Genetics and Genomics

Expression of Most Retrotransposons in Human Blood Correlates with Biological Aging

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Abstract

Retrotransposons (RTEs) have been postulated to reactivate with age and contribute to aging through activated innate immune response and inflammation. Here, we systematically analyzed the relationship between RTEs expression and aging using published transcriptomic and methylomic datasets of human blood. Despite no observed correlation between RTEs activity and chronological age, most RTE classes and families except short interspersed nuclear elements (SINEs) correlate with age-associated gene signature scores. Strikingly, we found that the expression of SINEs is linked to upregulated DNA repair pathways in multiple cohorts. DNA hypomethylation with aging was observed across RTE classes and associated with increased RTEs expression in most RTE classes and families except SINEs. Additionally, our single-cell transcriptomic analysis suggests a role for plasma cells in aging mediated by RTEs. Altogether, our multi-omics analysis of large human cohorts highlights the role of RTEs in biological aging and suggests possible mechanisms and cell populations for future investigations.

eLife assessment

This study investigates associations between retrotransposon element expression and methylation with age and inflammation, using multiple public datasets. The study is **valuable** because a systematic analysis of retrotransposon element expression during human aging ishas beenlacking. However, the data provided are **incomplete** due to the sole reliance on microarray expression data for the core analysis of the paper.

Introduction

Transposable elements (TEs) are genetic elements that can move within the genome and are categorized into DNA transposons and retrotransposons (RTEs), which depend on cDNA intermediates to function. RTEs include three classes: long terminal repeat (LTR) retrotransposons, which integrate into the genome like the retroviruses, and long and short interspersed nuclear

elements, known as LINEs and SINEs, respectively. LINEs and SINEs employ target-primed reverse transcription for genome integration, with SINEs depending on the proteins encoded by LINEs[1]. Although most of the RTE sequences are dormant in the host genome, they continue to play vital roles in human evolution and physiology[2,3].

RTEs are silenced through heterochromatinization and DNA methylation in the early developmental stages as part of the host defense mechanism[3,4]. However, increased RTE activity that occurs with aging can lead to genome instability and activation of DNA damage pathways[4–7]. Additionally, accumulation of cytoplasmic RTE cDNAs detected in aging organisms and senescent cells can activate type I interferon (IFN-I) response and inflammation[3,4,8–10], that contributes to inflammaging[11]. Furthermore, chromatin remodeling and cellular senescence also contribute partially to the RTE reactivation[10]. Cellular senescence is a non-proliferative state elicited by stress factors such as DNA damage and is closely intertwined with inflammation and aging[12]. Through the expression of senescence associated secretory phenotype (SASP), senescent cells promote inflammation and senescence in other cells, including immune cells, leading to a compromised immune system and a vicious cycle of inflammation[13]. Understanding how RTEs expression as a factor that might affect the SASP, cellular senescence, and inflammaging could offer important insights into strategies against aging and age-related diseases (ARDs).

Recent studies have documented the relationship between RTEs activation and age-related events, but comprehensive and large-scale studies are lacking, mainly due to the paucity of RNA-seq data for large non-cancerous human cohorts. Most of the existing transcriptomics datasets are microarray based, where the computational methods were not sufficiently developed to analyze repetitive elements. By overlapping the Illumina Microarray expression and methylation probes with RepeatMasker[14], we were able to identify enough probes to calculate the expression of RTEs classes and families. Building on this methodology, we explored how RTEs expression contributes to biological aging using publicly available transcriptomics microarray data derived from human blood samples. More specifically, we first investigated if RTEs expression was correlated with chronological age, and then we analyzed the relationship between RTE and age-related events including cellular senescence, inflammation, and IFN-I response with published gene signatures. Using microarray methylomic data, we also investigated the DNA methylation level of RTEs in blood samples of multiple non-cancerous human cohorts and examined the relation between DNA methylation and RTEs expression and aging. Furthermore, with annotated single-cell transcriptomic data of the peripheral blood mononuclear cells (PBMCs) of 21 healthy human samples from a recent study of aging immunity[15], we identified cells that could be implicated in the process of RTEs reactivation and aging. Lastly, the single-cell transcriptomic data of supercentenarians[16] was analyzed to examine the interplay between RTEs expression and aging as a factor of aging and longevity (**Fig. 1**).

