Identification of a miR-146b-Fas ligand axis in the development of neutropenia in T large granular lymphocyte leukemia

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SUPPLEMENTARY MATERIALS AND METHODS

Flow cytometry analysis
T-LGL phenotype was assessed by flow cytometry. Briefly, cells were stained with the following antibodies: anti-CD3-PE (SK7), anti-CD3-APC (SK7), anti-CD4-FITC (SK3), anti-CD8-APC (RPA-T8), anti-CD16-PECy7 (B73.1), anti-CD56-PE (B159), anti-CD57-FITC (NK-1), and analysed using a FACSCanto analyser and the BD FACSDiva software (everything from BD Bioscience). TCR Vβ repertoire analysis was performed using the IOTest Beta Mark TCR-Vβ Repertoire kit (Beckman Coulter).

STAT3 and STAT5b mutations analysis
For the screening of STAT3 and STAT5b mutations we used the set of primers reported by Koskela et al1 and Rajala et al2, respectively, to amplify the hot spot regions for STAT3 and STAT5b mutations (exons 19-24 of STAT3 and exons 16-18 of STAT5b). DNA was extracted from purified T-LGL and remaining autologous PBMCs using the Puregene Cell Kit Plus (Qiagen) and sequenced on the ABI 3130 sequencer (Applied Biosystem). The presence of D661Y and Y640F STAT3 mutations undetectable by direct sequencing, was also analysed by a DNA tetraprimer amplification refractory mutation system assay (ARMS-PCR), as previously reported3.

miRNA and Gene-Expression Analysis
High-throughput and single-miRNA expression analyses were performed by TaqMan as previously described4. Briefly, the small RNA fraction was purified by using the miRCURY RNA Isolation Kit (Exiqon) according to the manufacturer’s instructions. High throughput and single miRNA analysis were carried out by using the TaqMan® Human microRNA Array (Card Set v3.0, Applied Biosystems) and the TaqMan® microRNA Human Assays (Applied Biosystems), respectively. Array data were analysed by Gene Expression Suite software (Applied Biosystems). miRNA expression values both from arrays and from single assays were calculated according to the comparative threshold cycle method using U6 as endogenous control. Relative miRNA expressions have been reported as fold change (FC). miRNAs samples with a FC>2 or FC<0.5 and P<0.05 were considered as differentially expressed.

mRNA and primary transcript data were calculated with LinReg PCR 7.0 (http://LinRegPCR.nl) and Q-Gene software (http://www.gene-quantification.de/download.html) and then expressed as mean normalized expression (MNE) units after GAPDH or RPL32 normalization, unless otherwise indicated.
**Methylated DNA Immunoprecipitation (meDIP) assay**

Genomic DNA was firstly treated with 20 μg/mL RNAsel A (Sigma Aldrich) for 30 min at 37°C and subsequently shared to generate fragments between 300 bp and 1000 bp using the Bandelin Sonopuls HD 2070 ultrasonic homogenizer (Bandelin). Fragmented DNA (4 μg) was immunoprecipitated with 5 μg anti-5-methylcytosine mouse mAb (clone 33D3, Millipore). Immunoprecipitated DNA was purified using the QIAquik PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. qPCR data were expressed as percentage over input.

**Western blot**

The blots were incubated with antibodies anti-STAT3 (79D7, Cell Signaling), anti-phospho-STAT3 (Tyr705) (STAT3-YP; D3A7, Cell Signaling), anti-GAPDH (clone 6C5, Millipore), anti-HuR (19F12, Thermo Scientific) and anti-β-actin (Sigma Aldrich) Abs. Detection was carried out with an horseradish peroxidase–conjugated anti–rabbit and anti–mouse immunoglobulins (Amersham International Biotechnology) and an enhanced chemiluminescent detection system (Thermo Scientific) for STAT3 and STAT3-YP. Goat anti-Mouse IgG antibody DyLight™ 800 conjugated (Rockland antibodies & assay) and AlexaFluor® 680 goat anti-Rabbit IgG (Molecular Probes, Thermo Scientific) and were used for HuR, GAPDH and β-actin detection, respectively. Detection was performed using the ImageQuant LAS 500 and quantified by ImageQuant TL v8.1 software (GE Healthcare) for STAT3 and STAT3-YP, and using the Odyssey infrared imaging system (LI-COR Biosciences) for HuR, GAPDH and β-actin.

