Dysfunctional subsets of CD39+ T cells, distinct from PD-1+, driven by leukemic extracellular vesicles in myeloid leukemias

The immunosuppressive milieu is a hallmark of cancer, including acute and chronic myeloid leukemia (AML/CML). One of the main features of the immune tumor microenvironment is exhaustion/dysfunction of conventional CD4+ and CD8+ T cells, which hampers their effector function and immune killing of cancer cells. Targeting and reinvigoration of such cells by immune checkpoint blockade in solid tumors has revolutionized cancer therapies. However, in myeloid leukemias, immune checkpoint therapies are yet to yield successful results.[1,2] Therefore, the identification of novel subsets of exhausted T cells characterized by the expression of targetable markers could facilitate the development of successful therapies. Here, we demonstrate the emergence of extracellular vesicle-driven, dysfunctional CD39+ T cells in peripheral blood in myeloid leukemias. The identified CD39+ subsets were distinct from PD-1+ T cells and were therefore unlike CD39+ cells in solid tumors, where exhausted T cells co-express these markers.[3] The identified subsets could be implemented into diagnostic-immune monitoring schemes or could indicate specific immune checkpoint blockade strategies in myeloid leukemias.

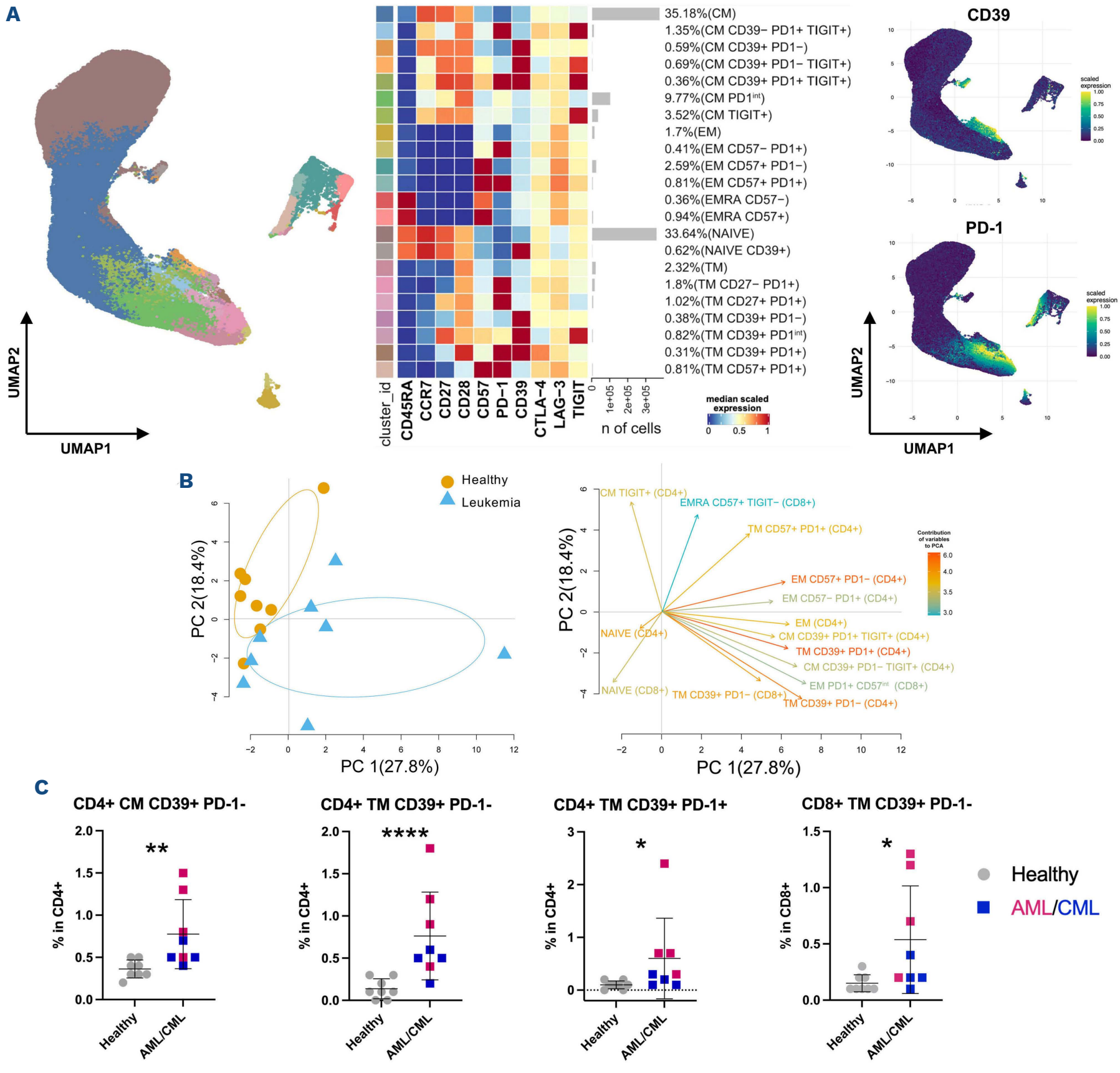
AML and CML exhibit similar immunosuppressive landscapes, despite distinct, and heterogenous molecular backgrounds. Striking similarity of immunological parameters in CML and AML across all hematological malignancies has been evidenced in a direct comparative immunogenomic study[4] and includes several features of T-cell exhaustion, especially in the bone marrow (BM) of patients at diagnosis. These include increased abundance of PD-1-, TIM-3-, CTLA-4- and TIGIT-expressing CD4+/CD8+ T cells.[5–8] The presence of CD8+PD-1+TIGIT+ cells has been associated with a lack of response to induction chemotherapy in AML.[9] On the other hand, several studies have found these subsets, especially PD-1-expressing T cells, to be less abundant in the peripheral blood (PB) than in the BM of patients[6,10] and to be unchanged or only moderately higher than in healthy controls.[8,9,11] Lack of distinct T-cell exhaustion features in PB hinders immune monitoring and the identification of AML/CML patients who could respond to immunotherapies.

