行政院國家科學委員會專題研究計畫　成果報告

自然弒細胞發生學的雙系模式：白血球介質 15 及 21 分別導引 CD56+Kir±lectis+ 及 CD56±Kirlectin 自然弒細胞 (3/3)

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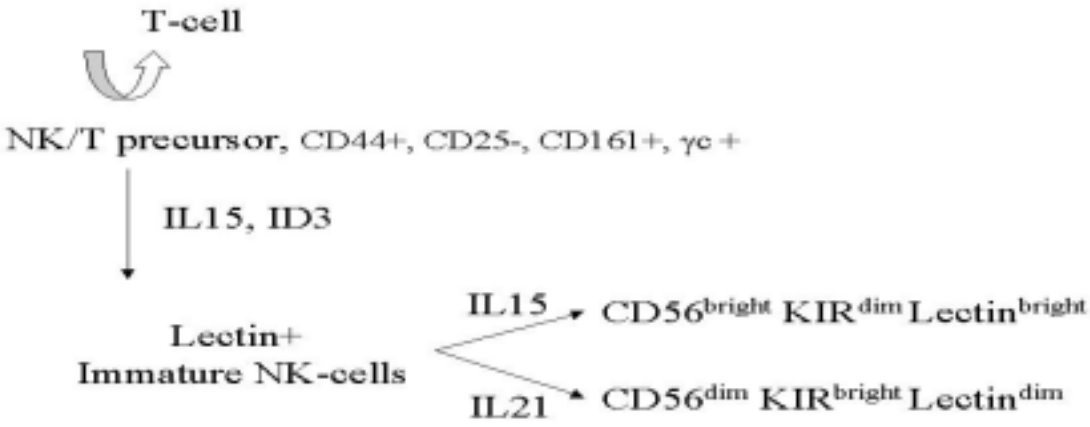
Introduction

We have proposed a moedl for NK-cell development



Mature human NK-cells are heterogeneous, and their developments are incompletely understood. We want to prove that interleukin 15 and interleukin 21 drive respectively the development of $CD56^{brigtht}KIR^{dim}Lectin^{bright}$ type I (IFN-γ+) NK-cells and $CD56^{dim}KIR^{bright}Lectin^{dim}$ type II (IL-13+) NK-cells. Biologically, this model can be used to further analyze the interrelationship between various NK-cell subsets. Clinically, this model can be used to further sub-classify NK-cell lymphomas or to analyze the role of NK-cells in transplantation pathology. Thus this model will have both biological and clinical significance. As summarized in the abstract and to be expanded in details in the Background section, we will use a developmental approach to integrate previous functional-structural studies. More specifically, we will achieve the following:

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for the study of NK-cell development. These rare bipotent T/NK precursors should be CD44+, CD25-, CD161+, γc +. They will be positively selected for interleukin 15 receptor and Id3, and negative selected for interleukin 7 receptor and E2A, and used as the starting materials for NK-cell development.

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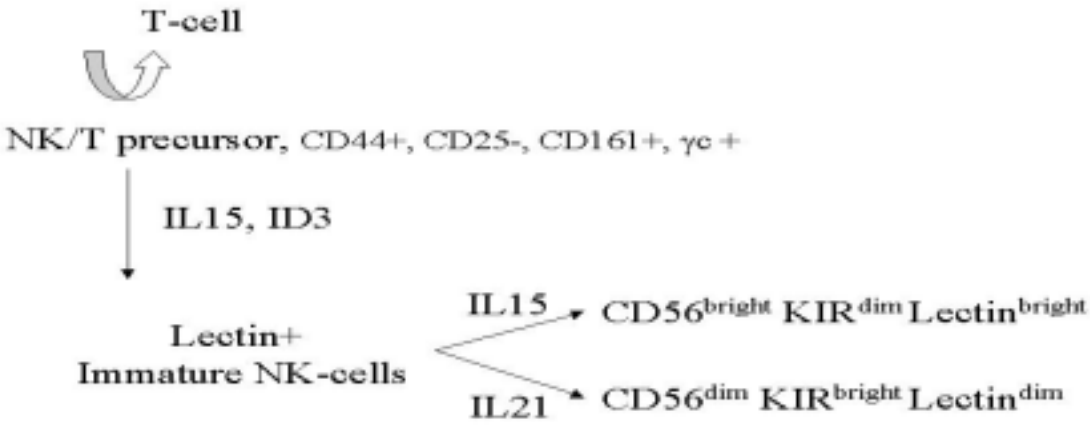

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CD94 1A transcripts characterize lymphoblastic lymphoma/leukemia of immature natural killer cell origin with distinct clinical features

Chung-Wu Lin,[1] Ting-Yun Liu,[1] Shee-Uan Chen,[2] Kun-Teng Wang,[1] L. Jeffrey Medeiros[3] and Su-Ming Hsu[1]

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Author contribution statements:

Chung-Wu Lin proposed that CD94 1A transcripts might be used by immature NK cells and wrote the initial manuscript. Ting-Yun Liu and Kun-Teng Wang carried out most of the experiments. Shee-Uan Chen collected the cord blood and isolated the progenitor cells. L. Jeffrey Medeiros reviewed the data and participated in the writing of the manuscript. Su-Ming Hsu supervised the whole project.

Abbreviations:

β2M: beta-2-microglobulin; DN: double negative; E2A: E2A immunoglobulin enhancer binding factor; EBER: EBV-encoded small RNA; EBV: Epstein-Bar virus; ID2: inhibitor of differentiation 2; IL-2: interleukin 2; IL-2Rα: interleukin-2 receptor alpha; IL-15: interleukin-15; IL-15Rα: interleukin-15 receptor alpha; KIR: killer immunoglobulin-like receptor; LBL: lymphoblastic lymphoma; LCM: laser capture microdissection; Lin: lineage; NK: natural killer; NK-LBL: LBL of the NK lineage; rLCM: reverse LCM; T-LBL: LBL of the T lineage; TCF-1: T-cell factor 1; TCR: T-cell antigen receptor; TdT: terminal deoxynucleotidyl transferase; WHO: World Health Organization

Abstract

Most lymphoblastic lymphomas (LBLs) are regarded as neoplasms of immature T-cells because they express cytoplasmic CD3 and frequently carry T-cell receptor (TCR) gene rearrangements. Immature natural killer (NK)- and T-cells, however, have a common bipotent T/NK-cell precursor in the thymus and NK-cells also express cytoplasmic CD3. Thus, some LBLs could arise from immature NK-cells. Mature NK-cells express two CD94 transcripts: 1A, induced by IL-15, and 1B constitutively. Since immature NK-cells require IL-15 for development, CD94 1A transcripts could be a marker of NK-LBL. To test this hypothesis, we used laser capture microdissection to isolate IL-15 receptor α^+ lymphoid cells from the thymus and showed that these cells contained CD94 1A transcripts. We then assessed for CD94 transcripts in 21 cases of LBL that were cytoplasmic $CD3^+$, nuclear TdT^+, and $CD56^-$, consistent with either the T- or NK-cell lineage. We found that 7 LBLs expressed CD94 1A transcripts without TCR gene rearrangements, suggesting NK-cell lineage. Patients with NK-LBL were younger than patients with T-LBL (15 years *versus* 33 years; p=0.11) and had a better 2-year survival (100% *versus* 27%; p<0.01). These results improve the current classification of LBL and contribute to our understanding of NK-cell differentiation.

