)</td>
<td>0/18 (0)‡</td>
<td>1/11 (9)</td>
</tr>
<tr>
<td>CD2-, cCD3+, or CD7- (%)</td>
<td>0/12 (0)‡</td>
<td>5/13 (38)</td>
<td>2/17 (12)</td>
<td>0/15 (0)‡</td>
</tr>
<tr>
<td>PTCRA/ABL more than 10%, TEA-Cα/ABL more than 10%, or TCR rearrangement (%)</td>
<td>0/17 (0)†</td>
<td>9/15 (60)</td>
<td>0/18 (0)†</td>
<td>0/9 (0)‡</td>
</tr>
</tbody>
</table>

Statistical significance between M3v and M3, M1/2, or M4/5 AML is indicated as † if P < .001, § if P < .05, and ‡ if P ≤ .001. The 2 final rows summarize data. The number of cases expressing at least 2 of the stated phenotypic or genotypic characteristics is shown.

NA indicates not applicable.

APL cohort showed that PTCRA expression correlated with CD34 expression (P = .03) and M3v morphology (P = .06).

In T-ALL, RAG1 and PTCRA expression levels are similar and are coordinately regulated.14 This was not the case in classic or variant APL (Figure 2B), in which RAG1 was not expressed at significant levels.

**M3v APLs express TEA-Cα sterile transcripts.** TEA-Cα sterile transcripts were quantified in all 36 APL cases and 34 of the 36 AML controls. For comparison, TEA-Cα levels in 36 T-ALLs that retained at least 1 TCRD allele and, by extrapolation, the TEA locus were quantified (Figure 2C). TEA-Cα was expressed at levels similar to those seen in T-ALL in M3v APLs, at low levels in M1/M2 AML, and at a much lower level in classic APL and M4/M5 (Figure 2C). As for PTCRA, levels in M3v APL were higher than those observed in 10% dilutions of normal blood and bone marrow. Univariate analysis of the entire APL cohort showed that TEA-Cα expression correlated significantly only with M3v (P < .001). Notably, neither TEA-Cα nor PTCRA correlated with CD2 or CD117 expression, or the presence of a PML-RARA bcr3 breakpoint, or FLT3-ITD (data not shown).

These data show that M3v APLs express PTCRA and TEA-Cα sterile transcripts from the TCRα locus at levels similar to those seen in T-ALL. Unlike T-ALL, these occur in the absence of RAG1 transcription. Classic M3 APLs express lower-level PTCRA but not TEA-Cα.

**Expression of TdT, cCD3, and TCR rearrangements**

Cytoplasmic CD3 expression was detected in the leukemic gated population in 2 of 13 M3v APL cases (Figure 1A) and TdT in 3 of 13, without coexpression of cCD3 (Table 1). Cytoplasmic CD3 expression was considered positive if relative fluorescent intensity (RFI) was more than 2 (Figure 1A) but was lower than that observed in contaminating T cells, identified by their higher level of CD45 expression (Figure S1A). Cytoplasmic CD3 was also expressed at lower levels than MPO within the same gated population (Figure 1A). Nonspecific intracytoplasmic staining was excluded by the absence of coexistent cCD3 and TdT expression (Figure 1B). Cytoplasmic CD3 and TdT expression was also seen in M1/M2 AML (RFI for cCD3, 2.3, 6.4, and 7.6 compared with an isotype control; Figure S1B) but not in classic M3 or M4/M5 AML. Surface TCR and CD8 expression by blast cells was not seen (data not shown). All 5 cCD3 or TdT-positive APL cases expressed PTCRA (19% to 373%) and TEA-Cα (3% to 66%). Four had a PML-RARA bcr3 breakpoint and were FLT3-ITD positive.