Our previous findings have demonstrated that leukemic extracellular vesicles (EV) drive the progression of myeloid leukemias by promoting heterogenous subsets of regulatory T cells.[12] Therefore, we hypothesized that leukemic EV could also promote the emergence of dysfunctional subsets of effector T cells present in the PB of myeloid leukemia patients. We aimed at finding traits relevant to both AML and CML, due to their similar immunological landscape and to potentially implicate new therapeutic targets that could complement the entire spectrum of tyrosine kinase inhibitors and checkpoint inhibitors applicable in myeloid leukemias. First, we performed deep profiling of T-cell maturation and exhaustion in the PB of acute and chronic myeloid leukemia patients at diagnosis (*Online Supplementary Table S1*) by high resolution, 15-color flow cytometry, followed by unsupervised FlowSOM clustering to assess changes in T-cell subsets in an unbiased manner (Figure 1A, B; *Online Supplementary Figure S1Aa-d* and *Ba-f)*. Principal component analysis based on the 14 most differential clusters of CD4+ and CD8+ T cells clearly distinguished leukemic patients from healthy controls. Among the subsets contributing to the separation of leukemic samples were T-cell populations expressing CD39, an ectonucleosidase previously identified on dysfunctional T cells in solid tumors, but not leukemias[3,13] (Figure 1B). Statistical analysis of FlowSOM clustering revealed that five subsets of CD4+ cells (*Online Supplementary Figure S1Aa*) and one cluster of CD8+ cells (*Online Supplementary Figure S1Ba-c)* were significantly upregulated in leukemic patients. The upregulated clusters were predominantly CD39-expressing populations (Figure 1C; *Online Supplementary Figure S2Aa* to *d)*. The upregulated CD39 expressing subsets included T cells of different maturation/memory statuses: CCR7+CD45RA-CD28+CD39+PD-1- central memory (CM) CD4+ cells, CCR7-CD45RA-CD28+CD39+PD-1- and CCR7-CD45RA-CD28+CD39+PD-1+ transitional memory (TM) CD4+ cells and CCR7-CD45RA-CD28+CD39+PD-1- TM CD8+ cells. The two TM subsets of CD4+ cells and one TM subset of CD8+ cells were nearly undetectable in healthy donors, being considered present only in leukemic patients (Figure 1C; *Online Supplementary Figure S2Aa-d)*. Contrary to what is usually observed in solid tumours,[3] several CD39-expressing T-cell subsets that were expanded in myeloid leukemias did not express PD-1, a canonical marker of T-cell exhaustion (Figure 1A, C).