Results

Analyzing DNA methylation and expression levels of RTEs using microarray data

We collected three published microarray datasets from large-scale human studies including the Multi-Ethnic Study of Atherosclerosis[17] (MESA, aged 44 to 83, n=1202), the Grady Trauma Project[18,19] (GTP, aged 15 to 77, n=359), and Genetics, Osteoarthritis and Progression[20] (GARP, aged 43 to 79, n=139), which are from peripheral monocytes of healthy human, whole blood of healthy humans, and PBMCs from primary osteoarthrosis patients, respectively (**Supplementary Table 1** and **Supplementary Information**). The studies were conducted using either Illumina HumanHT-12 V3 or Illumina HumanHT-12 V4 expression microarray kits. After comparing the probes of the two microarray versions, we adopted the more comprehensive probe

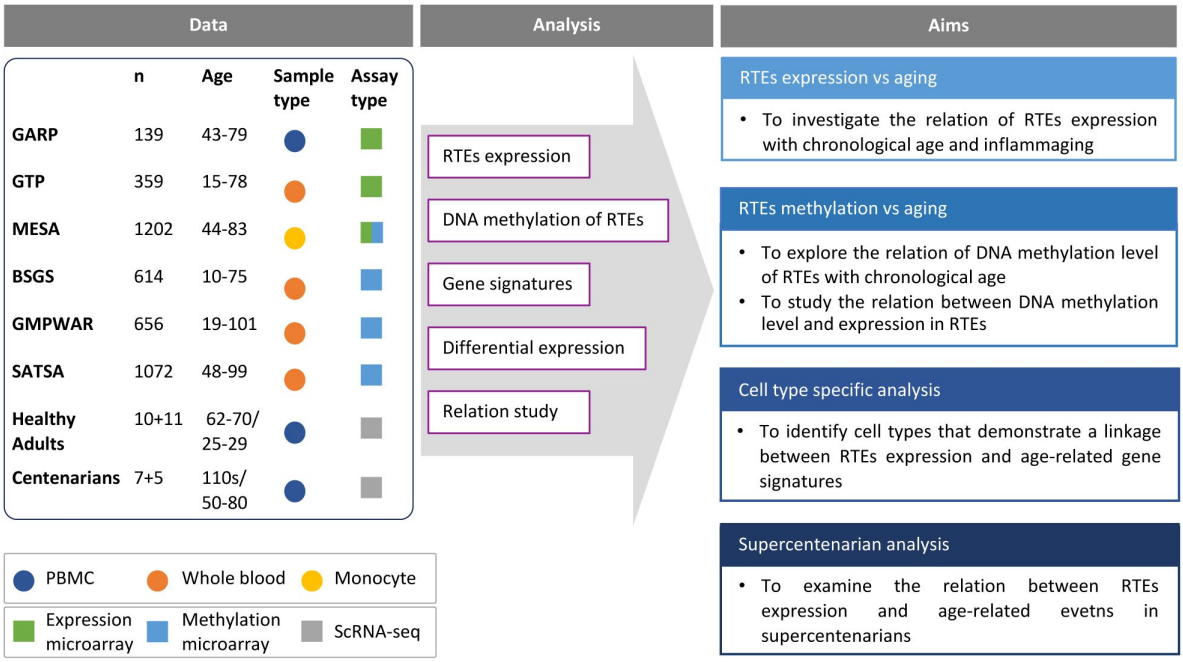

Figure 1.

Conceptual framework and the study design.

We collected published datasets of human blood samples for gene expression, DNA methylation, and single-cell transcriptomic data. The analysis aimed to study the relation between the expression and DNA methylation of RTEs versus chronological and biological aging in large human cohorts. The single-cell transcriptomic datasets were employed for cell type-specific analysis of RTEs in PBMC to identify the relation between RTEs expression and ageing events for annotated cell types within old versus young PBMC samples.

list of V4, which contains the full intersection of the two lists. To generate the RTEs expression, we mapped the probe locations to RepeatMasker to extract a list of intergenic probes that cover the RTE regions. We included three main RTE classes: (1) the LINE class, which encompasses the L1 and L2 families; (2) the SINE class, with Alu and MIR as the two main families; and (3) the LTR class which comprises the ERV1, ERVl, ERVL-MaLR, and ERVK families. Most of the RTE-covering probes available on Illumina HumanHT-12 V4 are present in MESA and GARP, while fewer are available in GTP (**Supplementary Fig. 1a**, **Supplementary Tables 2-4**).

Four methylation datasets were analyzed, including MESA, Swedish Adoption/Twin Study of Aging[21] (SATSA, aged 48 to 98, n=1072), Brisbane Systems Genetics Study[22] (BSGS, aged 10 to 75, n=862), and Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates[23] (GMPWAR, aged 19 to 101, n=656). SATSA, BSGS, and GMPWAR are genome-wide DNA methylation studies of whole blood samples from healthy individuals generated with Illumina Infinium 450k array (**Supplementary Table 1** and **Supplementary Information**). The DNA methylation probes from the Illumina Infinium 450k array kit were aligned to RepeatMasker to identify the probes overlapping the RTE regions (**Supplementary Table 5**). More than 90% of the RTE-covering probes are present in MESA and GMPWAR, while fewer, but more than half, are available in SATA and BSGS datasets (**Supplementary Fig. 1b**).

