In search of a master regulator of cytotoxicity: Transcriptional control of cytolytic effector function in human lymphocytes

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TRANSCRIPTION FACTOR EXPRESSION IN LARGE GRANULAR LYMPHOMA

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Abstract

Large granular lymphoma (LGL) is a clonal disease that can originate from cells of either the NK cell or the ab or γδ lineages of T cells. All LGL subtypes have large granules containing cytolytic effector molecules such as Granzyme B. The transcription factors (TFs) T-bet, Eomes and Blimp-1 govern molecular and cellular differentiation processes of cytolytic lymphocytes. We here investigated whether the cytolytic phenotype of LGL is related to the expression of these key regulatory factors. By using quantitative PCR we investigated TF expression in a cohort of patients with ab T cell, γδ T cell or NK LGL and made a comparison to different CD8⁺ T cell subsets of healthy controls. Irrespective of their origin, all LGLs showed high expression of T-bet, Eomes and Blimp-1 mRNA that exceeded the expression levels of these TFs in normal cytolytic CD8⁺ T cells. Furthermore, a recently identified homologue of Blimp-1, named Hobit, was also strongly expressed in the lymphoma cells. Interestingly, a linear relationship between GzmB expression and that of T-bet, Eomes and Blimp-1 was found. Since the transcriptional regulators can alter expression of various factors, we assessed whether a correlation existed between these TF and (pro-)apoptotic genes. In LGL, Bim and Noxa expression was diminished, while Bid expression was elevated compared to the transcription levels detected in peripheral NK cells of healthy donors. Thus, we show that transcripts of TF T-bet, Eomes, Blimp-1 and Hobit are abundant in LGL cells and correlate with their functional traits.
Introduction

Large granular lymphoma (LGL) is a rare heterogeneous disorder that is characterized by a strong and persistent expansion of mature malignant lymphocytes. LGL disorders can be classified into CD3^+ T cell LGL or NK-LGL, based on the clonal expansion of these cell types1. T-LGL which is the most frequently occurring form can be derived from cells of either the αβ (being either CD4^+ or CD8^+ T cells) or γδ lineage of T cells. Both the T and NK cell malignancies can clinically present as indolent or aggressive disease, and the NK cell marker CD56 is typically detected in aggressive LGL leukemia2-4. Usually elderly people are affected and the median age at diagnosis is 60 years5. Approximately 60% of the patients are symptomatic with recurrent bacterial infections secondary to neutropenia as the most prevalent manifestation6,7. T-LGL leukemia is strongly associated with several autoimmune diseases, especially rheumatoid arthritis and auto-immune cytopenias8,9. LGL cells show the characteristics of mature NK cells or primed cytotoxic T cells. They are large in size with a cytoplasm that contains coarse azurophilic granules, in which effector molecules such as Granzyme B (GzmB), Perforin and Interferon gamma (IFNγ) are found10-12. CD57, an adhesion molecule specific for primed CTLs, is expressed on the cell surface of T-LGL leukemia cells. Furthermore, the finding that monoclonal TCRab+CD4^+ T-LGL cells have a restricted Vβ repertoire suggested that LGL cells could be derived from specific T cell clones that arise after antigenic stimulation13. Indeed, Crompton et al. showed that expanded Vb13.1 CD4^+ T-LGL cells were directed against the HLA-DR7-restricted HCMV epitope, providing compelling evidence that HCMV can act as an antigenic stimulus for the initiation or maintenance of the LGL expansion14,15.

In CD8^+ T cells, lineage commitment and effector function such as cytokine production are tightly regulated by transcription factors to ensure that the formation of malignancy or auto-reactive T cells is prevented. This process is regulated by a set of key transcription factors, amongst which the T-box family members T-bet and Eomes (reviewed in 16,17). Compound deletion of the *Tbx21* (encoding T-bet) and *Eomesodermin* genes leads to a loss of virtually all memory CD8^+ T cells, NK cells and NKT cells18,19. T-bet and Eomes can directly activate IFNγ transcription by binding to its promoter20-23. Furthermore, ChIP analysis has shown that Eomes can directly bind to the GzmB promoter and that ectopic expression of Eomes induces GzmB in polarized TH2 cells, a cell type that is normally devoid of cytolytic activity20,21.