**Oligonucleotide used in cell transfection**

miR-146b mimic (PM10105), pre-miR Negative Control#2, ELAVL1 Silencer Select (si-HuR, s4610) and Silencer Select negative control #2 (all from Ambion, Thermo Scientific).

**Enzyme-linked immunosorbent assay (ELISA)**

Absorbance was immediately read at 450 nm using the Victor Multilabel plate (PerkinElmer).

**Statistics**

Hierarchical Clustering Analysis (HCA) of TaqMan Human microRNA Array data was performed by using the Multi Experiment Viewer (MEV, mev.tm4.org/) software with the following parameters: *Pearson Correlation and complete linkage*. For all the statistical analysis *P*<0.05 was considered significant.
Supplementary Figure S1. miRNAs differentially expressed in CD8 as compared to CD4 T-LGLL.

Volcano plot showing miRNAs differentially expressed in CD8 as compared to CD4 T-LGLs. For each miRNA the –log10 (P value) is plotted against the average log2 (Fold Change). Vertical dashed lines represent the 0.5 and 2 boundary values for the Fold Change (FC); horizontal dashed line represents the 0.05 P value boundary.
Supplementary Figure S2. Schematic representation of the human miR-146b promoter.

Arrows represent the three primer pairs used in the MeDIP analysis. Primer position in the miR-146b locus is reported as bp upstream (-) or downstream (+) the miR-146b transcriptional start site arbitrarily indicated as 0. Positions of methylated and unmethylated cytosine as well as predicted STAT3 binding site are also reported.
Supplementary Figure S3: Base pairing comparison between mature miR-146b and HuR 3’ UTR.

Base pairing comparison between mature miR-146b and HuR 3’ UTR putative target site is shown according to microRNA.org (http://34.236.212.39/microrna/home.do).