The chronological age is not linked with RTEs expression

Firstly, we examined the relationship between RTEs expression and chronological age in the MESA, GARP, and GTP cohorts. The ages of the individuals enrolled in these studies range from 15 to 83 years, with the mean being 70.2 (MESA), 60.1 (GAPR), and 42.5 (GTP) (**Supplementary Table 1**). Strikingly, no significant relationship (P value < 0.05) is found between chronological age and expressions of the three RTE classes across the datasets (**Fig. 2a**). Similarly, apart from a weak correlation observed between a few RTE families and chronological age, we could not identify any strong relation between chronological age and expression of RTE families across the three cohorts (**Supplementary Fig.2a**).

A similar correlation analysis was carried out in a scRNA-seq data of the PBMCs of 21 non-obese healthy men annotated into 25 cell types by Mogilenko et al.[15] (**Supplementary Table 6** and **online methods**). When comparing the RTEs expressions of the young (aged 25 to 29, n=11) group with the old (aged 62 to 70, n=10) group, we could not find any significant difference or trend in any cell types between the two groups (**Supplementary Figs. 3-5**). Overall, RTEs expression did not correlate with chronological aging across our microarray and scRNA-seq datasets.

RTEs expression positively correlates with age-associated gene signature scores except for SINEs

In mammals, aging is characterized by an intricate network of inflammation, IFN-I signaling, and cellular senescence[3]. To measure the level of biological aging from the transcriptomic data, we focused on six gene sets retrieved from widely cited studies: IFN-I[24], inflammatory cytokines[25], inflammatory chemokines[26], inflammaging[27], SASP[28], and cellular senescence[29] (**Supplementary Table 7**). The scores of these six age-associated gene sets were calculated for each individual in the MESA, GTP, and GARP cohorts by applying the *singscore* package in Bioconductor[30] (**online methods**). Except for GTP, in which 12 genes were missing from the gene sets in total (**Supplementary Table 8**), MESA and GARP microarray datasets had the expression of all the genes in the gene sets.

Then, we analyzed the relation between RTEs expression and the six age-associated gene signature scores. Positive correlations were observed between the expressions of LINEs and LTRs and age-associated signature scores across three datasets, while inverse correlation was present between the expression of SINEs and these scores in the PBMC samples from the GARP cohort (**Fig. 2a and b**). The discrepant pattern of LTRs and LINEs versus SINEs in PBMC is more pronounced in the