Furthermore, the significant expansion of CD39-expressing cells among the total CD4+ and CD8+ T lymphocytes was confirmed by manual analysis of phenotyping data (Figure 1D; *Online Supplementary Figure S2Ae, f*). Consist-

ent with previous reports[6,9,11] we did not observe expansion of T cells expressing other exhaustion markers in PB of patients, such as PD-1, CTLA-4 and TIGIT (*Online Supplementary Figure S1Ae*), or cytotoxic T lymphocytes expressing proinflammatory mediators IFN-γ and TNF-α, also relevant in immune checkpoint blockade (*Online Supplementary Figure S1Af*). Moreover, in order to confirm functional effects and activity of CD39 expressed on T cells, we analyzed plasma levels of adenosine – an immunosuppressive metabolite generated from ATP due to activity of CD39/CD73 axis. Plasma of leukemic patients has contained significantly more adenosine (Figure 1E; *Online Supplementary Figure S2Ag*), confirming functional relevance of CD39-expressing cells in myeloid leukemias (though adenosine generation does not have to be limited to CD39/CD73-expressing T cells). Crucially, the abundance of total CD4+CD39+ T cells and the three CD39-expressing TM subsets of CD4+ and CD8+ T cells correlated with the amount of leukemic CD34+ cells in AML/CML patients, revealing a link between these subsets and disease burden (Figure 1F).

Previous work suggested that while exhausted T cells may not be highly abundant in the blood of myeloid leukemia patients, they still make up a significant fraction of T cells



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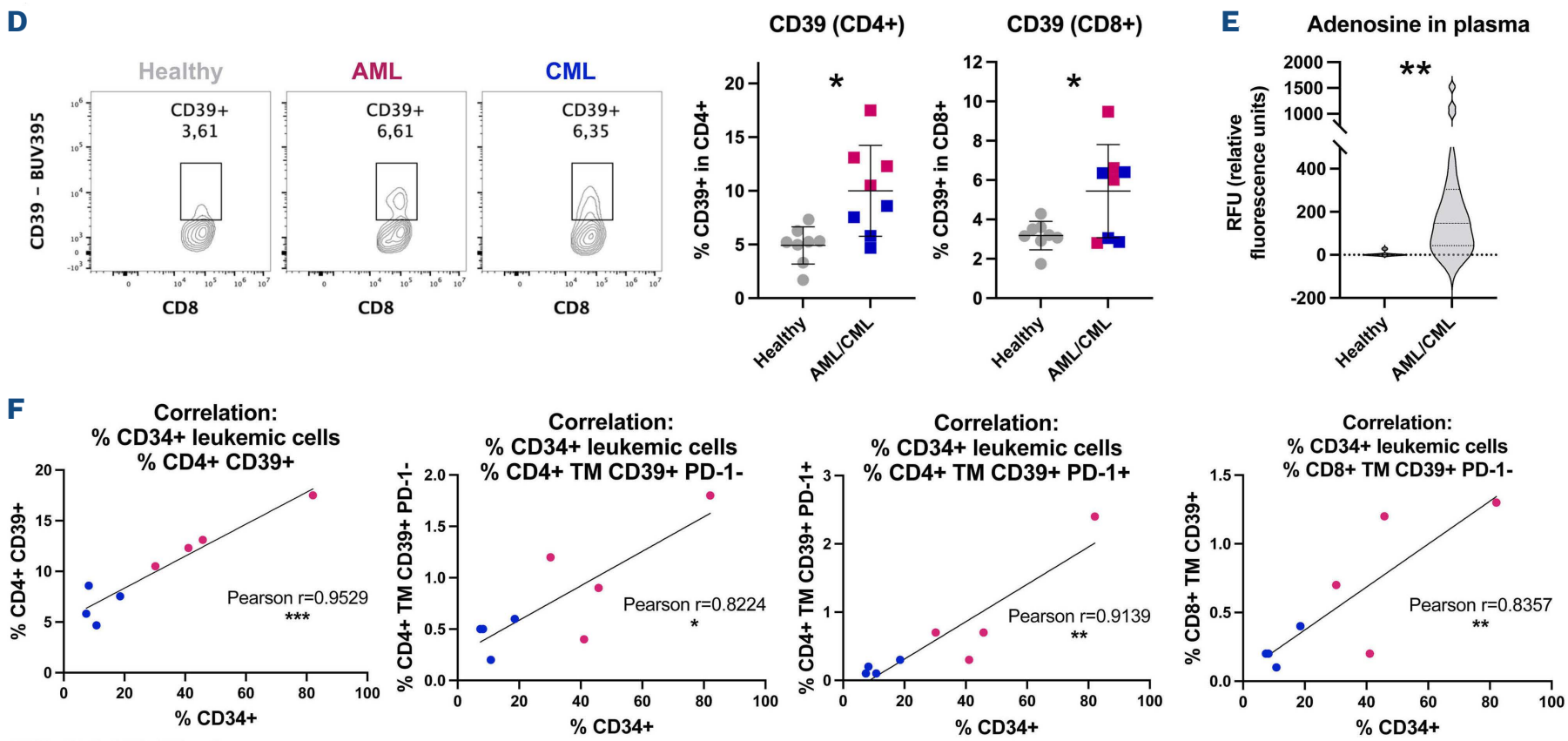