Introduction

Precursor lymphoblastic lymphoma/leukemia (LBL) frequently presents as a mediastinal mass in young patients. Immunophenotypically, most neoplasms are positive for cytoplasmic CD3 and nuclear terminal deoxynucleotidyl transferase (TdT) and carry T-cell receptor (TCR) rearrangements.[1] Thus, most LBL cases are thought to be of T-lineage and represent lymphomas of immature T-cells from the thymus. However, it is difficult to distinguish immature T-cells from immature NK-cells because both are derived from bipotent T/NK-precursors in the thymus and share similar immunophenotypes.[2-5] Cytoplasmic CD3 can be found in both immature T- and NK-cells.[6] Similarly, nuclear TdT is expressed by lymphoid precursors of various lineages and is not specific for T-cells.[7] In addition, TCR gene rearrangement, despite being a good marker for T-cells, can be found in cases of acute myeloid leukemia and precursor B-cell lymphoblastic leukemia due to the phenomenon of lineage promiscuity.[8] These traditional criteria, therefore, do not exclude the possibility that lymphoblastic lymphoma/leukemia of the NK-lineage exists.[9] Although CD56 (neural cell adhesion molecule; NCAM) has been recognized as a sensitive marker for NK-cells, particularly mature NK-cells,[10] CD56 is not specific and can be expressed by neoplastic myeloid, lymphoid, plasmacytoid dendritic,[11-13] and plasma cells. Other than CD56, CD161 and CD44 could be used as markers for NK-[14-17] and T-cells,[18,19] respectively. but their sensitivity and specificity in distinguishing NK-LBLs from T-LBLs have yet to be tested. The lack of suitable markers for immature NK-cells thereby makes it difficult to identify NK-cell LBL.

For the NK-cell lineage to diverge from the T-cell lineage, interleukin (IL)-15 and transcription factor ID2 are essential. By contrast, IL-2 and transcription factor E2A are important for T-cell development.[20-26] ID2 and E2A are highly homologous, and both belong to the helix-loop-helix

family of transcription factors. ID2 is a dominant-negative inhibitor of E2A and promotes NK-cell development at the expense of T-cell development.[27,28] Likewise, IL-2 and IL-15 are highly homologous, and their receptors are heterotrimers that share the same β and γ chains, but differ in the α chains.[29] The IL-2 receptor α (IL-2Rα) is also known as CD25, whereas the IL-15 receptor α (IL-15Rα) does not yet have a cluster designation (CD). Accordingly, NK-cell precursors most likely express IL-15Rα and ID2, and T-cell precursors are positive for CD25 and E2A.

T-cell precursors undergo TCR gene rearrangement and become immature T-cells that are committed to the T-cell lineage.[30] Expansion and survival of immature T-cells depend upon another transcription factor, TCF-1,[31,32] that is also present in a subset of mature NK-cells.[33] For NK-cell precursors, their subsequent development and lineage commitment to immature NK-cells is unclear. It is known only that mature human NK-cells use both the C-type lectin-like CD94 and killer immunoglobulin-like receptors (KIRs) as antigen receptors.[34-36] Recently, it was found that CD94 has both a distal 1A promoter and a proximal 1B promoter.[37,38] These promoters generate transcripts with identical coding regions. In mature NK-cells, the 1B promoter is constitutively active and produces the predominant transcript, whereas the 1A promoter is activated only by IL-15. The finding of an IL-15-regulated CD94 1A promoter raises the possibility that CD94 1A might be the predominant form expressed in immature NK-cells.

In this study, we used laser capture microdissection to isolate IL-15Rα$^+$ lymphoid cells from the thymus, and we confirmed that these immature NK cells are indeed CD94 1A$^+$. We also studied a group of LBL cases that were positive for cytoplasmic CD3 and nuclear TdT and distinguished two immunophenotypic groups. One group that we propose is of immature NK-cell origin is characterized by a CD94 1A$^+$/CD56$^-$/ID2$^-$ immunophenotype and lacks TCR gene rearrangements. In

contrast, the second group had a CD94 $1A^{-}$ immunophenotype and carries TCR gene rearrangements supporting T-cell lineage. The identification of a subset of LBL cases of immature NK-cell origin has both clinical and immunologic implications.

Materials and Methods

Tissue samples

The study was approved by the Institutional Review Board and informed consent was obtained according to the Declaration of Helsinki. Twenty-one LBL samples were collected. Diagnoses were made by histologic and immunophenotypic examination of lymph nodes or mediastinal biopsy specimens in the Pathology Department of the National Taiwan University Hospital between 1993 and 2001. All diagnoses were established on pre-treatment biopsy specimens at the time the patients presented initially. Histologically, all specimens showed a characteristic diffuse infiltration of lymphoblasts positive for cytoplasmic CD3 and nuclear TdT and were negative for CD56 and CD20. All neoplasms were negative for Epstein-Barr virus (EBV) small-encoded small RNA (EBER), and thus they were not associated with EBV infection. Clinical data were obtained from the medical records.

For comparison, other specimens were examined including 10 cases of extranodal nasal-type NK/T-cell lymphoma as defined according to the criteria specified in the WHO classification; 6 samples of the thymus obtained from thymectomy specimens of 2 adults who had myasthenia gravis, and from autopsy specimens of 4 infants who died of prematurity; 3 spleens obtained removed for cirrhosis and gastric cancer; 2 samples of endometrium obtained from curettage specimens for dysfunctional uterine bleeding; 1 tonsil excised for chronic tonsillitis; and 4 peripheral blood specimens obtained from volunteer normal donors.

Also studied were 4 samples of umbilical cord blood obtained from healthy newborns according to a protocol approved by the institutional review committee. Umbilical-cord mononuclear cells

were isolated by density gradient centrifugation with use of Ficoll-Paque at a density of 1.077. $CD34^+$ progenitor cells were then purified by an immunomagnetic cell separation system using multisort microbeads conjugated to a monoclonal mouse anti-human CD34 antibody (QBENDD/10, Miltenyi Biotec, Auburn, CA). The beads were dissociated from the purified $CD34^+$ progenitors after enzymatic release. The $CD34^+$ progenitor cells were further incubated with a mixture of biotin-conjugated antibodies against lineage (Lin) markers, including CD2 (clone LT2), CD3 (clone UCHT-1), CD14 (clone UCHM-1), CD16 (clone LNK16), CD19 (clone LT19), CD33 (clone WM53), CD41 (clone PM6/248), CD56 (clone MEM188), and glycophorin (clone AC107.3). All antibodies were obtained from Serotec (Raleigh, NC), except for CD3 from BD Biosciences (San Jose, CA) and glycophorin from Miltenyi Biotec (Auburn, CA). The $CD34^+Lin^+$ progenitors and $CD34^+Lin^-$ progenitors were separated by streptavidin-conjugated microbeads. Flow cytometry was used to check the purity of the cells. For flow cytometry, the $CD34^+Lin^-$ progenitors or $CD34^+Lin^+$ progenitors were labeled with a PC5-conjugated CD34 antibody (clone 581, Coulter Immunotech, Marseille, France).