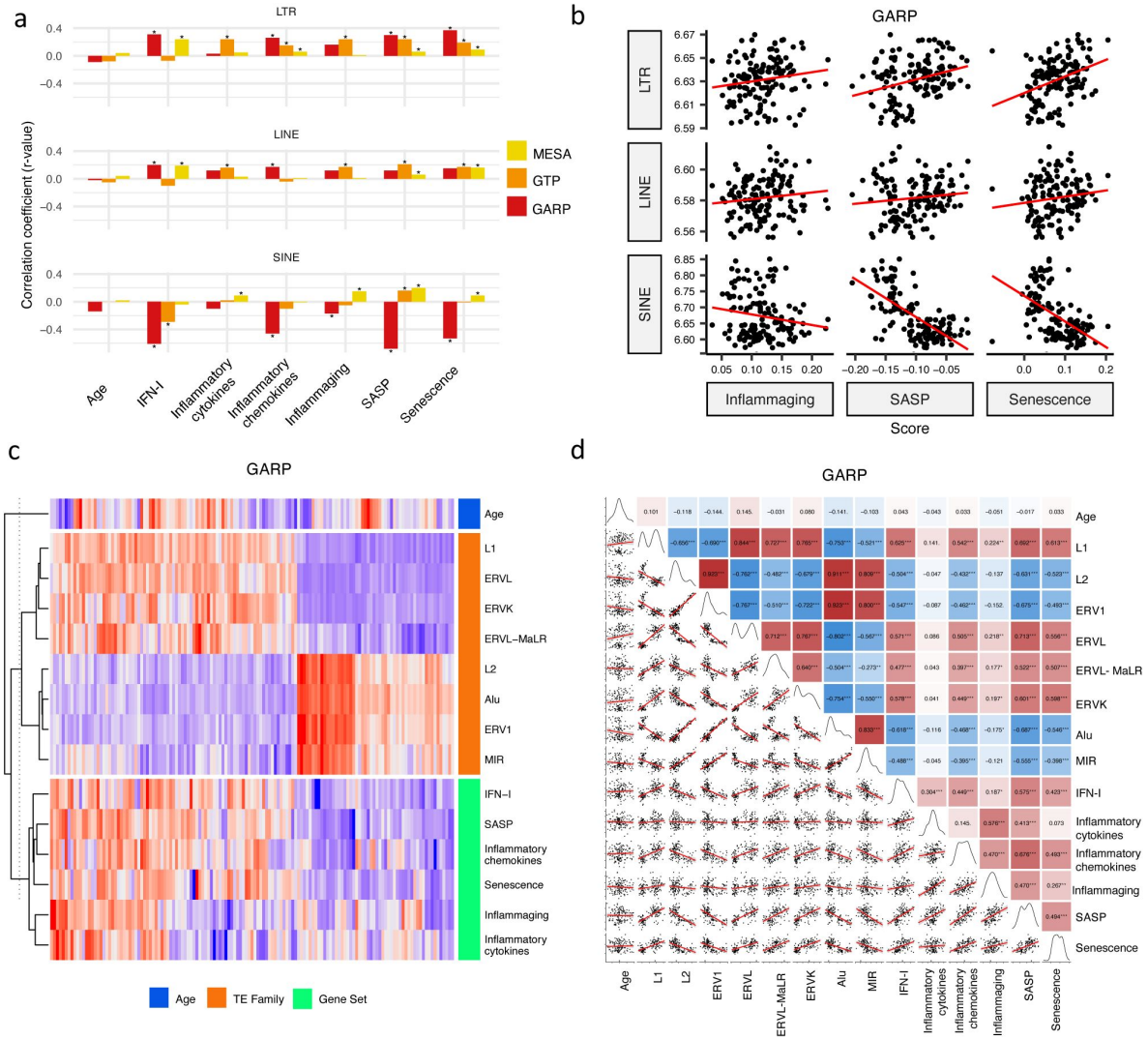

Figure 2.

Correlation analysis between RTEs expression, chronological age, and age-associated gene signature scores. a,

No correlation between RTEs expression and chronological age versus positive correlations between age-associated gene signature scores and LINE and LTR expressions. Pair-wise correlation coefficients were calculated between the expression of different RTE classes (LTR, LINE & SINE) and chronological age and six age-associated gene signature scores in monocytes (MESA), PBMCs (GARP), and the whole blood (GTP). **b**, Scatter plots displaying a positive correlation between LINE and LTR expressions and inflammaging, SASP, and senescence gene signature scores in PBMCs. **c**, Different families of RTEs were divided into two major groups based on their correlation and inverse correlation with age-associated signature scores in PBMC samples. **d**, Correlation matrix depicting all pair-wise combinations to identify the correlation between chronological age, RTE family expressions, and six age-associated signature scores in PBMCs. ** $P \leq 0.01$, *** $P \leq 0.001$, Pearson's correlation. MESA, n=1202; GARP, n=139, GTP, n=359.

correlation analysis between the expression of RTE families and the age-associated gene sets (**Fig. 2c and d**). Interestingly, the expressive patterns of RTE families are divided into two groups that also reflect their positive or inverse correlations with age-associated signature scores in the GARP cohort. In the GARP cohort, the strongest positive correlation with age-associated scores was observed in L1, ERVK, and ERVL. By contrast, the expressions of Alu and MIR of SINE, L2, and ERV1 were inversely correlated with age-associated signature scores in the GARP cohort (**Fig. 2c and d**). This pattern is also observed in PBMCs in the MESA monocyte samples for L1, ERVL, and ERVK, but not other RTE families (**Supplementary Fig.2a and c**). The whole blood GTP cohort showed divergent result to the other two cohorts perhaps because of the absence of certain genes in the six age-associated gene sets (**Supplementary Fig.2a-b** and **Supplementary Table 8**).

To study the association between RTEs expression and age-associated gene signatures, we also split the samples in each cohort based on quartiles of RTEs expression into high, low, and medium (top 25%, bottom 25%, and middle 50%) groups and compared the age-associated gene signature scores across these three groups. Across the datasets, there is an overall trend of increased scores of age-associated gene signatures in high versus low LINE and LTR expression groups (**Supplementary Fig. 6**). In fact, amongst all gene sets, we found the most significant increase for SASP and senescence and to a lesser extent for inflammaging signatures. We observed higher SASP, inflammatory cytokine, senescence, and inflammaging in the healthy monocytes (MESA) in high SINE- and Alu-expressing groups (**Supplementary Fig. 7**). However, in the PBMCs samples of the GARP cohorts, groups of high SINE and Alu expressions demonstrate lower expressions of SASP, inflammatory chemokine, senescence, and inflammaging, that reflects the inverse correlation we observed before (**Fig. 2c**). There are no specific trends for SINE expression in the whole blood samples from the GTP cohort (**Supplementary Fig. 7**). Taken together, SINEs display different pattern than LINEs and LTRs in terms of the association between their expressions and age-related gene signatures. This prompted us to conduct a gene set variation analysis (GSVA)[31] to identify the pathways that are regulated in the high versus low RTE expression groups in the three cohorts (**online methods**).