3’-ucggauaccuuaagUCAAGAGU- 5’ hsa-miR-146b

1067: 5’-agauuaacccucaAGUUCUCu - 3’ HuR
### SUPPLEMENTARY TABLES

**Supplementary Table S1. Laboratory and clinical features of thirty patients affected by T-LGLL.**

| Patient ID | T-LGLL | T-LGL Phenotype | Sex/Age, y | Ly, % | LGLs, % on Ly | ANC, x10^9/L | Hb, g/L | Pts, x10^9/L | STAT3 mutation | STAT5 mutation | TCR gene analysis | Relevant Vβ expression | Associated neoplasia |
|------------|--------|-----------------|------------|------|--------------|---------------|---------|--------------|---------------|----------------|-----------------|---------------------|----------------------|---------------------|
| LGL 1      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/57      | 72   | 74           | 0.95          | 151     | 282          | MUT (Y640F)   | WT             | C                | NF                  | no                  |
| LGL 2      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/79      | 66   | 52           | 1.43          | 142     | 338          | WT            | WT             | C                | NF                  | no                  |
| LGL 3      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/73      | 74   | 86           | 1.10          | 121     | 199          | MUT (Y640F)   | WT             | C                | 5.2                 | no                  |
| LGL 4      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/53      | 90   | 86           | 0.80          | 144     | 135          | WT            | WT             | C                | NF                  | no                  |
| LGL 5      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/82      | 53   | 51           | 5.70          | 139     | 198          | WT            | WT             | C                | 13.1                | yes                 |
| LGL 6      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/85      | 60   | 78           | 2.29          | 150     | 183          | WT            | WT             | C                | 13.6                | no                  |
| LGL 7      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/48      | 48   | 70           | 4.30          | 159     | 204          | WT            | WT             | C                | 5.1                 | no                  |
| LGL 8      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/51      | 56   | 55           | 2.40          | 151     | 183          | WT            | WT             | C                | NF                  | no                  |
| LGL 9      | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/72      | 43   | 50           | 3.18          | 149     | 252          | WT            | MUT (N642H)    | C                | 17                  | yes                 |
| LGL 10     | CD4+   | CD3+CD4+CD8+CD16+CD56+CD57+ | F/76      | 73   | 74           | 1.50          | 126     | 160          | MUT (Y640F)   | WT             | C                | 21.3                | no                  |
| LGL 11     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/79      | 69   | 59           | 0.50          | 125     | 66           | MUT (N647I)   | WT             | C                | 17                  | no                  |
| LGL 12     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/62      | 59   | 53           | 2.86          | 132     | 238          | WT            | WT             | C                | NF                  | no                  |
| LGL 13     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/39      | 92   | 88           | 0.83          | 152     | 175          | WT            | WT             | C                | NF                  | no                  |
| LGL 14     | CD4+   | CD3+CD4+CD8+CD16+CD56+CD57+ | M/69      | 57   | 48           | 3.28          | 163     | 175          | WT            | WT             | C                | 8                   | yes                 |
| LGL 15     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/66      | 73   | 85           | 0.71          | 134     | 204          | MUT (D661Y)   | WT             | C                | 2                   | no                  |
| LGL 16     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/68      | 86   | 66           | 0.46          | 128     | 104          | MUT (D661Y)   | WT             | C                | 13.6                | no                  |
| LGL 17     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | F/85      | 37   | 49           | 0.65          | 129     | 233          | MUT (Y640F)   | WT             | C                | 3 and 20             | no                  |
| LGL 18     | CD8+   | CD3+CD8+CD16+CD56+CD57+ | M/54      | 64   | 91           | 0.70          | 155     | 111          | WT            | WT             | C                | 8                   | no                  |
| LGL 19     | CD4+   | CD3+CD4+CD8+CD16+CD56+CD57+ | M/71      | 73   | 83           | 1.82          | 154     | 218          | MUT (Y665F)   | WT             | C                | 13.1                | no                  |
| LGL 20     | CD4+   | CD3+CD4+CD8+CD16+CD56+CD57+ | M/73      | 63   | 63           | 3.72          | 135     | 263          | WT            | WT             | C                | NF                  | no                  |
| LGL 21     | CD4+   | CD3+CD4+CD8+CD16+CD56+CD57+ | M/63      | 56   | 55           | 3.20          | 143     | 237          | WT            | WT             | C                | 9                   | no                  |
| LGL 40 | CD8+ | CD3+CD8+CD16+CD56+CD57+ | F/57 | 89 | 87 | 0.79 | 124 | 239 | WT | WT | C | 17 | no |
| LGL 41 | CD8+ | CD3+CD8+CD16+CD56+CD57- | M/56 | 72 | 57 | 0.94 | 130 | 193 | MUT (D661V) | WT | C | 2 | no |
| LGL 42 | CD8+ | CD3+CD8+CD16+CD56+CD57- | F/49 | 83 | 50 | 0.51 | 147 | 126 | MUT (Y640F) | WT | C | 7 | no |
| LGL 43 | CD4+ | CD3+CD4+CD8+CD16+CD56+CD57 | M/55 | 57 | 50 | 3.00 | 153 | 192 | WT | WT | C | 13.1 | no |
| LGL 44 | CD4+ | CD3+CD4+CD8+CD16+CD56+CD57 | F/45 | 48 | 50 | 4.90 | 121 | 334 | WT | WT | C | NF | yes |
| LGL 45 | CD8+ | CD3+CD8+CD16+CD56+CD57+ | F/69 | 56 | 45 | 1.08 | 145 | 186 | MUT (D661Y) | WT | C | 3 | no |
| LGL 46 | CD8+ | CD3+CD8+CD16+CD56+CD57+ | M/69 | 45 | 40 | 0.41 | 146 | 159 | WT | WT | C | 17 | no |
| LGL 47 | CD8+ | CD3+CD8+CD16+CD56+CD57- | F/56 | 65 | 88 | 0.61 | 113 | 194 | MUT (D566N) | WT | C | 22 | no |
| LGL 48 | CD8+ | CD3+CD8+CD16+CD56+CD57+ | F/58 | 78 | 54 | 1.4 | 154 | 219 | MUT (D661Y) | WT | C | 9 | no |

ANC indicates absolute neutrophil count, C indicates clonal rearrangement of TCR, Hb indicates haemoglobin, LGLs indicates large granular lymphocytes, Ly indicates lymphocytes and Plts indicates platelets.
Supplementary Table S2. Primers used in RT-qPCR analysis.