CD94 transcripts by RT-PCR

The sequences of the primers used for reverse transcription-polymerase chain reaction (RT-PCR) analyses are listed in Table 1. The table also provides the GenBank accession number of the CD94 cDNA, the positions of the primers, and the sizes of the PCR products. The "RS" in Table 1 is a random sequence, 5´-TGACAAACTGTGTTCACTAGC-3´, used for increased PCR specificity and incorporation of fluorescent labels.

Reverse transcription was performed in a 20-μl mixture at 40^oC for 50 minutes. The mixture

included 0.1 μg of RNA purified from formalin-fixed, paraffin-embedded tissue blocks, 0.5 μM antisense primer, 200 units of reverse transcriptase, 50 mM Tris-HCl at pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 μM of each dNTP. The cDNA from 2 μl of the reverse transcription mixture was amplified with the forward primer and a fluorescence-labeled primer of the random sequence 5´-tet-CTTCTGACAAACTGTGTTCACTAGC-3´ in a standard PCR reaction.

PCR was done in a 20 μl mixture including the DNA template, 0.3 μM of each primer, 15 mM Tris-HCl at pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM of each dNTP, and 1 unit of Taq polymerase. The reaction mixture was subjected to 35 cycles of PCR. Each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 45 seconds.

At the end of 35 cycles, a portion of the PCR products was separated by a high-resolution electrophoretic system (ABI377 with GeneScan software, Perkin-Elmer, Foster City, CA). The sizes of the RT-PCR products of CD94 are listed in Table 1. A reaction for beta-2-microglobulin (β2M) was run as a positive control, and a reaction under the same conditions except for the omission of reverse transcriptase was run as a negative control. The RT-PCR is deemed positive if its peak is higher than 10% of β_2M.

Killer immunoglobulin-like receptor repertoire analysis

This analysis was done according to a protocol developed in our laboratory.[39] Briefly, killer immunoglobulin-like receptors (KIRs) can be categorized in one of three splicing groups, KIR2DL4, KIR2D, or KIR3D. Three pairs of group-specific primers were designed and used in RT-PCR reactions to evaluate the presence or absence of the KIR transcripts. The primers were 5´-GCTGAGAGAGAAGGTTCTCATAT-3´ and 5´-CACTCCCCCACTGGGTGGTCGGC-3´ for KIR

2DL4, 5´-TGGGTGGGCCAGGAGGAAGGTTT-3´ and 5´-CATGGCGTGTGTTGGGTTCTTC TTG-3´ for KIR2D, and 5´-TGGGTGGGCCAGGAGGAAGGTTT-3´ and 5´-CACTCCCCCCACT GGGTGGTCGGC-3´ for KIR3D.

T-cell-receptor γ rearrangement

Standard PCR was performed to assess for T-cell receptor (TCR) γ chain gene rearrangements according to a protocol developed in our laboratory.[40] A monoclonal TCR rearrangement is considered to be present if a dominant clone, making up more than 30% of the total clones, is found.

CD161 transcripts by RT-PCR

RT-PCR for CD161 was performed using the primers listed in Table 1. The reaction conditions were identical to those for CD94. The RT-PCR result is considered to be positive if the amplified peak is greater than 20% of β_2M.

Immunohistochemistry

Immunohistochemical stains for CD44, E2A, ID2, and TCF-1 were performed on formalin-fixed, paraffin-embedded tissue sections of all 21 LBL cases. The antibodies used were CD44 (clone 2C5, R&D Systems, Minneapolis, MN), E2A (polyclonal, Santa Cruz Biotech, Santa Cruz, CA), ID2 (polyclonal, Santa Cruz Biotech, Santa Cruz, CA), and TCF-1 (7H3, Exalpha, Watertown, MA). The staining was done using conditions recommended by the vendor.

Lineage assignment of LBLs according to CD94 1A transcripts and TCR gene rearrangement

The 21 LBLs were divided into three groups, according tothe patterns of CD94 transcripts and TCR gene rearrangement. The T-cell lineage was characterized by the presence of monoclonal TCR gene rearrangement. The NK-cell lineage was characterized by the presence of CD94 1A transcripts without monoclonal TCR gene rearrangement. The uncommitted cases had neither a monoclonal TCR gene rearrangement nor CD94 1A transcripts.

Comparison between LBL of T- and the NK-cell lineage

The uncommitted group was excluded from this comparison because of the small sample size (n=3). Two-sample comparisons between the LBLs of T- and NK-cell lineage were performed with the binomial test for categorical data and the non-parametric Mann-Whitney U-test for continuous data.

Correlation with 2-year survival

The 21 LBLs were divided into two groups according to the 2-year survival. Two-sample comparisons were done with the binomial test for categorical data and with the non-parametric Mann-Whitney U-test for continuous data.

Isolation of IL-15Rα^+ immature NK cells from thymus by reverse laser capture microdissection

IL-15Rα^+ lymphoid cells, stained by a polyclonal goat antibody (R&D Systems, Minneapolis, MN), were isolated from 5 μm frozen sections of the thymus of infants who died of prematurity. In conventional laser capture microdissection (LCM), the laser hits the target cells directly, thereby causing disruption of the cells, loss of their histopathology, and damage to the nucleic acids. To

circumvent these difficulties, we used a reverse LCM (rLCM) that removed IL-15Rα^- cells from the slides. This approach preserved the original histopathology of the intact IL-15Rα^+ cells and allowed better recovery of RNA.

Results

Immature NK-cells from thymus are predominantly CD94 1A$^+$, but mature NK-cells from peripheral tissues or extranodal nasal-type NK/T-cell lymphomas are predominantly CD94 1B$^+$

We evaluated the distribution patterns of CD94 transcripts in benign and neoplastic tissues known to have either mature or immature NK-cells, such as umbilical cord blood, peripheral blood, tonsil, spleen, endometrium, thymus, and extranodal nasal-type NK/T-cell lymphomas (Table 2).

Umbilical cord progenitor cells, either $CD34^+Lin^+$ or $CD34^+Lin^-$, had neither CD94 1A nor CD94 1B transcripts. In 10 samples from peripheral blood, tonsil, spleen, and endometrium, 9 had predominantly CD94 1B transcripts, including 2 samples with only CD94 1B transcripts and 7 samples in which CD94 1B transcripts were much more numerous than CD94 1A. Only 1 of these samples, a spleen from a cirrhotic patient with hypersplenism and pancytopenia, showed only CD94 1A transcripts. In 10 extranodal nasal-type NK/T-cell lymphomas assessed, CD94 transcripts were identified in 7 neoplasms, and in 6 tumors CD94 1B transcripts were more numerous than CD94 1A transcripts. By contrast, all 6 samples of thymus had predominantly CD94 1A transcripts, including 5 with only CD94 1A transcripts and one in which CD94 1A transcripts were more numerous than CD94 1B transcripts. Representative patterns of CD94 transcripts were shown in Figure 1.