Elevated SINE expression is linked with the upregulation of the DNA repair pathways, while elevated LINE and LTR expressions are associated with the inflammatory response

We conducted GSVA for high versus low expression groups of RTE classes and families to identify the pathways that might be affected by RTEs expression. We specifically focused on the pathways (gene sets) related to the inflammatory and DNA repair responses retrieved from the Molecular Signatures Database (MSigDB)[32] with “inflammatory” and “dna_repair” as search keywords.

We found that a significant fraction of gene sets related to DNA repair were downregulated in the group of monocyte samples (MESA cohort) highly expressing LINE and LTR classes but upregulated in the monocyte and PBMC samples (MESA and GARP cohorts, respectively) highly expressing SINEs (**Fig. 3a**). At the family level, there is a unique increase in DNA repair activity in the monocytes of the cohort with high Alu expression. In the PBMC samples of the GARP dataset, DNA repair pathways are upregulated in groups highly expressing L2, ERV1, Alu, and MIR, while most of the other RTE classes demonstrate decreased DNA repair activity. In contrast, the inflammatory response is upregulated in the monocyte and PBMC samples of groups expressing LINE and LTR classes and families at high levels, and such upregulation is less common in SINE (**Fig. 3a**). These observations provide insight into the perpetuating contrast between SINE versus LINE and LTR observed in our analysis, as SINE might contribute to aging via genome instability instead of inflammation that is associated with LINE and LTR activities. Overall, our observations are consistent across MESA and GARP cohorts but less so in the GTP cohort (**Fig. 3b**), which might be attributed to less RTE-covering probes and the missing genes from the age-related gene signatures. (**Supplementary Fig. 1a, Supplementary Table 8**).