Figure 1. CD39+ dysfunctional T cells are a hallmark of myeloid leukemias. (A) UMAP (left) and heatmap (middle) representation of the CD4+ T-cell landscape, with different subsets and clusters (unique colors assigned and indicated next to the heatmap), identified by FlowSOM. The percentage and name of each cluster in the analysis are shown next to the heatmap. Heatmap colors represent the median expression of specified markers for each cluster, with blue representing low expression and red representing high expression. UMAP graphs (right) showing the relative expression of CD39 and PD-1 on CD4+ T cells. Differential analysis and distribution of subsets in individual samples of patients are shown in *Online Supplementary Figure S1Aa-d*. (B) Principal component analysis (PCA) showing different clustering of samples of healthy donors (Healthy) and acute and chronic myeloid leukemia (AML/CML) patients (Leukemia) based on immunological parameters (top 14 contributing populations of CD4+ and CD8+ T cells identified by FlowSOM). The first 2 PC explained a combined 47.1% of the variance, with 28.7% and 18.4% for the first and second PC, respectively. The graph on the right represents the contributions of specified parameters (immune subsets) to the observed differences, the length and direction of the arrows correlating with the movement of samples across the PC1 and PC2 axes. Parameters in one part of the graph negatively correlate with samples in the contrary region of the PCA graph. (C) Abundance of specified subsets of CD4+ and CD8+ T cells, as identified by unsupervised analyses, in AML and CML patients and healthy donors. Mean ± standard deviation (SD) is presented (n=8 AML/CML patients [4 AML, 4 CML] and 8 healthy donors). (D) Representative expression of CD39 on CD8+ T cells from healthy donors and AML/CML patients. Expression of CD39 on CD4+ and CD8+ T cells in AML and CML patients and healthy donors. Mean ± SD is presented, unpaired *t*-test with Welch's correction (n=8 AML/CML patients [4 AML, 4 CML] and 8 healthy donors). (E) Adenosine level in plasma of AML/CML patients and healthy donors, shown as relative fluorescence units (RFU) in a fluorometric assay, following fluorescence subtraction of endogenous background samples. Data is plotted as violin plots, with median and quartiles marked with dashed lines, unpaired *t*-test with Welch's correction (n=19 AML/CML patients [12 AML, 7 CML] and 12 healthy donors). (F) Linear correlation between the percentage (%) of CD34+ leukemic cells and the percentage (%) of total CD4+CD39+ T cells and the percentage (%) of 3 TM CD39 expressing T-cell subsets in AML/CML patients. The Pearson correlation coefficient was used as a measure of linear correlation between parameters. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