| RT-qPCR target | Sequence | Forward primer | Reverse primer |
|----------------|----------|----------------|----------------|
| DNMT1          | CCAAAGCCCGAGAGAGTGCCTCAG | CCTAGCAGCTTCCTCCTCCTT |
| FasL           | CCAACCCCTGAAAAAAAGGAG | ATAGGTGTCTTCCCATTCAG |
| FasL-PT        | GCTGCCACCCCTGAAGAAG | CCCTCCATCCCCCTATGCC |
| HuR            | TTTGGGCAGATCATCAACTC | ATGGGCTCAGAGGAACCTG |
| pri-miR-146b   | AACAGGGAGACGATTCACAG | CTTTGCCATTGATGTGTAGC |
| GAPDH          | AACAGCCTCAAGATCATCAGC | GGATGATGTCTGGAGAGCC |
| RPL32          | AGGGTTCTGAGAAGATTCAGG | GAACACATTGTGAGCGATCTC |

| meDIP-qPCR target | Sequence | Forward primer | Reverse primer |
|-------------------|----------|----------------|----------------|
| miR-146b +44bp +315bp | ATGCCCTGTGGACTCAGTTT | AGGATGCGATGGAAGATCAG |
| miR-146b –149bp +98bp | TCAGACCCTCCCTGGAATAG | CCTTGCCATTGATGTGTAGC |
| miR-146b –687bp -496bp | CCTCTCTCTGGATTCAAGC | GAAGTACCTGGGATAGTG |

Supplementary Table S3. miRNAs differentially expressed in CD8 and CD4 T-LGLs.

| miRNA           | FC    | P    |
|-----------------|-------|------|
| hsa-miR-146b    | 0.028 | 0.030 |
| hsa-miR-501     | 150.621 | 0.030 |
| hsa-miR-1249    | 50.970 | 0.007 |
| hsa-miR-1303    | 18.288 | 0.004 |
| hsa-miR-1227    | 13.830 | 0.004 |
| hsa-miR-571     | 11.546 | 0.005 |
| hsa-miR-335#    | 10.839 | 0.015 |
| hsa-miR-566     | 10.807 | 0.011 |
| hsa-miR-1247    | 8.215  | 0.005 |
| hsa-miR-1285    | 6.586  | 0.010 |
| hsa-miR-33a#    | 6.194  | 0.010 |
| hsa-miR-591     | 5.927  | 0.000 |
| hsa-miR-623     | 4.937  | 0.047 |
| hsa-miR-636     | 4.719  | 0.049 |
| hsa-miR-625#    | 4.350  | 0.022 |
| hsa-miR-1267    | 3.198  | 0.040 |
| hsa-miR-516-3p  | 3.015  | 0.039 |
| hsa-miR-1253    | 3.002  | 0.023 |
| hsa-miR-197     | 2.993  | 0.038 |
| hsa-miR-331-5p  | 2.970  | 0.045 |
| hsa-miR-1233    | 2.946  | 0.008 |
| hsa-miR-1825    | 2.808  | 0.026 |
| hsa-miR-484     | 2.796  | 0.008 |
| hsa-miR-630     | 2.572  | 0.006 |
| hsa-miR-939     | 2.445  | 0.019 |

FC indicates Fold Change.
Supplementary Table S4. Correlation analysis of miRNA expression, Absolute Neutrophil Count and STAT3-YP