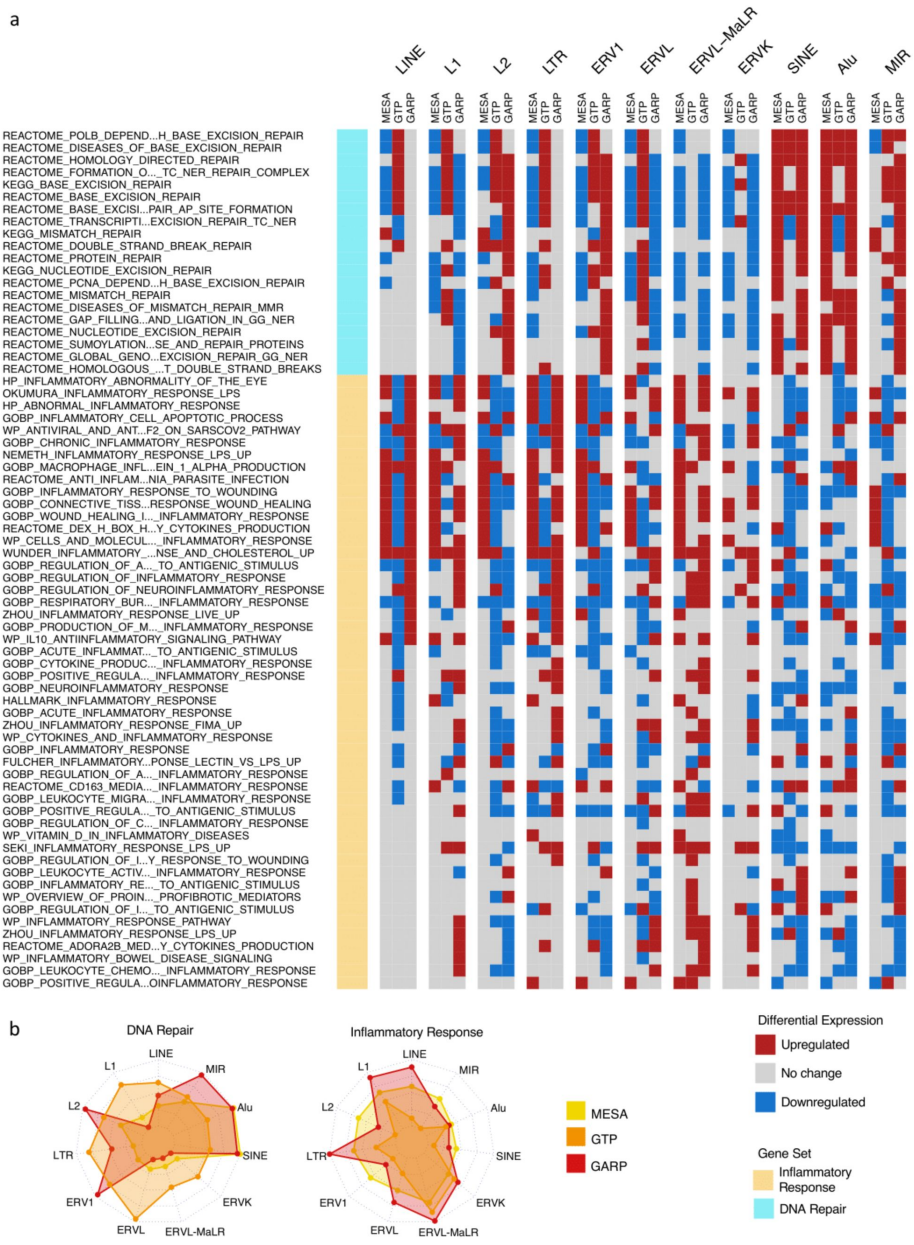

Figure 3.

Upregulation of DNA repair vs inflammatory responses for samples with high expression of SINE vs LTR and LINE in the MESA and GARP cohorts.

a, GSVA demonstrates increased activity of DNA repair pathways in the group of samples with high vs low SINE expression in the MESA and GARP cohorts. In contrast, the inflammatory response is upregulated in the sample groups highly expressing LINE and LTR classes and families in the MESA and GARP cohorts. The samples in each cohort were divided into low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) expression groups based on the expression of RTE classes or families. GSVA was applied on high vs low groups for each class and family of RTEs. The threshold for differential expression is set at $|\log FC| > 0.1$ and $P < 0.05$ (**online methods**). **b**, The Radar plot shows the difference between the number of upregulated versus downregulated gene sets related to DNA repair and inflammatory response in each cohort. While high expression of SINE and Alu is significantly associated with high number of up-regulated DNA-repair gene sets, LINE and L1 expressions are highly related to high number of activated gene sets related to inflammatory response in the MESA and GARP cohorts. This result is not highly supported by the GTP cohort, more likely due to the low number of probes in this cohort.

DNA methylation levels of RTEs inversely correlated with the chronological age and the RTEs expression except for SINE expression

The demethylation of RTEs is suggested to contribute to aging[3,5,7]. To examine the effect of RTEs demethylation on chronological aging, we analyzed DNA methylation data from multiple cohorts including monocytes from the MESA cohort and the whole blood data from GMPWAR, BSGS, SATSA. Our analysis showed an inverse correlation between the methylation level of RTEs and chronological age across all RTE classes and families in the whole blood and monocytes (**Fig. 4a-b** and **Supplementary Fig. 8**). This observed trend is in line with previous reports of demethylation of RTE with age in gene-poor regions[5,21,23,33].

To elucidate the relationship between the expression and methylation level of RTEs, we divided the MESA samples, with matched RTEs DNA methylation and expression, into three groups based on the level of expression of RTEs in different classes and families: low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) expression groups of monocyte samples from the MESA cohort. The DNA methylation levels of LINEs and LTRs significantly decrease in groups of high LINE and LTR expressions, respectively, but SINEs and the satellite repeats (as negative control) do not display the same pattern (**Fig. 4c**). LINE families, MIR, and LTR families except ERVK display lower levels of methylation in higher expression groups, while such pattern is absent in Alu, CR1, and ERVl (**Supplementary Fig. 9**). Although the DNA methylation level of SINEs positively correlates with the methylation levels of LINEs and LTRs, the expression of SINEs is not significantly correlated with the expression levels of other RTE classes (**Fig. 4d**). Overall, we found a distinct pattern for SINEs versus LTRs and LINEs by exploring a relationship between DNA methylation and expression levels of RTEs in monocytes, consistent with our other findings showing different patterns for SINEs versus LTRs and LINEs in PBMCs (see above). This result also suggests a dispensable role of DNA methylation for SINE silencing in monocytes, which is in line with previous studies showing SINEs are not derepressed by deletion of DNA methyltransferases or treatment with DNA demethylating agents and are primarily regulated by histone modifications[34].

Elevated level of RTEs in plasma cells of healthy PBMC samples is associated with the high SASP and inflammaging gene signature scores

As described above, we did not identify any association between RTEs expression and chronological aging for any human cohorts we analyzed. However, the positive correlation between LINE and LTR expressions with age-associated gene signature scores in the PBMC samples of the GARP cohort prompted us to investigate PBMC samples in more detail using PBMC scRNAseq data of 21 samples annotated into 25 cell types by Mogilenko et al.[15](**online methods** and **Supplementary Table 6**). The scRNA-seq samples were divided based on low and high age-associated gene signature scores to compare against the expression of RTE classes for each cell type (**online methods**). Among the 25 annotated cell types, plasma cells consistently demonstrate significantly elevated RTEs expression in cells of high SASP expression that is not observed in the other cell types (**Fig. 5a** and **Supplementary Figs. 10-12**). Increased LINE and SINE expressions were also observed in the plasma cells of the samples with high inflammatory chemokines and inflammaging gene signature scores (**Fig. 5b and c**). Although not statistically significant, elevated RTEs expression was also seen in the groups with high inflammatory cytokines and senescence but not IFN-I scores (**Fig. 5d-f**). Overall, this finding indicates that plasma cells might play an important role in the process in which reactivation of RTEs regulates aging.