These data suggest that immature NK-cells use predominantly CD94 1A transcripts, whereas mature NK-cells use predominantly CD94 1B transcripts. This pattern also indicates that CD94 1A transcripts can be used to identify LBLs of the NK lineage.

Lineage assignment of LBLs according to antigen receptor patterns: transcripts of CD94 1A,

but not KIRs identify an NK-LBL

We used the presence of TCR gene rearrangements or expression of CD94 1A transcripts as criteria for T-cell or NK-cell lineage commitment, respectively (Table 3). According to this scheme, 11 LBLs carried monoclonal TCR gene rearrangements and were classified as T-LBL, and 7 LBLs had CD94 1A transcripts and were classified as NK-LBL. The remaining 3 cases had neither TCR gene rearrangement nor CD94 1A transcripts and were considered to be of uncommitted lineage. The T-LBLs were negative for CD94 1A and frequently expressed cytoplasmic ID2, whereas the NK-LBLs were positive for CD94 1A and negative for ID2.

The immunohistochemical patterns of ID2 and E2A are shown in Figure 2. E2A exhibited nuclear staining in both normal thymus and T-LBL (A & B). For ID2, there is nuclear staining in the normal thymus, but cytoplasmic staining in T-LBL (C and D).

As shown in Table 4, except for CD161 which had borderline significance at p=0.05, the presence or absence of CD44, E2A, TCF-1, and KIRs was not lineage-specific and did not correlate with the lineage assignment based on the presence of CD94 1A transcripts. In particular, the differences in the distributions of KIR2DL4, KIR2D, and KIR3D between the T- and NK-cell lineages, useful in lineage assignment in mature NK-cell lymphomas, were not significant statistically.

NK-LBL had a better 2-year survival rate than did T-LBL

Patients with NK-LBLs (CD 94 1A positive) had a better prognosis than did those with T-LBLs (2-year survival, 100% *vs* 27%, $p< 0.01$, Table 4). NK-LBL patients also tended to be younger (15 yr *vs* 33 yr, p=0.11, Table 4), but, because of the small sample size, a firm conclusion cannot be drawn regarding the age difference. Lineage assignment based on antigen receptors, using the pres-

ence of CD94 1A or TCR gene rearrangement, therefore, had both biological and clinical significance.

As a confirmatory test (Table 5), we divided the LBL patients into two groups, depending on whether or not the patients survived beyond 2 years. Among various clinical and biological parameters tested, we found that 2-year survival correlated most strongly with presence of CD94 1A transcripts and absence of TCR gene rearrangement (p=0.01).

CD94 1A transcripts could be detected in immature IL-15Rα^+ lymphoid cells from thymus

The existence of a bipotent T/NK precursor in the thymus is well known, but has not been well characterized histologically. Immunohistochemistry on the thymus from an infant of 29-week gestational age who died of prematurity showed either focal aggregates of IL-15Rα^+ lymphoid cells (Figure 3, A&B) or scattered interstitial staining (Figure 3, C&D). We isolated these cells by rLCM, and found that these cells had a CD94 1A transcript pattern similar to that shown in Fig 1A.

Discussion

In this study, we have attempted to improve the classification of LBLs by recognizing a subset of neoplasms that are of immature NK-cell lineage. We started with 21 LBLs, all of them positive for cytoplasmic CD3 and nuclear TdT, and negative for CD56. Because CD3 is part of the TCR complex and TdT is the enzyme that creates junctional diversity during TCR gene rearrangement, it is not surprising that immature T- or T/NK-cell precursors express cytoplasmic CD3 and TdT. It should be noted, however, that neither cytoplasmic CD3 nor TdT implies commitment to T-cell lineage. For example, immature or fetal NK-cells also express cytoplasmic CD3,[6] and TdT can be detected in the earliest $Lin^{-}/Sca\text{-}1^{+}/ILR7^{-}/C\text{-}Kit^{hi}/TdT^{+}$ common lymphoid precursors.[7] Therefore, the cytoplasmic CD3 and nuclear TdT immunophenotype is not lineage specific.

With the use of CD94 1A expression and TCR gene rearrangement, our data support a reassessment of lineage assignment of LBLs, based on recent advances in the developmental biology of T- and NK-cells, both of which are derived from a common T/NK-cell thymic precursor. For the T-cell lineage,[30] development proceeds from a bipotent T/NK-cell precursor through T-cell precursors double negative (DN) stages 1 through 4, to CD4/CD8 double-positive immature T-cells, and finally to CD4 or CD8 single positive mature T-cells. The process of lineage commitment takes place in $CD44^{+}/CD25^{+}$ T-cell precursors at stages DN2/DN3, characterized by TCR β gene rearrangement that is promoted by the transcription factor E2A. For the NK-cell lineage, development depends on IL-15[20,21] and the transcription factor ID2.[22,23] One of the earliest surface markers associated with the NK-cell lineage is CD161, but the intermediate stages preceding mature NK-cells are still unknown. It is known only that mature NK-cells use the CD94/NKG2 family and KIRs in humans as antigen receptors, but details on the regulation and acquisition of these receptors are lim-

ited.

In accordance with these data, we present a model for NK-cell development that can be integrated into the classification of LBLs (Fig 4). In this three-stage model, the bipotent T/NK-cell precursor develops into a uni-potent precursor, then to immature stages, and finally to mature cells. CD94 1A transcripts and TCR rearrangements determine lineage commitment and have clinical significance. The presence of CD94 1A without monoclonal TCR gene rearrangement identifies NK-LBL, most of which are negative for cytoplasmic ID2 .

The absence of ID2 in NK-LBLs and the presence of cytoplasmic ID2 in T-LBLs is of interest. One might expect expression of nuclear ID2 to be associated with NK-LBL, and E2A to be associated with the T-LBL, because these molecules are required for NK- and T-cell development, respectively.[27,28] However, quite unexpectedly, we found that cytoplasmic ID2 was associated with T-LBL, and E2A was not lineage-specific. How the loss of ID2 leads to NK-LBL is unknown, but it is known that redistribution of ID2 from the nucleus to the cytoplasm is associated with differentiation of murine hematopoietic cells,[41] and that loss of E2A can cause T-lymphoblastic leukemia.[42-44] Thus, the biologic significance of cytoplasmic ID2 is worthy of further investigation.

