Natural killer (NK) cells represent a class of innate lymphocytes with large granular morphology and cytotoxic functions, characterized by the CD3–/CD16+/CD56+ phenotype. According to CD56 expression, two major NK cell subsets can be recognized, CD56high/CD16dim/CD56neg NK cells with the ability to release cytokines and CD56dim/CD16high NK cells displaying cytotoxic ability toward virus infected or neoplastic cells. NK cells are traditionally considered part of innate immunity but evidence has been recently provided that a distinct NK cell subset may respond to specific antigens like adaptive immune cells. These NK cells, also known as “NK memory”, are induced by the chronic stimulation of viral infections or by cytokines (IL-12, IL-15 and IL-18) and are included in the CD56dim/CD16high NK cells subgroup equipped with CD57 but lacking CD62L. Chronic lymphoproliferative disorder of NK cells (CLPD-NK) is a provisional entity, recognized by the 2016 WHO classification, characterized by chronic expansion of at least 500/mm³ NK cells with restricted killer immunoglobulin-like receptor (KIR) pattern, whose assessment is of crucial relevance due to the lack of T-cell receptor rearrangement and clinical CLPD-NK patients. By flow analysis, NK cells of 25 patients affected by CLPD-NK were analyzed for CD16 and CD56 expression (Supplementary Methods), recognizing two major NK cell subsets, that is, patients with CD56dim/CD16dim NK cells (4/25, 16%) and patients with CD56neg/CD16high NK cells (21/25, 84%) (Figs. 1a–c). All patients were evaluated for clinical and hematological characteristics. Median age was 62 years (range 42–79) with no significant difference between CD56dim/CD16dim and CD56neg/CD16high subgroups. As expected, neutropenia (absolute neutrophil count (ANC) < 1500/mm³) was the most relevant feature, detected in 10 out of 25 patients (40%), with 5 patients (20%) presenting severe neutropenia (ANC < 500/mm³). Anemia and thrombocytopenia have been detected only in a minority of patients (2/25 and 3/25, respectively) and were generally mild (Table 1). In terms of clinical presentation, almost all symptomatic patients were included in the CD56neg/CD16high subgroup with 8 out of 21

**Correspondence:** Gianpietro Semenzato (g.semenzato@unipd.it) or Renato Zambello (r.zambello@unipd.it)

1Department of Medicine, Hematology and Clinical Immunology Section, Padua University School of Medicine, Padua, Italy

2Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

© The Author(s) 2018

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.
(38%) patients presenting neutropenia and 5 out 21 (24%) severe neutropenia. Only three patients required treatment (low-dose cyclophosphamide in two cases and methotrexate in one) during the natural history of the disease, all belonging to CD56<sup>neg/dim</sup>/CD16<sup>high</sup> subset.

Considering the clinical heterogeneity of CD56<sup>neg/dim</sup>/CD16<sup>high</sup> subset, we analyzed the expression of CD57 on...
cells of these patients identifying a 51 ± 13.5% mean expression positivity. Of notice, all five patients who experienced severe neutropenia and all symptomatic patients requiring treatment presented a significantly lower CD57 mean expression toward other CD56^neg/dim/CD16^high patients (12.48% ± 2.87 vs 60.43% ± 4.38, p < 0.0001, Fig. 1d). As a consequence CD57~/CD57+ ratio was significantly higher in symptomatic patients as compared with remnant CD56^neg/dim/CD16^high patients (10.84 ± 5.24 vs 0.84 ± 0.33, p = 0.0007, Fig. 1e). Taken these data together, through immunophenotype, three major NK cell subgroups of patients can be identified. CD16 levels discriminated between CD56^dim/CD16^dim subgroup and CD56^neg/dim/CD16^high subgroup. Among the latest, CD57 expression identified patients who experienced severe neutropenia characterized by a “Cytotoxic” phenotype with CD57 negativity from less symptomatic patients characterized by CD57 expression, resembling the “NK Memory” phenotype (Figs. 1f, g, respectively). All the three subgroups displayed high CD94 expression, whereas only CD56^dim/CD16^dim NK cells expressed discrete amount of CD62L. No statistically significant differences in CD94-NKG2A/C and KIR expression were found among the three subgroups although CD94/NKG2C phenotype and KIR restriction for CD158b and CD158e were almost exclusively distinct features of NK “Memory” subgroup (8/16 and 3/16, respectively), whereas CD56^dim/CD16^dim and NK “Cytotoxic” subgroups displayed CD94-NKG2A phenotype and presented a skewed KIR pattern characterized by lack of KIR expression (3/4 and 5/5, respectively). All these features are reported in Table 1.