The Zinc-finger containing transcription factor Blimp-124 is also involved in the regulation of effector formation in T cells. Blimp-1 deficient mice display a severe defect in the generation of short-lived effector cells (SLECs) and instead predominantly form memory pre-cursor T cells25-28. Ectopic expression of Blimp-1 in activated T cells enhances GzmB expression29 and Blimp-1 deficiency in mice leads to reduced GzmB production but enhanced IFNγ production26. Thus T-bet, Eomes and Blimp-1 are all regarded as crucial regulators of effector T cell differentiation. Moreover, they directly or indirectly regulate the cytolytic activity of these cells by influencing GzmB and IFNγ expression. Recently, a Homologue Of Blimp-1 In T cells (Hobit) was discovered, which has restricted expression in cytolytic cells such as NK cells, NKT cells and CD8^+ T cells. Hobit was abundantly expressed specifically in
CD8+ T cells directed against human CMV epitopes as well as in effector phenotype CD8+ T cells. We have shown that ectopic expression of Hobit induced IFNγ in a human CD4+ T cell line, while blockage of Hobit expression in the NK cell line NK92 led to down regulation of IFNγ expression 10.

In this study we show that T-bet, Eomes, Blimp-1 and Hobit are all highly expressed in LGL leukemia and that expression of all of these TFs with the exception of Hobit correlated with GzmB expression. In addition, we analyzed pro- and anti-apoptotic factors in LGL and identified Bim and Noxa as pro-apoptotic molecules that were significantly lower in LGL cells compared to NK cells of healthy donors. Thus, we have identified TFs involved in effector T cell differentiation as possible new targets that may contribute to deregulation of differentiation and apoptosis of transformed NK cells or T cells in LGL disease.

Results

Clinical and laboratory features of LGL samples

To analyze transcription factor expression in various LGL subtypes, we selected a cohort of patients with typical αβ CD4+ or CD8+ T cell, γδ T cell or NK cell lymphoproliferation (Table 1). The patients enrolled in this study had a median age of 68 years (range 11–84 years) with no male or female predominance, which is in line with normal LGL epidemiology. Four transcription factors, T-bet, Eomes, Blimp-1 and Hobit, were analyzed that have been implicated to function as transcriptional regulators of cytotoxic T cells and NK cells (Fig. 1A). The LGL samples were compared to naïve, effector phenotype 31 and memory phenotype CD8+ T cells. As previously shown, effector CD8+ T cells and to a lesser extent memory CD8+ T cells displayed elevated expression levels of these transcription factors 32 and contained effector molecules such as IFNγ and GzmB 33,34 (Fig. 1A and B).

A striking and consistent observation was that when compared to naive CD8+ T cell samples, all LGL subtypes contained a high quantity of T-bet, Eomes, Blimp-1 and Hobit mRNA transcripts (Fig. 1A). LGL cells showed variety in TF expression levels between the different subtypes (Fig. 1A). In γδ and NK-LGL cells, the relative abundance of T-bet, Eomes and Blimp-1 significantly exceeded the levels found in the cytotoxic T-cell LGL subset. The relatively high abundance of Blimp-1 was unexpected since this protein may act as a tumor suppressor in lymphoid malignancies, and is mutationally inactivated in various lymphoma types 35. Hobit expression levels were elevated in all LGL samples, although a clear discrimination between the subtypes as seen for the other TF was not observed (Fig. 1A). In conclusion, the data from figure 1 show that LGL cells possess the transcription factors that are involved in the generation of cytoltyc molecules.

To test a possible correlation between TFs and effector molecules, GzmB and IFNγ expression was measured by quantitative PCR (Fig. 1B). All samples contained substantially higher amounts of these molecules than naïve CD8+ T cells, consistent with the higher levels of the IFNγ and/or GzmB inducing TFs T-bet, Eomes, Blimp-1 and Hobit. γδ-LGL samples had higher amounts of IFNγ mRNA compared to the other LGL samples. Both γδ-LGL and NK-LGL samples had higher amounts of GzmB than CD8+ LGL cells. The expression levels of these
effector molecules suggest that γδ-LGL cells have more explicit effector function and NK-LGL cells have more explicit cytotoxic function than CD8+ LGL cells.