TCRD or TCRD rearrangements were found in 27% of M3v, 6% of M3, and in a single MLL-rearranged AML, in which IGH/TCR rearrangements are relatively common8 (Table 1). Neither partial TCRD D μδ rearrangements nor IGH D μδ were seen in the 15 M3v’s tested (data not shown). Although all TCR rearrangements were clearly visible using heteroduplex polyacrylamide gel electrophoresis (PAGE) analysis, which has an approximate 1% to 5% level of sensitivity of detection, the intensity of rearrangements was in keeping with their presence in significant subclones (Figure 3). The single TCRD-rearranged classic M3 was PTCRA and TEA-Cα negative and demonstrated an isolated, incomplete TCRD D62-D63 rearrangement corresponding to non–T-restricted TCRD rearrangements, which are often classified as illegitimate.29 In contrast, 4 M3v APLs had undergone TCRD rearrangement, with 1 also demonstrating an incomplete monoallelic V62-D63 rearrangement. No rearrangements involving J6 were seen. These profiles are in contrast to immature T-ALLs, where early evidence of TCRD rearrangement is always associated with TCRD rearrangement, with most involving J61.14 Among the 5 rearranged TCRD alleles (2 rearrangements were found in 1 case of M3v), 4 involved 3’ Vγ (1 Vγ10-Jγ1/2 or 5’ Jγ (3 Vγ7-JP1/2) segments and, as such, were classified as immature,14 whereas only 1 involved mature Vγ8-Jγ1/2 (5’Vγ3-Jγ1) segments. All 4 M3v cases with TCRD rearrangement were FLT3-ITD positive, but most were TdT and cCD3 negative.

Taken together, these data demonstrate that M3v APLs express specific markers of lymphoid/T-cell differentiation, including PTCRA and TEA-Cα transcripts; CD2, cCD3, or TdT expression; or immature TCR rearrangements. Although often found independently, the coexistence of T-cell lymphoid transcripts and TCR rearrangement and T-associated proteins was significantly higher in M3v (Table 1). Notably, however, M1/M2 AML demonstrated T-cell features more frequently than M4/M5 cases. This was notably due to a case of CD7+ CD2+ TdT+ CD13+ CD33− M2 AML with 12% myeloperoxidase by cytochemistry, for which MPO and cCD3 were not evaluated (UPN1042), and to a case of...
PTCRA⁺ cCD3⁺ CD56⁺ CD13⁺ CD33⁺ MPO⁺ CD4⁻ CD7⁻ CD2⁻ M1 AML (UPN1525).

Transcriptional profiling of selected transcripts

To determine whether other T-cell–associated transcripts were preferentially associated with APL, we analyzed published gene expression data using Affymetrix U133 microarray in an independent series of 19 classic M3, 16 M3v, 79 M1/M2 (37 M1, 42 M2), and 47 M4/M5 AML (30 M4, 17 M5) cases with a normal karyotype and 25 adult T-ALLs, including 9 early and 16 cortical cases. Transcripts were selected either because they are known to play a role in T-cell lymphopoiesis or to function in the regulation of the early stages of human hematopoiesis (Table S1). No attempt was made to undertake an exhaustive analysis, which has been performed independently on this set of AML. Normalized background intensities varied from 50 to 90, allowing reliable detection of transcripts with values above these levels. Despite this, we concentrated on values above 1000 in APL other than for significant negatives. For significant T-cell lymphoid–associated transcripts with mean intensities between 100 and 1000, only probe sets that were considered to be present in at least 34 of 35 APL cases were taken into consideration (data not shown).

We initially compared transcripts that were differentially expressed between M3 and M3v. In keeping with immunophenotypic data, median CD2, CD34, and CD45 mRNA levels were higher in M3v compared with classic M3. Notably, both CD2 and CD34 transcript levels are relatively low in M3v despite the frequent expression of the corresponding protein at the cell surface (Figure 4; Table 2). SMARCA4/BRG1 was expressed at significantly higher levels in M3v and T-ALL than classic M3 and other AML subgroups. SMARCA4/BRG1 encodes a component of the SWI-SNF chromatin remodeling complex and plays a role in T-cell development.29

We then compared APL in general with other AMLs and T-ALLs. The most strikingly overexpressed T-cell lymphoid transcript in both classic and M3v APL (Figure 4; Table 2) was the T/NK-specific transcript, NKG7/GMP17, and transcripts from the TCRG locus, labeled as TCRGV9 but specific for the TCRG segment. This was not associated with significant transcription of TCRD, TCRB, TCRγ, or CD3 transcripts (Table S2) and as such is unlikely to represent transcription from contaminating normal T cells. IKAROS (ZNFM1A1) transcripts showed a trend for higher levels in M3v, which reached statistical significance (P = .005) when compared with M1/M2 AML (Table 2). MYB was expressed at similar levels in APL and T-ALL, which were significantly higher than those from M4/M5 and M1/M2 AML. Relevant transcripts that were not identified at significantly higher levels in APL included RAG1, Notch1, HES1, GATA3, IL7R, HEB, E2A, CD4, CD5, CD7, and CD56/NCAM1 (Table S2).