in the leukemic BM.[1] In order to gain further insight into dysfunctional CD39+ T cells, we analyzed CD39 expression on non-Treg CD4+ and CD8+ T cells in the BM and spleen in a mouse model of CML-like disease. We also included analyses in our previously developed[12] model of Rab27a-deficient CML, with attenuated secretion of EV (Figure 2A). While healthy BM and spleen contained almost no CD39+ cells, a significant fraction of CD39+ T cells appeared in mice with CML-like disease (Figure 2B, C), making up approximately 50% of effector T cells in BM (Figure 2C). Consistent with our data from AML/CML patients, these cells predominantly exhibited an antigen-experienced CD44+ memory phenotype (Figure 2D). Strikingly, expansion of CD39+ cells in mice with CML-like disease was regulated by EV secretion by leukemic cells (in Rab27a knock-out leukemia), pinpointing the involvement of leukemic EV in the development of this dysfunctional subset (Figure 2C), although other factors, including cytokines (IL-6, IL-12, TGF-β), may also potentially contribute to expansion of these cells. This would corroborate our previous data on the proleukemic influence of leukemic EV and the role of Rab27a in disease progression.[12] In order to study whether leukemic EV directly drive the expansion of dysfunctional CD39+ T cells, we sorted human non-Treg CD4+ and CD8+ T cells from healthy donors and treated them with EV released by either CML-K562 or AML-MOLM-14 cells. Leukemic EV significantly upregulated the expression of CD39 on both CD4+ and

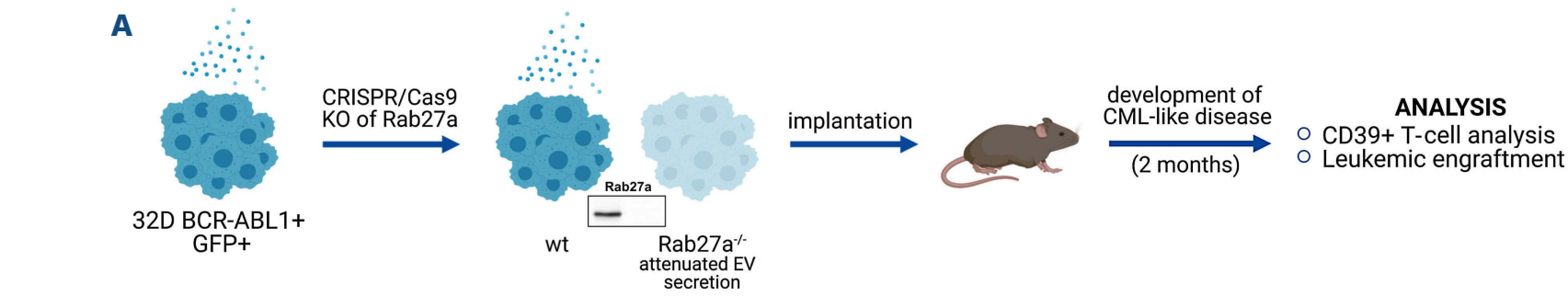

Figure 2. CD39+ dysfunctional T cells expand in the bone marrow and spleen of mice with chronic myeloid leukemia-like disease. (A) Experimental scheme of experiments in an *in vivo* model of chronic myeloid leukemia-like (CML-like) disease. (B) Unsupervised tSNE clustering of CD3+ Foxp3- effector T cells from the spleens of control mice, healthy mice (CTRL) and animals with leukemia-like disease. The bottom graph shows the localization of CD4+ CD39+ or CD8+ CD39+ cells on the tSNE map. Data from 3 mice (per group) from a single experiment were used as representative groups. In each group, 30,000 viable CD3+ T cells were clustered, 10,000 from each animal (obtained by downsampling in FlowJo). (C) Expression of CD39 on T cells in the bone marrow (BM) and spleen (SPL) of mice bearing leukemia-like disease. In each graph, data are presented as mean ± standard deviation, one-way ANOVA with Tukey's post-test. N=6-8 animals per group, from 3 different experiments (different litters/groups of animals and leukemic cell injections). (D) Distribution of naïve and memory subsets of T cells among CD39+ T cells in the BM and SPL of mice with CML-like disease (n=6 animals from 3 different experiments). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