γδ cells are a small subset of T cells that develop in the thymus like conventional T cells. In contrast to αβ T cells, γδ T cells have a very restricted TCR usage, limited clonal diversity and a uniform effector phenotype. They can respond quickly to danger signals after triggering of their TCR receptor in a similar fashion to innate cells by their Pattern Recognition Receptors (PRRs). γδ cells are able to instantly generate effector molecules without the need for proliferation. Therefore, the larger extent of transcripts encoding effector molecules such as IFNγ and GzmB and TF involved in their production in γδ LGL cells is consistent with the phenotype of conventional γδ T cells. In NK-LGL the prominent increase in expression of GzmB rather than IFNγ corresponds with the high levels of GzmB rather than IFNγ in conventional NK cells. These data show that the transcription factors identified as transcriptional regulators of GzmB and IFNγ expression as well as the effector molecules themselves are abundantly expressed in NK, γδ and ab CD8+ LGL.

We next investigated whether there was a correlation in LGLs between the transcripts of effector molecules and TFs that regulate these effector molecules in regular CD8+ T cells and NK cells. The CD8+ T LGL samples (n=7) were selected because of group homogeneity.

Table 1. Clinical and laboratory features of LGL leukemia. Summary is provided of the most relevant clinical and hematological findings at diagnosis of the 15 LGL leukemia patients enrolled in this study. The immunophenotype was determined by analysis of the cell membrane expression of T- and NK-cell associated antigens by immunofluorescence stainings. The percentage of tumor cells was calculated as the proportion of LGL cells as part of the total amount of mononuclear cells present in the peripheral blood. (1): LGL cells were purified from the total fraction of mononuclear cells using FACS cell sorting. (p): LGL cells were partially stained by the monoclonal antibody.

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A clear correlation of T-bet, Eomes and Blimp-1 with GzmB of $R^2 \geq 0.80$ was observed (Fig. 2A). However, no correlation was detected between Hobit and GzmB. A comparable correlation was measured in the complete LGL set including γδ-LGL and NK-LGL cells, albeit with a reduced fit (Data not shown; T-bet, Eomes and Blimp-1 resp. $R^2=0.69, 0.56, 0.62$; n=15). It has been shown in mice that T-bet, Eomes and Blimp-1 are involved in the transcriptional regulation of GzmB production in NK cells and CD8$^+$ T cells$^{18,36}$. Our data shows that in human LGLs T-bet, Eomes and Blimp-1 correlated with GzmB expression, and that this correlation is most apparent in T-LGLs derived from the CD8$^+$ T cell subtype. A similar correlation was not found between this set of transcription factors and IFNγ (Fig. 2B). Although when all LGL subtypes were taken into account, Blimp-1 showed a moderate correlation of 0.72 (Fig. 2B). This was surprising, since all of these transcription factors are reportedly involved in the control of IFNγ production in other cell types. This suggested that other factors are involved in the regulation of IFNγ expression in LGLs.

Figure 1. Transcription factor levels in three subtypes of LGL cells. Levels of the depicted transcription factors (A) and effector molecules (B) were determined using qPCR in CD8$^+$, γδ and NK LGL cells obtained by isolating mononuclear cells from peripheral blood. As a healthy control cytotoxic T-cells were FACS cell sorted into naive, effector and memory cells based on CD45RA and CD27 staining. The mRNA expression in each sample was calculated relative to naive T-cells isolated from healthy donors.
Correlation between expression levels of Serpin B9 and TF T-bet, Eomes and Blimp-1

The expression of the transcription factors T-bet, Eomes and Blimp-1 has an impact on the cytotoxic potential of LGL cells. Since these TF are potent repressors and/or activators of extensive differentiation pathways, we investigated whether expression levels of T-bet, Eomes, Blimp and Hobit could be used to predict the survival and persistence of LGL cells in human patients. Failure to undergo apoptosis leads to the formation of malignant cells, either by aberrant expression of pro-apoptotic genes or down regulation of anti-apoptotic proteins. By MLPA, the expression profile of apoptosis and survival inducing molecules was performed for all LGL subtypes. The transcript levels of 33 genes were determined that are regarded as key molecules in apoptotic pathways (Fig. 3A). No major differences in the relative expression of apoptotic markers were detected between the NK, CD8+ T cell and γδ T cells. This was in contrast to figure 1 in which the LGL samples displayed a distinct pattern of TF usage. Therefore, high expression of either of the four TF analyzed does not correlate to the relative expression of pro- or anti-apoptotic genes.

Comparison of apoptotic profile of NK and LGL cells.