To validate the expression of TCRG seen in array data, TCRG transcripts were quantified by RQ-PCR using JγP1/2- and Cγ-specific primers, because JγP1/2 segments were those found to be rearranged most commonly. Transcript levels were highest in M3v APL, significantly higher than in classic M3 and M4/M5 but not M1/M2 (Figure 5), and were similar to those seen in immature (IM) T-ALLs that have not yet rearranged the TCRG locus. As expected, JγP1/2-Cγ TCRG transcripts were lost in T-ALLs with TCRG VγJγ rearrangement (preβ and TCRγ-δ; Figure 5).
Discussion

In this paper, we demonstrate that APL, despite being considered the most differentiated subtype of AML, exhibits several features of a T-cell lymphoid program. This raises several issues regarding the cellular origins of APL that in turn impact on our understanding of normal hematopoiesis and its disruption by leukemic oncogenes.

T-cell lymphoid features included expression of CD2, cCD3, or TdT proteins; PTCRA, TEA-α, or TCRγ1β transcripts; and immature TCRγ rearrangements. Most were more pronounced in M3v, in which PTCRA and TEA-α transcripts levels by RQ-PCR were comparable with those observed in T-ALL and higher than those found in immature cCD3+ CD7+ IM T-ALLs arrested prior to β selection. TCRγ sterile transcripts were similar to those observed in IM T-ALLs just about to undergo TCRγ rearrangement. Most M3v’s demonstrated more than one of these parameters, but it was striking that few exhibited a “full house” of T-cell features. The profiles seen also differed from those observed in T-ALL; in particular, PTCRA transcripts were not associated with RAG1 transcripts, TCRγ rearrangement was seen in the absence of TCRD rearrangement, and CD2 expression was not associated with other early cell-surface markers of T lineage, such as CD7 or CD5. The large number of T-cell features is in keeping with an activated T-cell lymphoid program, but the discrepancies both within individual cases and between cases suggest that this lymphoid program is not coordinately regulated. It is highly unlikely that these T-cell features result from contaminating normal T lymphocytes, because PTCRA and TEA are normally expressed only during thymic development; the transcript levels observed in M3v APL were too high to correspond to those of minor T-cell lymphoid contamination, and the asynchronous nature of the T-cell lymphoid profiles is difficult to reconcile with mature T cells, as is the absence of TCRβ and TCRα transcripts profiles. Furthermore, immunocytometric analysis of CD45 gated blasts with multiparameter analysis allows unequivocal identification of the cell expressing CD2 or TdT, as widely recognized in APL, and cCD3, as shown here.

Within the context of AML, to our knowledge, PTCRA has only been described in CD4+ CD56+ lin− plasmacytoid dendritic-cell AML. Notably, the highest level of PTCRA transcripts (PTCRA/ABL, 50%) seen in the M1/M2 AML control group occurred in a case of CD56+ CD13+ CD33+ MPO+ CD4− M1 AML, which may represent a variant of these dendritic AMLs. During normal development, PTCRA is expressed in murine Thy1+ circulating fetal cells, which include thymic precursors, but not in the Thy1+ CD117+ fraction that contains pluripotent precursors.32 In humans, low-level PTCRA transcripts have been variably identified in cord blood, bone marrow, and fetal CD34+ cells and in G-CSF-stimulated peripheral adult CD34+ cells, where they have been interpreted as evidence of circulating prethymic precursors. PTCRA transcripts in peripheral or hepatic cCD3+ CD33−/− CD2+− TCRβ Dμβ1− RAG1+ but CD34− CD7− CD56− D83− cells were interpreted as evidence of extrathymic T-cell differentiation. Because PTCRA expression in APL correlated with CD34 expression, it is possible that, in view of the low-level expression observed in normal bone marrow CD34+ cells; these transcripts originate from the PML-RARA M3v target stem cell. PTCRA expression in APL is not, however, merely a reflection of maturation arrest at a CD34+ stage, because approximately half of the M3v cases were PTCRA+ CD34+ and CD34 was expressed by 28% of PTCRA+ M4/M5 AMLs. Furthermore, we did not identify PTCRA transcripts in cDNA from sorted CD34+ cells from normal adult bone marrow (PTCRA/ABL, 0.1%). It is likely that PTCRA is only expressed by a minority of CD34+ bone marrow cells, which possibly overlap with one of the potential PML-RARA targets.