STAT3 exon 21 mutations analysis and STAT5b exons 16 to 18 analysis were performed in our cohort of patients (Supplementary Methods). As previously reported9, the frequency of STAT3 mutated patients was lower with respect to T-LGLL with only two (8%) mutated patients found. These latters were characterized by the “Cytotoxic” NK immunophenotypic signature without dominant KIR expression and NKG2A pattern. In addition, all mutated patients presented severe neutropenia and required treatment during the natural history of the disease. None STAT5b mutated patient was found in our cohort of CLPD-NK patients (Table 1).

With the aim of identifying biological features matching with clinical characteristics, we focused on the recognition of a specific NK cell immunophenotypic signature that eventually allows a biological and clinical classification of this rare disorder. We found that, independently from KIR expression, patients characterized by CD56^neg/dim/CD16^high/CD57- cytotoxic NK cell expansion represent a different phenotypic subgroup characterized by symptomatic disease and by the presence of STAT3 mutation, suggesting a more aggressive NK cell proliferation. These findings agree with the article of Morice et al.10 in which CLPD-NK patients were classified upon CD56 expression; CD56^neg NK cell patients were
characterized by CD16 expression, presence of cytopenia and treatment requirement. However, this analysis alone may not be enough considering that also among CD56\textsuperscript{neg/dim}/CD16\textsuperscript{high} patients, a different clinical behavior can be recognized. We found that patients who experience severe neutropenia and eventually require treatment belong to the CD56\textsuperscript{neg/dim}/CD16\textsuperscript{high}/CD57\textsuperscript{−} NK subset, thus conferring a prognostic role to CD57. As a further confirmation, the specific CD56\textsuperscript{neg/dim}/CD16\textsuperscript{high}/CD57\textsuperscript{−} immunophenotypic signature is also partially associated to a specific biological hallmark of LGL leukemia, which is the presence of STAT3 mutation. As we recently demonstrated in T-LGLL\textsuperscript{11}, also in this large cohort of CLPD-NK patients, immunophenotypical analysis can identify patients with symptomatic disease and might represent a suitable surrogate of STAT3 mutation sequencing. WHO 2016 classification confirmed CPLD-NK as a provisional entity, emphasizing the high heterogeneity of the disease\textsuperscript{4,7,12}. The frequency of STAT3 mutated patients found in our cohort is slightly lower as compared with what has been reported in previous studies by Jerez and Rajala\textsuperscript{4,7,12}. This feature can be explained by a higher frequency of symptomatic and treated patients in their study populations, possibly due to enrichment in NK “Cytotoxic” patients, as herein defined. Unfortunately, in the above articles\textsuperscript{4,7,12}, the frequency of CD57 NK cells was not reported.

Clonality assessment for NK cell neoplasms still represents an Achilles heel due to the lack of a clonotypic structure on these cells. Besides, only a small fraction of CLPD-NK reveals STAT3 mutation, the majority of patients displaying wild-type STAT3. For this reason, other biological features were assessed to prove the clonal proliferation. Historically, a skewed pattern of KIR expression was considered as a reliable surrogate of clonality to characterize pathological NK cell expansion\textsuperscript{13,14}. On the contrary, Bàrcena and colleagues\textsuperscript{9}, using HUMARA assay to distinguish monoclonal vs polyclonal proliferation, recognized a surrogate for NK clonality in CD94\textsuperscript{high}/HLA-DR\textsuperscript{+} signature. This approach was able to identify a concordance between the presence of STAT3 mutation and monoclonal NK cell proliferation but, surprisingly, not significant differences were found between monoclonal and polyclonal patients in terms of clinical features (specifically the presence of cytopenia), suggesting that a percentage of truly CLPD-NK symptomatic patients were missed by this classification. In addition, CD94 expression represents a specific marker of mature NK cells that is not suitable for distinction between polyclonal and monoclonal NK cell proliferations.