CD8+ T cells *ex vivo* (Figure 3A). Other markers of T-cell exhaustion, such as PD-1, CTLA-4 and LAG-3, were upregulated by leukemic EV (*Online Supplementary Figure S2Ba, b*), although PD-1 was not exclusively co-expressed with CD39 (Figure 3A), further corroborating the phenotype of T cells in the PB of leukemic patients (Figure 1C). Primary EV isolated from the plasma of AML/CML patients also triggered CD39 expression by T cells (Figure 3A). In order to evaluate whether phenotypic changes and expansion of CD39+ cells induced by leukemic EV are connected to diminished functionality, we analyzed the secretion of proinflammatory cytokines. T-cell production of the effector cytokines IL-6, IFN-γ (by CD4+ T cells) and TNF-α (by CD8+ T cells) and the myeloid chemoattractant chemokine CCL2 was downregulated following treatment with leukemic CML and AML EV (Figure 3B). This demonstrates that the functionality of effector T cells is hampered by leukemic extracellular vesicles, concurrently with the dysfunctional/exhausted phenotype.

Finally, we verified whether leukemic EV also affect the metabolic capacity of T cells. Activated T cells robustly utilize glycolytic metabolism, whereas exhausted T cells exhibit impaired glycolysis and metabolic dysfunction has been recognized as one of hallmarks of T-cell exhaustion.[14] First, we measured the glycolytic rate in non-Treg CD4+ and CD8+ T cells using Seahorse technology. Surprisingly, CD8+ T cells treated with leukemic EV exhibited significantly stronger glycolytic flux (Figure 3C). In order to gain deeper insight into the metabolic profile of T cells, we applied the SCENITH™ assay and flow cytometric barcoding to profile several metabolic processes with single-cell resolution[15] (*Online Supplementary Figure S2Bc*). T cells in