The T-cell lymphoid program described here in APL is difficult to reconcile with prevailing models of a myeloid-restricted nature for the cellular target of the t(15;17). According to classic hematopoietic models, which invoke early divergence of myeloid and lymphoid progenitors, the T-cell lymphoid program would suggest that APL arises in multipotent progenitors, in which gene expression data have revealed colocalization of myeloid and lymphoid transcripts.36 The fact that PML-RARA positivity has been demonstrated in the CD34+ CD38− fraction in APL is in keeping with this. Based on this model, M3v would arise in more immature progenitors than classic APL.2 The higher levels of CD34, CD117, and CD45RA expression in M3v are in accordance with this hypothesis.

The data presented here are also consistent with an alternative hematopoietic model as proposed by Katsura38 in which there is no early divergence of myeloid and lymphoid pathways but populations with either B- or T-cell lymphoid and myeloid potential. While the Katsura model is based on in vitro proliferation of murine fetal liver progenitors, recent evidence
Table 2. Affymetrix U133A microarray fluorescences in arbitrary units of selected genes with indication of the Affymetrix probe set number.

<table>
<thead>
<tr>
<th>Gene</th>
<th>U133A Probe set</th>
<th>Fluorescence median</th>
<th>P, Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45_PTPRC</td>
<td>212587_s_at</td>
<td>711 460 1381 978</td>
<td>&lt; .001 NS &lt; .001 .038 &lt; .001 &lt; .001 NS NS &lt; .001 NS &lt; .001 .008 .001</td>
</tr>
<tr>
<td>CD45_PTPRC</td>
<td>207238_s_at</td>
<td>808 582 1394 1038</td>
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</tr>
<tr>
<td>CD44</td>
<td>209543_s_at</td>
<td>117 111 154 335</td>
<td>.043 .001 &lt; .001 NS NS NS NS NS NS NS NS NS NS</td>
</tr>
<tr>
<td>MYB</td>
<td>204798_at</td>
<td>3642 3951 3469 2399</td>
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</tr>
<tr>
<td>SMARCA4</td>
<td>212530_s_at</td>
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</tr>
<tr>
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<td>SMARCA4</td>
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<td>NKG7</td>
<td>213915_at</td>
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</tr>
<tr>
<td>CD2</td>
<td>205831_at</td>
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<td>.016 &lt; .001 .002 &lt; .001 .036 NS .001 &lt; .001 .027 &lt; .001</td>
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<tr>
<td>PTCRA</td>
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<td>.010 NS .005 NS NS NS NS NS NS NS NS NS 0.24</td>
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</table>

Median values are indicated according to the leukemia category. Statistical analysis was performed using a Mann-Whitney U test for an identical hypothesis. NS indicates not significant (P > .05).
exists in favor of an initial separation of the erythroid/megakaryocyte lineage from a precursor with myeloid and lymphoid potential and for early T- or B-lymphoid orientation in cells that retain myeloid potential. Expression profiling of human cord blood CD34+ cells demonstrated that the most specific transcripts that allowed separation of CD45RA+ CD7+ cells with T/NK orientation, while maintaining expression of granulomonocytic lineage genes, from more immature CD45RA+ CD7+ progenitors with myeloid and erythroid potential included TCRG and NKG7, both of which were expressed at significantly higher levels by microarray analysis in APL when compared with other forms of AML. NKG7 is also known as GMP17 (granule-membrane protein, 17 kDa), GIG1 (G-CSF induced gene 1), or p15-TIA-1 and is expressed in cytotoxic granule (granule-membrane protein, 17 kDa), GIG1 (G-CSF induced gene 1), or p15-TIA-1 and is expressed in cytotoxic granule