To assess whether apoptotic genes were differentially regulated between cells of the same origin in health and in disease, LGL cells of the NK subtype were compared to NK cells derived from peripheral blood of healthy controls (Fig. 3, B and C). The expression profile of the majority of all genes was similar between CD3-CD16+CD56+ NK cells and CD3-CD56+ LGL cells. Specifically the transcript levels of Bim, Noxa and Bid were significantly altered in the various subsets of LGL subsets compared to NK cells (Fig. 3, B and C). The expression level of pro-apoptotic Bim was significantly lower in NK-LGL than in NK cells of healthy controls. Bim plays an important role in Caspase independent cell death during the contraction phase

Figure 2: Correlation between transcription factors T-bet, Eomes, Blimp-1, Hobit and GzmB or IFNγ expression. The mRNA expression of the indicated TFs was correlated to the expression of (A) GzmB and (B) IFNγ in CD8+ LGL cells. For each data-set the goodness of fit was calculated ($R^2$).
Moreover, Bim is essential for the cytokine deprivation-induced apoptosis of B- and T lymphocytes. In addition the pro-apoptotic BH3 family member, Noxa showed a significant down regulation in LGL cells. Noxa and Bim have shown to act together to induce NK cell apoptosis in mice after IL15 deprivation. Moreover, Noxa was also induced after IL15 withdrawal, and during glucose deprivation in human T cells. A combined loss of Bim and Noxa protects mouse NK cells against IL15 deprivation in vitro. Therefore, the down regulation of both Noxa and Bim may enable a pro-survival pathway for LGL cells to persist in vivo in humans.

The expression levels of Bid mRNA were stably increased in the LGL samples compared to NK cells (Fig. 3, B and C). This was an unexpected finding, since Bid is a pro-apoptotic molecule. It is unclear whether the upregulated levels of Bid affect the survival of LGL cells, since the expression of Bid protein is reported to be regulated at the translational level by proteasomal degradation. Therefore, the mRNA levels of Bid may not adequately reflect the protein levels of Bid. The data in figure 3 suggest that based on mRNA transcription levels, the apoptotic proteins Bim, Noxa and Bid are the most likely candidates that cause the deregulation of apoptosis in LGL cells. In contrast, Bcl-2 was expressed at higher levels in LGL cells compared to NK cells, although the difference did not reach statistical significance. Thus, the maintenance of LGL cells may depend on the down regulation of pro-apoptotic Bim and Noxa rather than upregulation of pro-survival molecules.

Serpin B9 (PI-9) is a serine protease inhibitor that binds and inactivates serine proteases and therefore may protect cytolytic effector cells from GzmB driven cell death. No clear fit was found between the mRNA expression of GzmB and PI-9 ($R^2=0.5$) (Fig. 4A). Subsequently, the relative expression of PI-9 was correlated to the abundance of TF in the same LGL cells to determine whether a relationship could be established. As shown in figure 4A, PI-9 expression is tightly linked to the expression levels of Eomes, and Blimp-1 and to a lesser extent T-bet ($R^2$: resp. 0.90, 0.87 and 0.63). No correlation was found between PI-9 and Hobit (Fig 4B). Since expression levels of Eomes and PI-9 are so closely regulated, these genes may have a similar induction pathway.

**Discussion**

T-bet, Eomes, Blimp-1 and Hobit regulate terminal differentiation and the production of molecules with cytolytic and immunomodulatory properties such as GzmB and IFNγ. In this paper we analyzed the expression of these TFs in CD8$^+$ T cell subsets and show that they are highly expressed in all LGL subtypes. In NK and γδ T and CD8$^+$ αβ T LGL cells, T-bet, Eomes and Blimp-1 are present in quantities that are comparable to or even exceed that of other cytotoxic lymphocytes such as effector-type CD8$^+$ T cells. This indicates that LGL cells share the effector phenotype of NK cells, γδ T cells or effector-type CD8$^+$ T cells that can quickly respond to danger signals, without the need for further proliferation or maturation. Indeed, in line with this notion, we also found high levels of GzmB and IFNγ in all LGL subtypes.

The expression of T-bet and Eomes in NK-LGL is in concordance with their expression in peripheral NK cells, of which these cells are likely derived. When comparing the relative expression of CD8$^+$ T cell LGL to the CD8$^+$ T cell subsets in healthy controls, it appears that
the mRNA expression level is more similar to the levels in effector than memory T cells. This indicates that the LGL subsets are more comparable to effector CD8+ T cells than to memory CD8+ T cells.