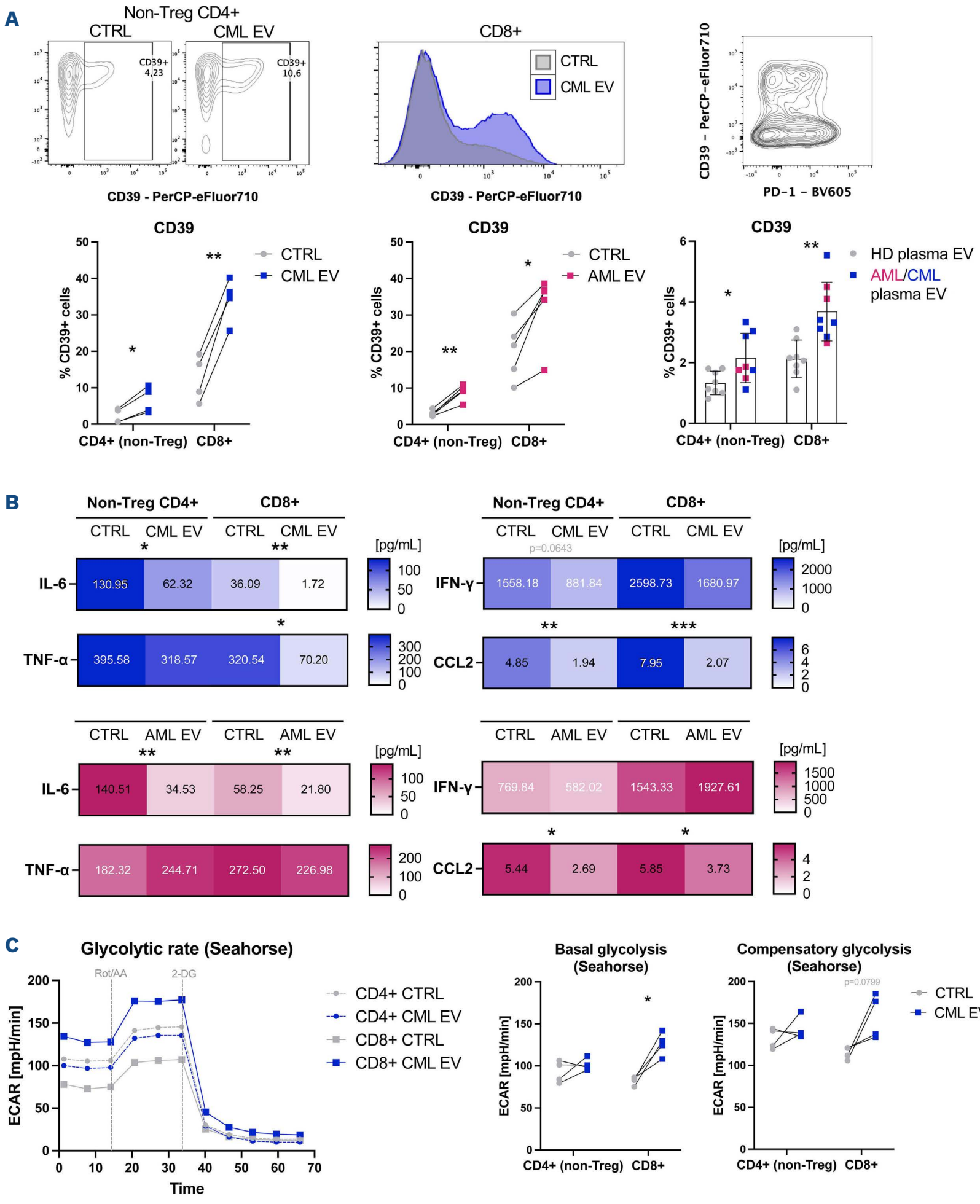

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Figure 3. Leukemic extracellular vesicles promote CD39 expression and dysfunction of effector CD4+ (non-Treg) and CD8+ T cells. (A) Representative expression of CD39 following treatment with chronic myeloid leukemia (CML) extracellular vesicles (EV) (for either non-Treg CD4+ or CD8+ T cells, as specified) and expression of CD39 *vs.* PD-1 following treatment of CD8+ T cells with acute myeloid leukemia (AML) EV (right). Expression of CD39 on CD4+ (non Treg) and CD8+ T cells cultured with leukemic EV: CML-K562 EV, AML-MOLM-14 EV and primary EV from the plasma of leukemia AML/CML patients. For CML-K562 and AML-MOLM-14 EV, data are from 4-5 experiments (n=4-5). For plasma EV, n=8 AML/CML patients (3 AML, 5 CML) and 8 healthy donors, the mean ± SD is presented, unpaired *t*-test with Welch's correction. (B) Secretion of effector cytokines (pg/mL, calculated per $1x10^5$ cells, final concentration shown on graphs) detected in culture medium of CD4+ (non-Treg) and CD8+ T cells cultured with CML-K562 EV and AML-MOLM-14 EV. Data are from 3-4 experiments (n=3-4, except CCL2 secretion by CD8+ cells treated with AML EV, where n=2). Two-tailed ratio paired *t*-test. (C) Representative curves of extracellular acidification rate (ECAR) of CD4+ (non-Treg) and CD8+ cells in control conditions and following treatment with CML EV. ECAR was measured under basal conditions and following treatment with rotenone/antimycin (Rot/AA) and 2-deoxyglucose (DG). Basal and compensatory (following Rot/AA) glycolysis/ECAR quantified for CD4+ (non-Treg) and CD8+ cells. (D) Representative histograms for puromycin staining in the SCENITH™ assay in control and CML EV-treated CD8+ T cells under control conditions (CO) and following treatment with DG, oligomycin (O) and deoxyglucose+oligomycin (DGO). Glycolytic capacity calculated for CD4+ (non Treg) and CD8+ T cells in the SCENITH™ assay. For (C, D), the data are from 4 experiments (n=4). For (A-D), samples were paired that were used to treat the same batch of (primary) T cells, two-tailed paired *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001. For experiments with plasma EV in (A), primary cells from 3 donors were used, so pairing was not performed.

ex vivo cultures exhibited almost complete dependence on glucose as an energy source, but in CD8+ cells, some of the energetic output was also due to mitochondrial respiration (*Online Supplementary Figure S2Bd, e)*. Following treatment with leukemic EV, CD8+ T cells no longer exhibited mitochondrial metabolism and were entirely glycolytic (Figure 3D), consistent with the changes observed in Seahorse experiments (Figure 3C). These results suggest that leukemic EV paradoxically promote a more effector-cell-like, glycolytic metabolism in dysfunctional T cells which might constitute a rescue mechanism to maintain an activated phenotype. On the other hand, increased dependence on glycolysis may render effector CD8+ T cells more susceptible to a glucose-deficient tumor/leukemia microenvironment. The observed increase in glycolytic capacity may also be explained by the fact that leukemic EV shuttle glycolytic enzymes (*Online Supplementary Figure S2Bf, g*), as observed in mass spectrometry analysis of CML EV proteome, performed initially in our previous study.[12]