The etiology of LGLL is still unknown but several authors hypothesized that chronic antigenic stimulation can trigger an initial LGL proliferation that subsequently carries on as a consequence of persistent cytokine stimulation or gain of somatic mutation (i.e., STAT3 mutation)\textsuperscript{5}. The evidence that some patients are characterized by NK cells expansion with memory-like phenotype, may support this hypothesis. In fact, several studies highlighted that viral stimulation by cytomegalovirus can trigger a CD57\textsuperscript{+}/NKNG2C NK cell expansion with memory properties and, in some cases, this process is stimulated by cytokines, like IL-12, IL-15 and IL-18, which have been proven to play a pathogenetic role in LGLL\textsuperscript{15}.

In conclusion, through NK cell subsets flow analysis, discrete subtypes of CLPD-NK can be identified. At variance with KIR expression, which does not correlate with clinical features, patients characterized by CD56\textsuperscript{neg/dim}/CD16\textsuperscript{high}/CD57\textsuperscript{−} cytotoxic NK cells expansion represent a unique phenotypic subgroup characterized by more symptomatic disease and the presence of STAT3 mutation, suggesting a more aggressive proliferation of NK cells.

Acknowledgements
The authors would like to thank Associazione Italiana per la Ricerca sul Cancro (AIRC, IG-15286), Cariparo, Cariverona e Ministero dell’Università e della Ricerca Scientifica e Tecnologica (MURST).

Authors’ contributions
G.B. designed the research, analyzed data and wrote the manuscript. A.T., G.C., P.F. contributed to analyze data. G.S. provided funding, participated to the design of the study, analyzed data, wrote the manuscript and supervised the study.

Conflict of interest
The authors declare that they have no conflict of interest.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41408-018-0088-1).

Received: 9 January 2018 Revised: 5 March 2018 Accepted: 27 April 2018 Published online: 05 June 2018

References
1. Campbell, K. S. & Hasegawa, J. Natural killer cell biology: an update and future directions. J. Allergy Clin. Immunol. 132, S36–S54 (2013).
2. Min-Oo, G., Kamimura, Y., Hendricks, D. W., Nabekura, T. & Lanier, L. L. Natural killer cell: walking three paths down memory lane. Trends Immunol. 34, 251–258 (2013).
3. Cerwenka, A. & Lanier, L. L. Natural killer cell memory in infection, inflammation and cancer. Nat. Rev. Immunol. 16, 112–123 (2016).
4. Semenzato, G., Marino, F. & Zambello, R. State of the art in natural killer cell malignancies. *Int. J. Lab. Hematol.* **34**, 117–128 (2012).
5. Lamy, T., Moignet, A. & Loughran, T. P. Jr. LGL leukemia: from pathogenesis to treatment. *Blood* **129**, 1082–1094 (2017).
6. Koskela, H. L. et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N. Engl. J. Med.* **366**, 1905–1913 (2012).
7. Jerez, A., Clemente, M. J., Makishima, H., Koskela, H. & Leblanc, F. Peng Ng K. et al. STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T-cell large granular lymphocyte leukemia. *Blood* **120**, 3048–3057 (2012).
8. Andersson, E. I. et al. High incidence of activating STAT5B mutations in CD4-positive T-cell large granular lymphocyte leukemia. *Blood* **128**, 2465–2468 (2016).
9. Barcena, P. et al. Phenotypic profile of expanded NK cells in chronic lymphoproliferative disorders: a surrogate marker for NK-cell clonality. *Oncotarget* **6**, 42938–42951 (2015).
10. Morice, W. G. et al. Chronic lymphoproliferative disorder of natural killer cells: a distinct entity with subtypes correlating with normal natural killer cell subsets. *Leukemia* **24**, 881–884 (2010).
11. Teramo, A. et al. STAT3 mutation impacts biological and clinical features of T-LGL leukemia. *Oncotarget* **8**, 61876–61889 (2017).
12. Rajala, H. L. et al. The analysis of clonal diversity and therapy responses using STAT3 mutations as a molecular marker in large granular lymphocytic leukemia. *Haematologica* **100**, 91–99 (2015).
13. Semenzato, G. et al. The lymphoproliferative disease of granular lymphocytes: updated criteria for diagnosis. *Blood* **89**, 256–260 (1997).
14. Zambello, R. et al. Expression and function of KIR and natural cytotoxicity receptors in NK-type lymphoproliferative diseases of granular lymphocytes. *Blood* **102**, 1797–1805 (2003).
15. Romee, R. et al. Cytokine activation induces human memory-like NK cells. *Blood* **120**, 4751–4760 (2012).