Our data shows that the T-box factors Eomes and T-bet are highly expressed in γδ-LGL and that IFNγ is specifically upregulated compared to LGL cells derived from other lymphocyte...
subsets (Fig. 1A). Chen et al. have previously shown that γδ T cells of healthy adults show a faster kinetics of IFNγ transcription compared with CD4+ and CD8+ T cells and that γδ T cells produce significantly greater amounts of IFNγ in a proliferation-independent manner when compared with other T cell subsets. We show that these characteristics of conventional γδ T cells may be retained in γδ derived LGL. Furthermore, the enhanced IFNγ expression in γδ T cells also corresponded with enhanced Blimp-1 levels, implicating this TF next to the T-box genes T-bet and Eomes in the transcriptional regulation of the explicit effector phenotype of γδ LGL cells.

Hobit is highly expressed in all LGL samples but no prevalence was seen in one of the subtypes. Furthermore, no correlation with the effector molecules GzmB and IFNγ was observed (Fig. 2). Hobit is restricted to cytotoxic cells and enforced expression induces IFNγ in CD4+ T cells. It is feasible that in LGL cells a basal Hobit expression is necessary for IFNγ production and augmentation of Hobit transcription does not lead to an increase in IFNγ production. Moreover, the influence of Hobit on IFNγ production might be indirect with the relative expression of other TF more strongly affecting the production of the cytokine. This would result in other TFs correlating more closely to the CTL effector molecules.

An unexpected finding was the high expression of Blimp-1 in all types of LGL cells. In previous reports Blimp-1 was detected in multiple B-cell neoplasms, but only rarely in T cell malignancies. Anaplastic large cell lymphoma (ALCL), a malignancy characterized by CD3 negative cells which express CD30 on their cell surface, has previously been shown to express Blimp-1. However, immunohistochemistry staining could not detect Blimp-1 protein in all ALCL patients. Since Blimp-1 is a negative regulator of cell cycle, a failure to upregulate Blimp-1 could lead to malignancy. Comparative genome hybridization showed that in NK

Figure 4 (A) Correlation between the relative expression of PI-9 and transcription factors T-bet, Eomes, Blimp-1 and Hobit. (B) Correlation between PI-9 and GzmB. The mRNA expression was measured in arbitrary units (AU) which is calculated relative to the expression of each probe set in non-cytotoxic naive CD8+ T-cells. For each data-set the goodness of fit was calculated ($R^2$).
malignancies Blimp-1 expression is often reduced by chromosomal deletion or methylation of the Blimp-1 gene\(^4\), consistent with a role for Blimp-1 as a tumor suppressor gene. In our study, we did not find low Blimp-1 expression in the samples of the NK LGL subtype, nor in the CD8\(^+\) T LGL or the γδ T LGL subtypes. In contrast, high levels of Blimp-1 were observed in all LGL subtypes. Therefore, it is unlikely that Blimp-1 has a role as a tumor suppressor gene in LGL malignancy. It remains to be investigated how LGL cells are able to maintain themselves despite the high levels of Blimp-1 that is known to drive terminal differentiation in normal effector T cells.

It is possible that LGL cells express the β-isoform of Blimp-1 (Blimp-1β). This isoform is generated by an alternative transcription initiation site that leaves the DNA binding Zn-finger region intact, but has a disrupted PR domain and reduced repressor activity\(^5\). Blimp-1β has been measured in myeloma cell lines\(^6\) and T-cell lymphoma\(^7,51\), although in mice the truncated form did not act as a dominant-negative regulator\(^8\). It would be interesting to measure if this isoform is more abundantly expressed in LGL. Alternative splice variants for Hobit have also been described in conventional CD8\(^+\) T cells and NK cells (Vieira Braga et al., manuscript in preparation). However, only the Hobit-L isoform was detected in our LGL cohort (data not shown).

When detecting changes in apoptotic genes in all LGL samples, a number of anti-apoptotic genes was highly expressed including PI9. PI-9 has been shown to be present in neoplastic cells of several lymphoma subtypes, including NKT-cell lymphoma\(^5\). Bladergroen et al. suggested that elevation of PI-9 provides malignant cells the opportunity to escape elimination mediated by the release of GzmB by cytotoxic T lymphocytes\(^54\).