| miRNA        | ANC p   | ANC P   | STAT3-YP p | STAT3-YP P |
|--------------|---------|---------|------------|------------|
| hsa-miR-630  | -0.866  | 0.030   | 1.000      | 0.003      |
| hsa-miR-146b | 0.866   | 0.030   | -0.866     | 0.033      |
| hsa-miR-939  | -0.829  | 0.058   | 0.943      | 0.017      |
| hsa-miR-33a#| -0.829  | 0.058   | 0.943      | 0.017      |
| hsa-miR-636  | -0.829  | 0.080   | 0.943      | 0.017      |
| hsa-miR-1285 | -0.657  | 0.173   | 0.829      | 0.058      |
| hsa-miR-1303 | -0.600  | 0.242   | 0.829      | 0.058      |
| hsa-miR-623  | -0.600  | 0.242   | 0.829      | 0.058      |
| hsa-miR-516-3p| -0.543 | 0.297   | 0.771      | 0.103      |
| hsa-miR-566  | -0.543  | 0.297   | 0.771      | 0.103      |
| hsa-miR-1233 | -0.543  | 0.297   | 0.771      | 0.103      |
| hsa-miR-1247 | -0.829  | 0.059   | 0.657      | 0.175      |
| hsa-miR-1267 | -0.600  | 0.240   | 0.657      | 0.175      |
| hsa-miR-591  | -0.543  | 0.297   | 0.657      | 0.175      |
| hsa-miR-1249 | -0.600  | 0.242   | 0.600      | 0.242      |
| hsa-miR-484  | -0.429  | 0.419   | 0.600      | 0.242      |
| hsa-miR-331-5p| -0.429 | 0.419   | 0.600      | 0.242      |
| hsa-miR-197  | -0.429  | 0.419   | 0.600      | 0.242      |
| hsa-miR-335#| -0.486  | 0.356   | 0.543      | 0.297      |
| hsa-miR-571  | -0.429  | 0.419   | 0.543      | 0.297      |
| hsa-miR-625#| -0.486  | 0.356   | 0.486      | 0.356      |
| hsa-miR-1253 | -0.486  | 0.356   | 0.486      | 0.356      |
| hsa-miR-1825 | -0.371  | 0.497   | 0.486      | 0.356      |
| hsa-miR-501  | -0.600  | 0.242   | 0.429      | 0.419      |
| hsa-miR-1227 | -0.429  | 0.419   | 0.429      | 0.419      |

ANC indicates absolute neutrophil count; \( p \) indicates the Spearman correlation coefficient.
Supplementary Table S5. Patients used in each analysis.

| Figure ID | CD8 | CD4 |
|-----------|-----|-----|
| **1C**    |     |     |
| 1         | LGL 1 LGL 3 LGL 5 LGL 9 | LGL 10 LGL 17 |
| **2A**    |     |     |
| 1         | LGL 1 LGL 3 LGL 5 LGL 9 LGL 22 LGL 23 LGL 26 LGL 32 LGL 33 | LGL 10 LGL 17 LGL 18 LGL 19 LGL 20 LGL 31 |
| **2B - LEFT** |     |     |
| 1         | LGL 1 LGL 3 LGL 5 LGL 9 LGL 22 LGL 23 LGL 26 | LGL 10 LGL 17 LGL 18 LGL 20 LGL 31 |
| **2B - CENTRE** |     |     |
| 1         | LGL 1 LGL 3 LGL 5 LGL 9 LGL 22 LGL 23 LGL 26 LGL 32 LGL 33 | LGL 10 LGL 17 LGL 18 LGL 19 LGL 20 LGL 31 LGL 39 LGL 43 LGL 44 |
| **2C - RIGHT** |     |     |
| 1         | LGL 1 LGL 3 LGL 5 LGL 9 LGL 22 LGL 23 LGL 26 | LGL 10 LGL 17 LGL 18 LGL 20 LGL 31 |
| **3A**    |     |     |
| 1         | LGL 40 LGL 41 LGL 42 | LGL 18 LGL 39 LGL 44 |
| **3B - LEFT** |     |     |
| 1         | LGL 32 LGL 36 LGL 40 LGL 45 LGL 47 LGL 48 |     |
| **3B - RIGHT** |     |     |
| 1         | LGL 23 LGL 32 LGL 40 LGL 42 LGL 45 LGL 46 LGL 48 |     |
| **4A**    |     |     |
| 1         | LGL 1 LGL 3 LGL 9 LGL 26 | LGL 10 LGL 17 LGL 20 LGL 31 |
| **4B**    |     |     |
| 1         | LGL 1 LGL 3 LGL 9 LGL 22 LGL 26 LGL 32 LGL 33 | LGL 10 LGL 17 LGL 19 LGL 20 LGL 31 |
| **4C**    |     |     |
| 1         | LGL 1 LGL 3 LGL 9 LGL 22 LGL 26 LGL 32 LGL 33 | LGL 10 LGL 17 LGL 19 LGL 20 LGL 31 |
| **4D**    |     |     |
| 1         | LGL 1 LGL 9 LGL 23 LGL 32 | LGL 19 LGL 39 LGL 43 LGL 44 |
| **4E**    |     |     |
| 1         | LGL 1 LGL 3 LGL 9 LGL 22 LGL 26 LGL 32 LGL 33 | LGL 10 LGL 17 LGL 19 LGL 20 LGL 31 |
| **6A**    |     |     |
| 1         | LGL 32 LGL 33 |     |
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