Figure 5. Quantification by RQ-PCR of the expression of TCRG J-γ-P1/2-γ relative to ABL in IM, pre-M1, and M2v T-ALLs and M3 variant (M3v), classic M3, and M1/M2 and M4/M5 acute myeloid leukemias. Results are presented as a box plot graph using a logarithmic scale. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Narrow horizontal bars above and below the box indicate the 90th and 10th percentiles. Outliers are indicated as dots. The number of cases quantified in each category is indicated below the category. Mann-Whitney statistical results comparing each category with M3v's are indicated by P-value.

also account for the demonstration of B-lymphoid features in AML1-ETO-positive AML. Overexpression of PAX5 has been specifically associated with AML1-ETO-positive AML. This is likely to account for the expression of CD19 and CD79, which are downstream targets of this B-lineage master regulator. By recruitment of corepressors, AML1-ETO represses E-box target genes such as E2A or HEB that regulate B and T lymphopoiesis to variable degrees and, as such, could prevent B-lymphoid development while being permissive for a certain degree of myeloid differentiation. By extrapolation, PML-RARA, which is also known to recruit corepressors, may prevent lymphoid differentiation while promoting, or at least being permissive for, progression along the myeloid pathway. The lymphoid repression might be only partial, leading to a residual T-cell lymphoid program, as identified here. Within this context, it is notable that PTCRA expression was seen in the only case with AML1-ETO within the M1/M2 control AML group (PTCRA/ABL, 19%). Studies have already shown that enforced expression of CEBPA and CEBPP in differentiated B cells induces reprogramming into macrophages by inhibiting PAX5, and enforced IL-2 signaling can induce myeloid differentiation in early murine thymic precursors. We looked for evidence of relative overexpression of genes encoding early T-cell lymphoid transcription factors, such as GATA3, Notch1, HEB, or E2A in APL, but no differences were observed compared with other forms of AML and levels were lower than those of T-ALL. The absence of a physiologic T-cell lymphoid transcriptional profile, together with the aforementioned discrepancies in the T-cell characteristics identified in APL, is more in favor of a deregulated T-cell lymphoid program, which cannot simply be explained by a lineage promiscuity model.

It is possible that APL transformation leads to deregulation of a master regulator that controls the loci that we show to be abnormally expressed. The CD2 locus consistently lies in an open chromatin configuration in APL. Similarly, sterile transcripts at rearranging loci, such as TCRα and TCRγ, reflect an open configuration that is thought to favor but not be sufficient for subsequent rearrangement. It is possible that PML-RARA may directly or indirectly modify chromatin configuration, although it is also possible that these loci were already in an open configuration at the time of initial transformation. It is notable that high levels of TCRγ sterile transcripts, but not TCRδ or TCRβ, were identified by transcriptional profiling and that the TCR rearrangements preferentially involved the TCRγ locus and corresponded to the type of rearrangements that can occur in the presence of low or undetectable RAG1 levels. Opening of the TCRγ locus is normally mediated by IL-7 binding, but this is unlikely to be operational in APLs, which do not express the IL-7 receptor, including in the present series. It is possible that this normal pathway is short-circuited by a downstream effect of the PML-RARA fusion protein or by other genetic abnormalities.

An alternative explanation for the lymphoid features in APL might be PML-RARA-related genomic instability, for example, due to disruption of PML nuclear bodies and delocalization of constituent proteins such as BLM. However, such a hypothesis is difficult to reconcile with the observation that T-cell lymphoid deregulation was particularly associated with the hypgranular variant form of APL, given that the PML-RARA fusion also lies at the heart of the classic form of the disease.

In conclusion, we present data that suggest that APLs, particularly M3v's, are associated with several parameters...
indicative of a partial, deregulated, T-cell lymphoid program in conjunction with undoubted myeloid differentiation. This may either reflect transformation of a precursor with T/myeloid potential, for which increasing evidence exists, or partial lineage reorientation of a T/NK precursor toward the myeloid lineage or vice versa as a direct or indirect consequence of PML-RARA expression. While such hypothesis-generating observations clearly require formal testing in appropriate cellular or animal models, the data presented here should help appropriate design of such models.

Acknowledgments

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Expression of T-lineage-affiliated transcripts and TCR rearrangements in acute promyelocytic leukemia: implications for the cellular target of t(15;17)

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