The anti-apoptotic protein Mcl-1 has been implicated to play a central role in a pathway that prevents LGL cells to undergo apoptosis\(^55\). Figure 5B showed that Mcl-1 transcripts were readily present, and no increase was detected over the levels measured in NK cells. However, the amount of mRNA in these cells may not be representative for Mcl-1 protein levels, since Mcl-1 is predominantly controlled posttranscriptionally.

MLPA analysis showed elevated expression of Bid transcripts in LGL cells, above levels observed for NK cells. This contrasted with recent findings that T-LGL and NK-LGL leukemias have a significantly reduced accumulation of Bid\(^56\). This decrease in Bid was not caused by alterations in RNA expression, but resulted from increased proteasomal degradation. Reversal of degradation by Bortezomib upregulated Bid protein, which induced apoptosis in the leukemic cells. The survival of the LGL cells in this study despite the enhanced expression of Bid mRNA could therefore be caused by similar mechanisms that regulate protein levels such as proteasomal degradation. More research is required to clarify whether Bid has indeed a central role in accumulation of leukemic cells.

The large quantities of LGL cells in the donors listed in this study indicate that the anti-apoptotic genes clearly outweigh the mRNA expression of pro-apoptotic genes. Indeed, the mRNA levels of the anti-apoptotic genes, Noxa and Bim were down regulated in NK-LGL cells when compared to NK cells of healthy donors. This was in line with earlier reports in which Bim played an essential role in the homeostasis of hemapoietic cells and cytokine deprivation-induced apoptosis\(^57-60\). Noxa and Bim have shown to act together when
inducing NK cell apoptosis in mice after IL15 withdrawal\textsuperscript{61}. A combined loss of Bim and Noxa in double knockout mice protected NK cells to a large extent against IL15 deprivation in vitro. Therefore, the down regulation of both Noxa and Bim might be a general way for LGL cells to persist in vivo in humans.

NK-LGL, which harbor CD56 on their cell surface, usually induce an aggressive disease with a poor prognosis\textsuperscript{62}. We investigated whether CD56 or any of the other cell surface markers depicted in table 1 that determine the immunophenotype, has a distinct expression profile for either the TFs, effector molecules, or apoptotic markers. In our cohort the presence of CD56 or any of the other cell surface markers did not result in a significantly altered TF, apoptosis or effector molecule profile (data not shown). Thus, TFs of effector differentiation do not predict severity of LGL disease.

It is important to understand the mechanisms that regulate the homeostasis of LGL cells. Expression patterns of key TFs as well as apoptotic molecules determine the survival and cytotoxicity of the leukemic cells. We here have demonstrated that expression levels of the proteins that are important transcriptional regulators of effector differentiation are highly expressed in LGL leukemia. Further analysis of these markers can ultimately give insight into the question what event drives development of malignant LGL cells and how LGL cells maintain themselves as fully differentiated effector cells.

**Material and methods**

**Patients and samples**

Patient samples (cDNA) were provided by Erasmus MC, University Medical Center\textsuperscript{63}. Patients were classified as described previously\textsuperscript{64}, according to clinical, laboratory, histological, cytomorphological, immunophenotypical and molecular data. In case of a confirmed hematological malignancy, the sample was classified according to the WHO classification of hematological malignancies. We obtained a total of 15 LGL malignancies, of which the patient characteristics are described in table 1. The high percentage of LGL cells present in the peripheral blood, allowed us to use unfractionated mononuclear cells of patients unless stated otherwise.

Human CD8\textsuperscript{+} T cell subsets were isolated from peripheral blood of four healthy donors. PBMC were isolated from heparinized blood using standard density gradient centrifugation and subsequently cryopreserved in liquid nitrogen until the day of analysis. To isolate naïve (CD8\textsuperscript{+} CD45RA\textsuperscript{high}CD27\textsuperscript{high}), effector type (CD8\textsuperscript{+}CD45RA\textsuperscript{high}CD27\textsuperscript{low}) and memory cells (CD8\textsuperscript{+}CD45RA\textsuperscript{low}CD27\textsuperscript{+}) from latently chronic HCMV infected healthy donors, PBMC were isolated from buffy coats using Ficoll centrifugation. Then, CD8\textsuperscript{+} T cells were isolated by CD8\textsuperscript{+} microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled with CD27-FITC (7C9)\textsuperscript{65}, CD45RA-RD1 (Beckman Coulter, Woerden, Netherlands), and CD8-allophycocyanin (BD Pharmingen, San Diego, CA, USA).