As summarized in Table 6, markers associated with precursors, such as CD161 and CD44, or those associated with mature cells, such as TCF-1 and KIRs, are not useful clinically. The lack of lineage specificity for CD161 is not totally unexpected, because CD161 is present in subsets of T-cells, including those residing in the intestines.[45] Markers associated with mature T- or NK-cells, such as TCF-1 and KIRs,[31-33,36] are not useful for delineating T- or NK-LBLs that are derived from immature lymphoblasts. Because KIRs are related to T-cell receptors, and both belong to the immunoglobulin super-family, one might suppose that the two evolutionarily conserved receptor fami-

lies could be used as criteria for lineage commitment in two developmentally related cellular lineages. We found, however, that KIRs are not useful in lineage assignment for LBLs (Table 4). As KIRs are found only in human NK-cells, lineage commitment probably does not depend on KIRs. This finding is also consistent with a previous report that CD94 expression precedes KIRs, and that lineage commitment is independent of KIRs.[46]

LBL expressing NK cell-associated antigens was first recognized in 1987.[47] By screening 38 cases of LBL, the authors found 6 cases expressing the NK-associated antigens CD16 (Leu11b) and CD57 (Leu7). As a group, these cases of so-called "NK-LBL" were TdT^+, CD2 $(T11)^+$, CD4 $(Leu3A)^+$, $CD16^+$, and $CD57^+$, and they had a worse prognosis than that of the T-LBL. However, the true nature of these neoplasms was never further addressed at the molecular level. Subsequently, CD56 became a popular marker of NK-cells even though CD56 is not specific and can be expressed by many other neoplasms. This has led to confusion in the literature regarding neoplasms of NK-cell lineage. For example, the entity described in the WHO classification as blastic NK-cell lymphoma[48] is, in fact, not a neoplasm of NK-cells and most likely is derived from type II plasmacytoid dendritic cells.[13] For these reasons, we did not consider the absence of CD56 expression in LBLs as evidence excluding NK-cell lineage, prompting us to search for other markers useful for distinguishing immature NK- from T-cells.

In conclusion, we propose that LBLs can be divided into those of NK- and T-cell lineage using CD94 1A and TCR gene rearrangement. Among 21 cases of LBL that were positive for cytoplasmic CD3 and nuclear TdT and negative for CD56, seven cases expressed CD94 1A transcripts and did not carry monoclonal TCR gene rearrangements supporting immature NK-cell lineage. These NK-LBLs occurred in younger patients who had a better prognosis than patients with T-LBL

after appropriate chemotherapy. The use of CD94 1A, instead of CD56, as a marker of NK-cell lineage reflects recent advances in NK-cell biology and emphasizes the importance of complete lineage commitment in the biology of NK-cells. Based on the identification of a true NK-LBL, we present a revised model of NK-cell development. This model not only stresses the importance of lineage commitment for clinical prognosis, but also implies further constraints on the ultimate model of T/NK-cell development.

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Figure legends

Figure 1. High-resolution electrophoretic tracings of mature and immature CD94 patterns. From the top to the bottom panels, two distinct patterns of CD94 transcripts are shown. The immature CD94 1A pattern, either CD94 1A alone or CD94 1A >> CD94 1B, was found in the thymus or LBLs. The mature CD94 1B pattern, either CD94 1B alone or CD94 1A <<1B, was found in peripheral tissues or extranodal nasal-type NK/T-cell lymphomas. (X-axis: in base pairs; Y-axis: intensity of the fluorescence.)

Figure 2. Immunohistochemistry for E2A and ID2 from LBLs and thymus of premature infants. Note the nuclear E2A stain in both the thymus (A) and a T-LBL (B). For ID2, the staining is nuclear in the normal thymus (C), but cytoplasmic in a case of T-LBL (D). (A-D, 400X, immunohistochemistry using formalin-fixed, paraffin-embedded tissue sections and hematoxylin counterstain.)

Figure 3. IL-15Rα^+ immature lymphoid cells isolated from thymus of a premature infant by reverse laser capture microdissection. The figure shows a nodular pattern of IL-15Rα^+ lymphoid cells in the thymus before and after reverse laser capture microdissection (rLCM) (A & B, with hematoxylin counterstain), and an interstitial pattern of IL-15Rα^+ lymphoid cells in the thymus before and after rLCM (C & D, phase-contrast view without hematoxylin counterstain). Note the well-preserved histopathology of IL-15Rα^+ lymphoid cells isolated from a 5 μm frozen section of thymus with use of rLCM.

Figure 4. A three-stage model of NK development. The cytoplasmic $CD3^+$ nuclear TdT^+ bipotent

T/NK-cell precursor may develop into either immature T-cells under the actions of E2A and IL-2, or immature NK-cells under the actions of ID2 and IL-15. Lineage commitment is fully established when immature T-cells complete TCR gene rearrangement, or, as our data support, immature NK-cells express CD94 1A transcripts. The model shows where the cytokines and transcription factors are critical, but does not exclude their possible roles at other developmental stages. For example, the assignment of ID2 before IL-15 is based on previous data[23] and a recent review.[49] According to this model, the loss of ID2 in NK-LBLs is due to abnormal differentiation. Whether the expression of IL-15Rα or, equivalently, the role of IL-15, might precede that of ID2 in the development of normal NK cells requires further investigation.