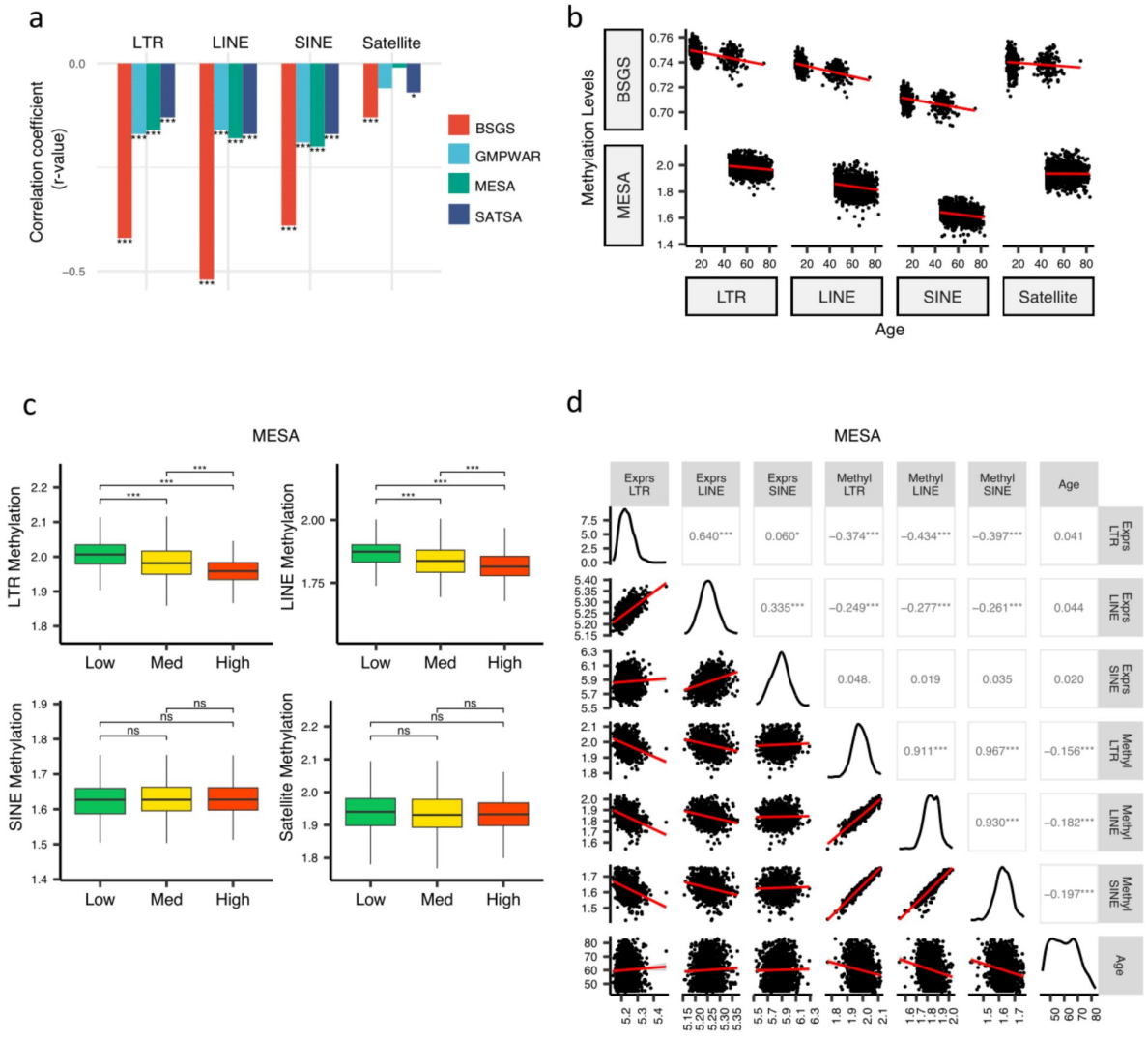

Figure 4.

Inverse correlation of DNA methylation levels of RTEs with the chronological age and the RTEs expression except for SINE expression.

a, b, Methylation levels of RTE classes inversely correlate with chronological age in monocyte (MESA) and whole blood (BSGS, SATSA, and GMPWAR) samples. Satellite DNA is included as a control group. **c**, Methylation levels versus low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) expressions of RTE classes in monocytes (MESA). Wilcoxon test; ns: not significant. **d**, Correlation matrix for RTE expressions and methylation levels, and chronological age. * $P \leq 0.05$, **** $P \leq 0.0001$, Pearson's correlation. MESA, n=1202; BSGS, n=614; GMPWAR, n=656; SATSA, n=1072.