NK cells were obtained by staining PBMCs isolated from the PBMC fraction of peripheral blood of healthy controls with CD3\textsuperscript{+}-FITC, CD16\textsuperscript{+}-PE and CD56\textsuperscript{+}-APC (BD Pharmingen, San Diego, CA, USA) and isolation of the CD3 CD16 CD56 fraction.
Flow cytometric cell sorting was performed using a FACS Aria (BD Biosciences, San Jose, CA, USA).

RNA isolation and Quantitative PCR

RNA was isolated using the nucleospin RNA isolation kit (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions. mRNA was amplified using the MessageAmp II Kit (Ambion, Austin, TX, USA). Amplification of specific targets was performed by quantitative RT-PCR using Lightcycler FastStart DNA Master SYBR Green I (Roche) and the following primers: Eomes Forw. (5’-ACTGGTTCCACTGGATGAG-3’), Eomes Revs. (5’-CCACGCCATCCTCTGTAACT-3’), Hobit (ZNF683) Forw. (5’-CATATGGCCAAGAGCTTTGG-3’), Hobit (ZNF683) Revs. (5’-AGAGCTTCACTCAAACCTGCCC-3’), Prdm1 Forw. (5’-CAACAATTTGGCCTCTTCC-3’), Prdm1 Revs. (5’-GCATTCACTGGCTTTCTTC-3’), T-bet Forw. (5’-GGGAAACTAAGCTCACAAC-3’), Revs. (5’-CCCAAAGGAATTGACAGTTG-3’), S18 Forw. (5’-GGCAACAAAGCTCGTGAAGA-3’), S18 Revs. (5’-CAGAAAGTGACGAGCGCCTCCTC-3’)

Data were analyzed using LightCycler Software, version 3.5 (Roche) and the program LinRegPCR, version 7.5 (Ramakers et al., 2003). To confirm the purity and specificity of the reaction, a melting curve analysis was performed at the end of the PCR by slowly increasing (0.1°C/s) the temperature of the reaction from 65 to 95°C. S18 was used as an internal reference.

Immunophenotyping

Cells were analyzed for cell membrane expression of T- and NK cell associated antigens, including CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD56, CD57 and HLA-DR antigens (BD Biosciences). Immunofluorescence stainings were performed as described and evaluated on either FACScan or FACSCalibur (BD Biosciences) flow cytometers. Data analysis was performed using either the CellQuest or Flowjo (BD Biosciences).

RT-MLPA Procedure and Analysis

Using MLPA (multiplex ligation-dependent amplification) a selection of apoptosis related target genes (Depicted in fig. 3, except for genes that were not shown because of no detectable expression: DR6, p21, OMI, Ciap1, Bcl-g, HRK, Livin, Bik, Boo) was analyzed at mRNA level. All probes used in the apoptosis gene probe set including an M13-derived MLPA probe that allows input normalization, have been previously described as well as detailed reaction conditions. In brief, RNA samples were first reverse transcribed by means of a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probes. Annealed oligonucleotides were covalently linked by Ligase-65 at 54°C (MRC-Holland). Ligation products were amplified by PCR (33 cycles, 30 s at 95°C; 30 s at 60°C; and 1 min at 72°C) via one unlabeled and one FAM labeled primer (10 pMol). After the PCR stage, aliquots of samples were mixed with Genescan-500 ROX size standards and run on an ABI 3100 capillary sequencer (Applied Biosystems, Warrington, UK) in Genescan mode.
Data was analyzed with Genescan and compiled in table format with Genotyper and exported for further analysis to Microsoft Excel spreadsheet software. The sum of all peak data was set at 100% to normalize for fluctuations in total signal between samples, and individual peaks were calculated relative to the 100% value. Signals below the detection limit in medium were assigned a value corresponding to the threshold value for noise cut-off in Genescan. The logarithm (base 2) of the resulting data sets were imported in the TIGR Multiexperiment viewer version 2.2 (http://www.tigr.org/software/tm4).

Statistical methods

For detection of differences between two populations the two-tailed unpaired t-test was used. Significance is depicted as * when P-value is 0.01 - 0.05 and ** when P-value is <0.01. Linear regression and goodness of fit (R²) were calculated by GraphPad Prism software.
Reference List


2006. TCRγδ+ large granular lymphocyte leukemias reflect the spectrum of normal antigen-selected TCRγδ+ T-cells. *Leukemia* 20:505.