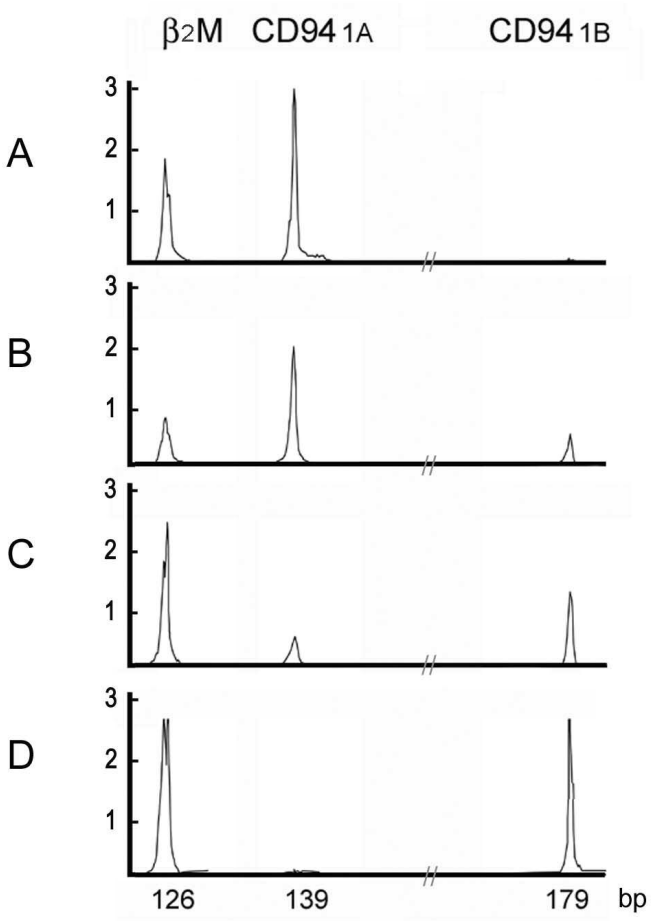

Figure 1

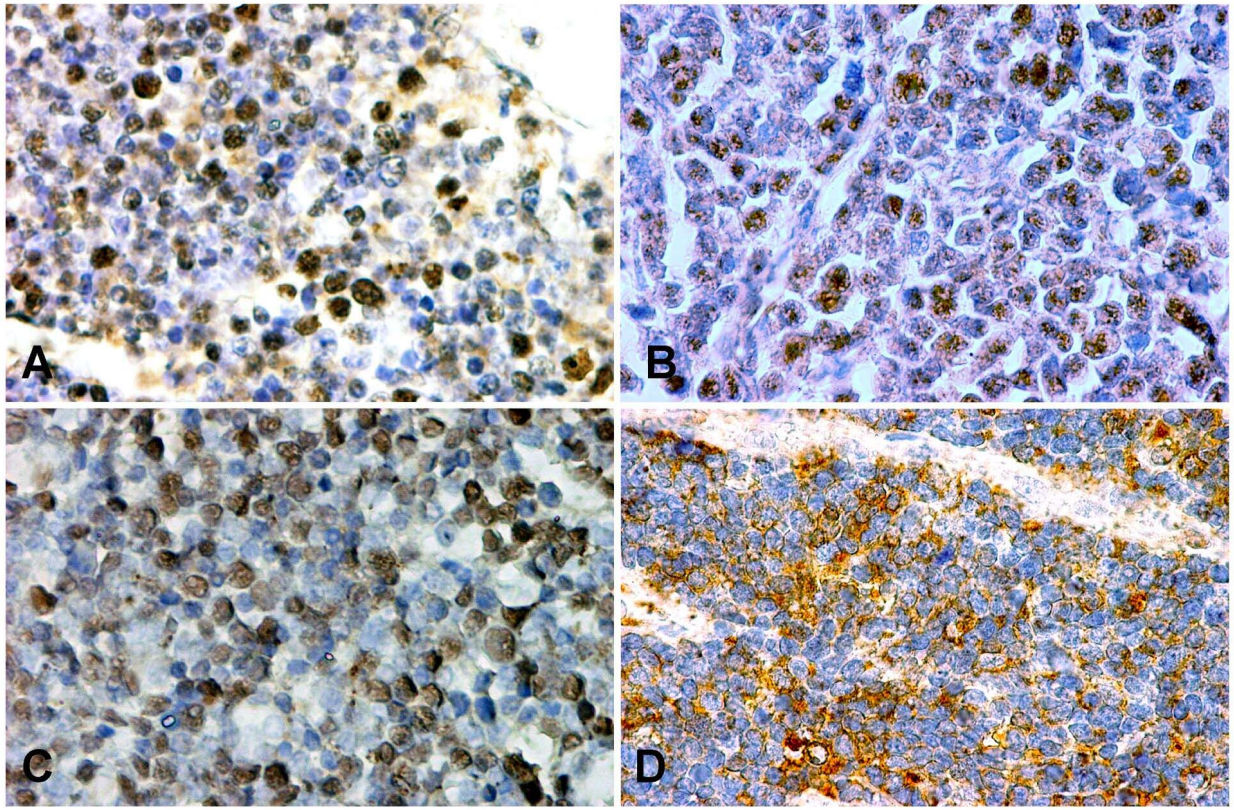

Figure 2

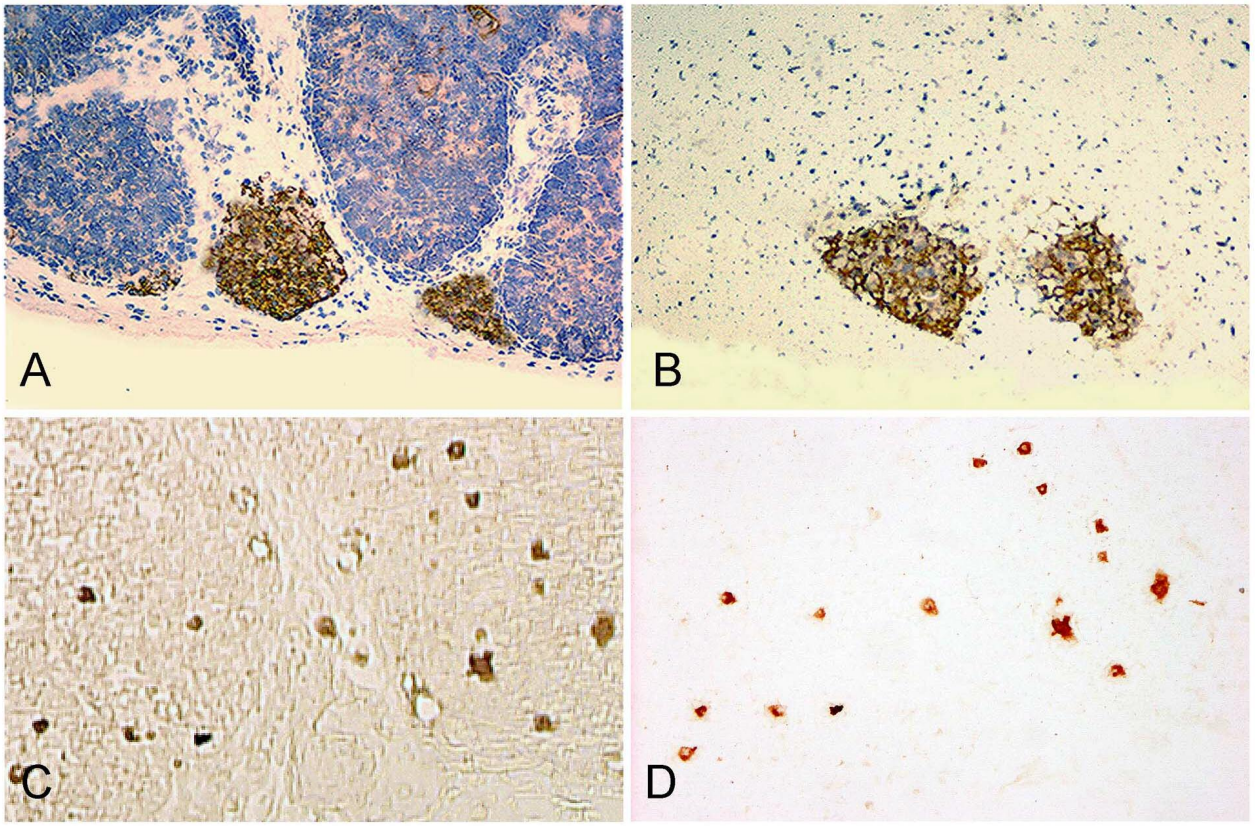

Figure 3

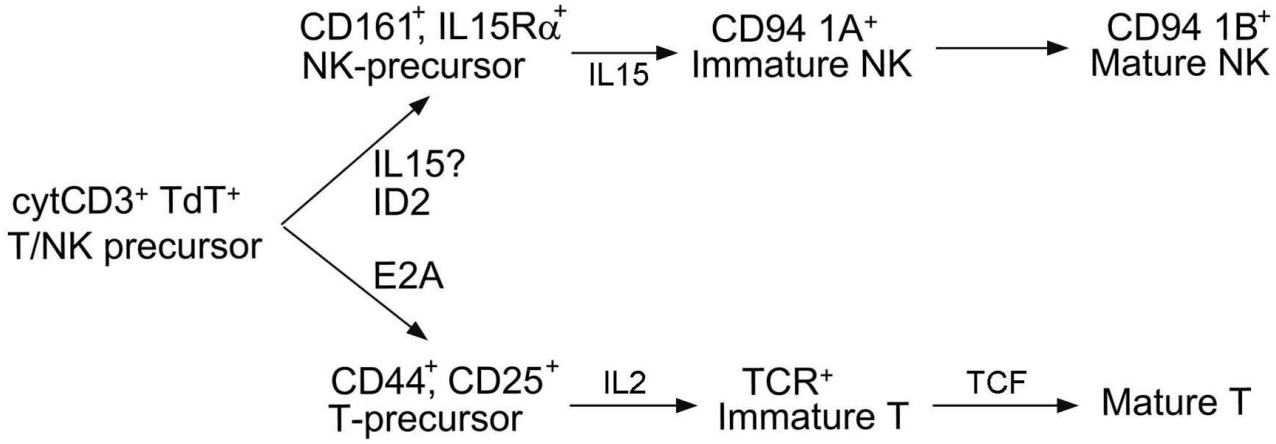
CD161+, IL15Rα+
NK-precursor
IL15
CD94 1A+
Immature NK
CD94 1B+
Mature NK
cytCD3+ TdT+
T/NK precursor
IL15?
ID2
E2A
CD44+, CD25+
T-precursor
IL2
TCR+
Immature T
TCF
Mature T

Figure 4

Table 1. Primers for CD94 and CD161

GenBank	Forward	5′-3′	Exon	Reverse	5′-3′	Exon	Size*
CD94 1A AY227806	5′-AGC-TCA-TCA-AGT-TCA-AGA-GAC-3′	40-60	1A	5′-RS TTT-TCA-GAA-AGA-ACA-GCG-TTG-3′	150-130	1	136
CD94 1B NM002262	5′-ATT-TCT-TCA-TAC-TCA-ACT-TTC-3′	138-158	1B	5′-RS ACC-TCC-ACA-GAG-TGG-TCT-TAA-3′	291-271	2	179
CD161 NM002258	5′-CTT-AGC-TGT-GCT-GGG-ATT-ATT-CTC-3′	187-210	2	5′-RS-CTG-CAT-TTT-TCT-ATT-GAT-GAT-TT-3′	284-262	3	123
β_2M NM004048	5′-CTT-TGT-CAC-AGC-CCA-AGA-TAG-3′	377-397	2	5′-RS-CAG-AAT-TTG-GAA-TTC-ATC-CAA-3′	477-457	4	126

RS=5’-TGA-CAA-ACT-GTG-TTC-ACT-AGC-3’

*Size: Size of the RT-PCR product in base pairs

Table 2. Tissue distribution of CD94 transcripts

CD34 progenitors and thymus					
	Age	**Sex**	**Tissue**	**Diagnosis**	**CD94**
1	0 d	M	$CD34^{+}Lin^{+}$ UC progenitors	normal newborn	neg
2	0 d	M	$CD34^{+}Lin^{-}$ UC progenitors	same case above	neg
3	0 d	F	$CD34^{+}Lin^{+}$ UC progenitors	normal newborn	neg
4	0 d	F	$CD34^{+}Lin^{-}$ UC progenitors	same case above	neg