Taken together, our data show that different subsets of CD39+ effector CD4+ and CD8+ T cells constitute hallmarks of acute and chronic myeloid leukemia. The three identified transitional memory subsets were present exclusively in leukemic patients and correlated with leukemic burden. This finding indicates their potential relevance for disease monitoring, especially as exhausted subsets of T cells expressing PD-1 and TIGIT were not observed in the PB of patients, either in this study or in others.[8,9,11] Importantly for immunotherapeutic solutions in AML/CML, three of four CD39-expressing subsets more abundant in myeloid neoplasms did not express PD-1. They could thus constitute a pool of dysfunctional T cells not responding to anti PD-1/PD-L1 antibodies that are therefore resistant to therapy. CD39 expression was also observed on AML blasts, which facilitated cytarabine resistance in immunodeficient models.[16] CD39 could therefore be a relevant therapeutic target to mediate both immune and non-immune eradication of leukemic cells. While our study was limited to a small group of leukemic patients and lacked longitudinal monitoring, we linked the expansion of dysfunctional CD39+ T cells with the burden of CD34+ cells in patients, as well as with Rab27a secretion of EV and disease progression *in vivo*, which further strengthens our conclusions. We have previously shown that diminished EV secretion (Rab27a deficiency) by leukemic cells attenuated engraftment of leukemic cells in a mouse model of CML-like disease, by promoting effector regulatory T cells, but not suppressive myeloid or B cells.[12] CD39-expressing T cells thus constitute another immune cell population that contributes to the observed phenomena. Our findings refer to both AML and CML, and could therefore benefit treatments of both myeloid leukemias, also in combination with tyrosine kinase inhibitors which target specific mutations. Collectively, CD39+ dysfunctional T cells and their specific subsets expand in the PB of patients with myeloid leukemias, due to the influence of leukemic extracellular vesicles. We postulate that the identified subsets of CD39+ T cells can thus both have diagnostic value and be a potential therapeutic target.

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https://doi.org/10.3324/haematol.2022.281713

Received: July 5, 2022.
Accepted: November 3, 2022.
Early view: November 17, 2022.

Disclosures

No conflicts of interest to disclose.

Contributions

JS and DLT performed phenotyping of T cells from patients; DLT, JS, RJA, SDB and AC analyzed and discussed flow cytometry data; JS, RB, MBO, AP, AN, LTK and MW performed *ex vivo* experiments; JS, LTK and EK performed *in vivo* experiments; JS, DC performed proteomic analysis; UW, WG-P and GB provided primary material; JS, SDB, AC and KP conceptualized and supervised the project and experiments; JS prepared figures and the manuscript draft; JS, SDB, AC and KP prepared and reviewed the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to thank Ewa Wąsiewicz for collecting clinical samples from leukemic patients and thank all the patients who donated blood for this study. The authors would like to acknowledge Magdalena Lebiedzinska-Arciszewska, PhD (Laboratory of Mitochondrial Biology and Metabolism, Nencki Institute) for help with adenosine measurements.

Funding

This work was supported by EMBO Scientific Exchange Grant (short-term fellowship) STF 8905 (to JS), Polish National Science Center grant 2018/29/N/NZ3/01754 (to JS) and Foundation for Polish Science grant TEAM TECH Core Facility Plus/2017-2/2 (POIR.04.04.00-00-23C2/17-00, co-financed by the European Union under the European Regional Development Fund) (to KP). SDB was the Marylou Ingram Scholar of the International Society for Advancement of Cytometry (ISAC) for the period 2016–2020. RJA is the Marylou Ingram Scholar of the International Society for Advancement of Cytometry (ISAC) for the period 2020–2023.

Data-sharing statement

The experimental methods and protocols are available upon request to the corresponding authors.

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