5	1 d	F	thymus	premature infant (GA: 29 weeks)	1A
6	1 d	F	thymus	premature infant (GA: 37 weeks)	1A
7	1 d	M	thymus	premature infant (GA: 27 weeks)	1A
8	1 d	M	thymus	premature infant (GA: 27 weeks)	1A
9	40	F	thymus	MG with LH	1A
10	17	F	thymus	MG with LH	1A>>1B

Peripheral tissues					
	Age	**Sex**	**Tissue**	**Diagnosis**	**CD94**
1	44	M	spleen	cirrhosis with hypersplenism & pancytopenia	1A
2	32	F	endo-metrium	reactive endometrium	1B
3	75	M	spleen	gastric cancer without splenic involvement	1B
4	45	F	endo-metrium	reactive endometrium	1B>>1A
5	74	M	spleen	cirrhosis with splenomegaly	1B>>1A
6	65	M	tonsil	chronic tonsillitis	1B>>1A
7	43	M	PB	normal volunteer	1B>>1A
8	24	M	PB	normal volunteer	1B>>1A
9	28	F	PB	normal volunteer	1B>>1A
10	27	F	PB	normal volunteer	1B>>1A

ENNLs					
	Age	**Sex**	**Tissue**	**Diagnosis**	**CD94**
1	60	M	nasal cavity	ENNL	neg
2	65	M	nasal cavity	ENNL	neg
3	45	F	nasal cavity	ENNL	neg
4	61	M	nasal cavity	ENNL	1A
5	42	M	nasal cavity	ENNL	1B>>1A
6	62	M	nasal cavity	ENNL	1B>>1A
7	55	M	nasal cavity	ENNL	1B>>1A
8	39	F	nasal cavity	ENNL	1B>>1A
9	69	M	nasal cavity	ENNL	1B>>1A
10	38	M	nasal cavity	ENNL	1B>>1A

Abbreviations: d: day; ENNL: extranodal nasal-type NK/T-cell lymphoma; F: female; GA: gestational age; Lin: lineage; M: male; MG with LH: myasthenia gravis with lymphoid hyperplasia; neg: negative; PB: peripheral blood; UC: umbilical cord. Age in years unless otherwise specified.

Table 3. Pathologic and clinical data for lymphoblastic lymphomas

	Sex	Age	CD161	CD44	E2A	ID2	TCF	TCR	1A	1B	KIR 2DL4	KIR 2D	KIR 3D	LDH	blast	WBC	HB	PLT	Med mass	stage	ALL-like therapy*	Sur
Uncommitted																						
1	F	52	–	+	–	c+	–	–	–	–	–	–	–	1298	70	22.1	10.4	32	–	IV	n	30
2	F	53	–	–	+	–	–	–	–	–	–	–	–	NA	0	7.2	15	194	+	II	n	120^{+}
3	F	29	+	–	+	c+	–	–	–	–	–	–	–	419	30	10.7	12.1	434	–	IV	n	14
T lineage																						
4	F	7	–	+	ND	–	–	+	–	–	–	–	–	494	0	7.1	12.4	255	+	III	y	12
5	M	23	+	–	+	c+	–	+	–	–	–	–	–	586	0	1.6	10.9	103	–	IV	n	21
6	F	69	–	–	–	c+	–	+	–	–	–	–	–	373	0	7.6	3.5	52	–	III	n	1
7	M	33	–	–	+	c+	+	+	–	–	–	–	–	2530	60	19.9	8.6	276	–	IV	n	18
8	M	43	–	–	–	–	ND	+	–	–	–	–	–	1970	57	4.7	3.5	44	+	IV	n	7
9	M	18	–	–	+	c+	+	+	–	–	–	–	–	2340	0	5.6	11	190	+	III	n	1
10	M	29	–	–	–	–	+	+	–	–	–	–	+	577	0	8.7	13.9	280	+	III	n	14
11	M	28	+	+	+	–	+	+	–	–	–	–	+	289	6	7.4	16.1	166	+	IV	n	33
12	M	9	+	+	–	c+	+	+	–	–	–	–	+	540	NA	2.8	8.2	125	–	IV	y	45
13	M	39	–	+	ND	ND	ND	+	–	–	–	+	+	908	0	5.6	14.1	313	–	IV	n	7
14	M	15	+	–	+	c+	–	+	–	–	–	–	+	210	0	7.6	14.3	334	–	III	y	102^{+}
NK lineage																						
15	M	3	–	–	+	–	+	–	+	+	–	–	+	1900	0	7.5	3.6	10	+	III	y	34^{+}
16	F	19	+	+	–	–	+	–	+	–	+	–	+	247	12	9.1	13.3	129	–	IV	n	78^{+}
17	F	12	+	–	–	–	+	–	+	–	–	–	+	NA	0	7.2	13.5	271	+	III	y	105^{+}
18	M	27	+	–	–	–	ND	–	+	–	–	+	–	1407	0	6.1	13.3	232	+	II	n	108^{+}
19	M	12	+	+	–	–	–	–	+	–	–	–	–	372	0	5.2	13	238	–	IV	y	108^{+}
20	M	15	+	+	–	–	+	–	+	–	–	–	–	2353	23	15.3	12.2	92	–	IV	y	50^{+}
21	F	28	+	+	+	–	+	–	+	+	–	–	–	318	3	6.6	10.6	266	–	IV	n	45^{+}

Abbreviations: 1A: CD94 1A; 1B: CD94 1B; age in years; blast: percentage of blasts in peripheral blood; c: cytoplasmic; F: female; HB: hemoglobin in g/dl; KIR: killer immunoglobulin-like receptor; LDH: lactate dehydrogenase, IU/L; M: male; Med: mediastinal; n: no; NA: not available; ND: not determined; PLT: platelets, $10^3/\mu l$; Sur: survival in months, where a + sign indicates a censored case; TCF: T-cell factor; TCR: T-cell receptor; WBC: white blood cells, $10^3/\mu l$; y: yes. * The patients were treated with either ALL-like or CHOP-based regimens.

Table 4. Lineage assignment according to CD94 1A and TCR rearrangement

	Uncommitted	T	NK	*p* value
Number of cases	3	11	7	
Median age	52	33	15	0.11
Sex (male: female)	0:3	9:2	4:3	0.28
CD161	1/3	4/11	6/7	0.05
CD44	1/3	4/11	4/7	0.39
E2A	2/3	5/9	2/7	0.27
c-ID2	2/3	6/10	0/7	0.02
TCF-1	0/3	5/9	5/6	0.28
KIR 2DL4	0/3	0/11	1/7	0.21
KIR 2D	0/3	1/11	1/7	0.73
KIR 3D	0/3	5/11	3/7	0.91
LDH	859	586	890	0.49
Blasts in blood	2/3	3/10	3/7	0.59
WBC	10.7	7.1	7.2	0.49
HB	12.1	11.0	13.0	0.89
PLT	194	190	232	0.55
Mediastinal mass	1/3	5/11	3/7	0.91
Stage IV	2/3	6/11	4/7	0.91
ALL-like regimens*	0/3	3/11	4/7	0.22
2-year survival	2/3	3/11	7/7	0.008

Abbreviations: age in years; c-ID2: cytoplasmic ID2; HB: hemoglobin in g/dl; KIR: killer immunoglobulin-like receptor; LDH: lactate dehydrogenase IU/L; PLT: platelets 10^3/µl; TCF-1: T-cell factor; WBC: white blood cells, 10^3/µl.
* The patients were treated with either ALL-like or CHOP-based regimens.

Table 5. 2-year survival correlates with CD94 1A & TCR rearrangement

	Died before 2 years	Alive after 2 years	*p* value
Number of cases	9	12	
Median age	29	17	0.11
Sex (male: female)	6:3	7:5	0.70
CD161	2/9	9/12	0.02
CD44	2/9	7/12	0.11
E2A	4/7	5/12	0.52
c-ID2	5/8	3/12	0.11
TCF-1	3/7	7/11	0.39
CD94 1A	0/9	7/12	0.01
CD94 1B	0/9	2/12	0.21
KIR 2DL4	0/9	1/12	0.38
KIR 2D	1/9	1/12	0.81
KIR 3D	2/9	6/12	0.20
T-cell receptor	8/9	3/12	0.01
LDH	586	456	0.19
Blasts in blood	3/9	5/11	0.59
WBC	7.1	7.3	0.64
HB	11	13.2	0.22
PLT	255	194	0.35
Mediastinal mass	4/9	5/12	0.90
Stage IV	5/9	7/12	0.89
All-like regimens	1/9	6/12	0.07

See Table 4 for abbreviations.

Table 6. Summary of candidate lineage markers

Marker	Earliest Detectable	Role in T/NK development	Role in mature T/NK lymphoma	Role in lineage or prognosis of NK-LBL vs. T-LBL
CD161	Before commitment	Present early during the development of NK-cell precursors; its expression persists into mature NK cells.[16] Also present in subsets of T cells, e.g., in IFN-γ-producing intestinal T cells[45]	Not available	Less useful than CD94 1A
CD44	Before commitment	One of the earliest markers on T-cell precursors at the DN1 stage of development[18]	Often lost in ENNLs, but present in peripheral T-cell lymphoma[40]	Not useful
ID2 & E2A	Before commitment	Transcription factors of the helix-loop-helix family; E2A induces the rearrangement of TCR; ID2 is a dominant-negative inhibitor of E2A and is essential for NK development.[22-28]	Loss of E2A leads to T-cell leukemia[42-44]	ID2 lost in NK-LBL
KIRs	After commitment	3 alternative splicing groups, KIR_{2DL4}, KIR_{2D}, and $KIR_{3D,}$ found on human NK cells only.[36] Expression of KIRs is preceded by CD94.[40]	A restricted KIR repertoire found in ENNLs[46]	Not useful
TCF-1	After commitment	A transcription factor with functions in the maintenance, survival, and expansion of T cells[31-33] A different isoform found in mature human NK cells, also known to induce Ly49 NK-cell antigen receptors in the mouse[50]	Loss of TCF-1 in a subset of peripheral T-cell lymphomas[51]	Not useful