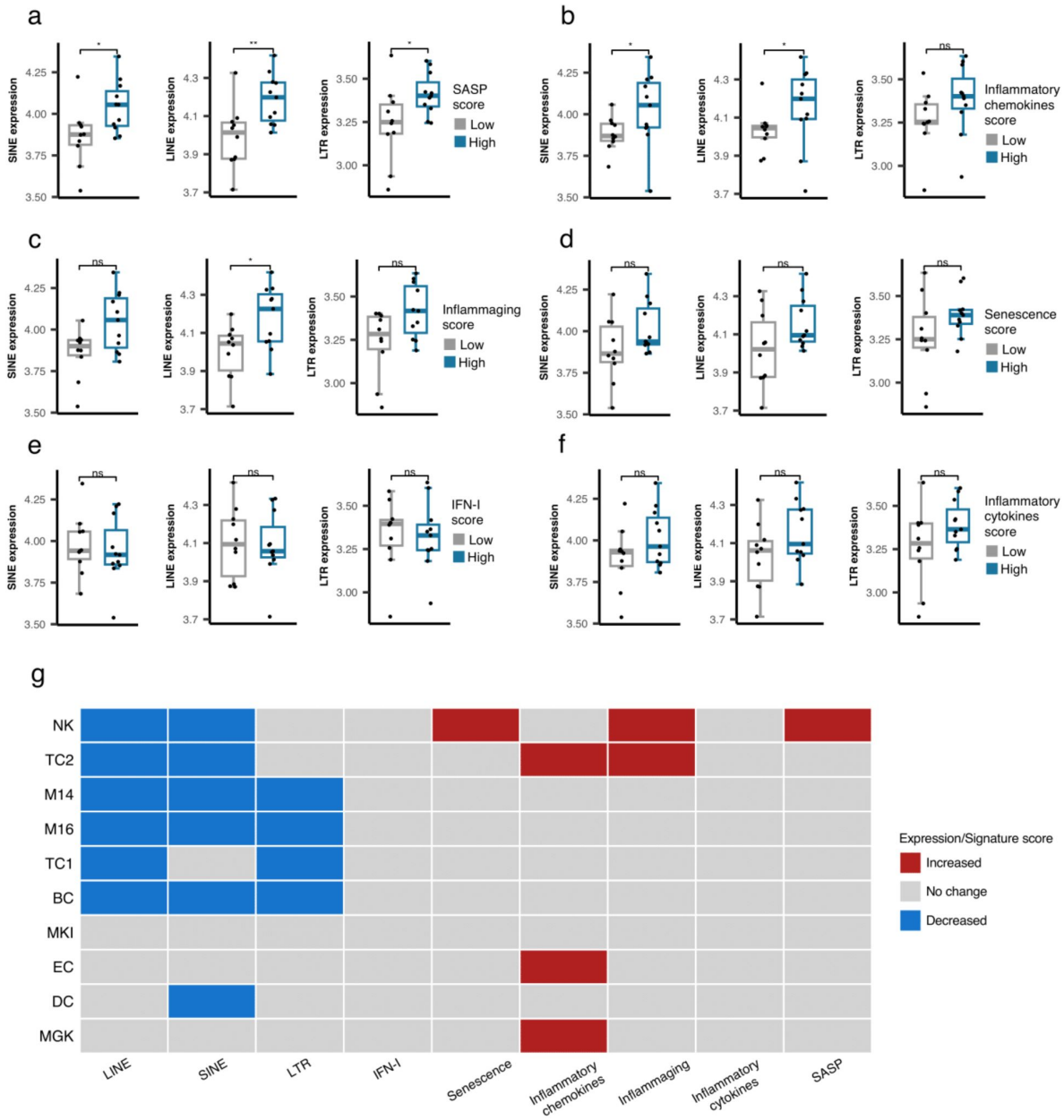

Figure 5.

Cell type-specific analysis of RTEs expression vs age-associated gene signature scores in two PBMC scRNA-seq cohorts.

a-f, Unique increased expression of RTEs in the Plasma B cells of the samples with high age-associated gene signature scores indicates the potential role of plasma B cells in ageing. Wilcoxon test. n = 21. **g,** Decreased expressions of RTE classes and increased age-associated gene signature scores in multiple annotated cell types obtained from the PBMCs of supercentenarians compared to ordinary elderlies as control. Supercentenarians, n=7; control, n=5, age 50s to 80s. Wilcoxon test was applied to identify the significant changes. NK, Natural killer cell; BC, B-cell; TC1, T-cell 1; TC2, T-cell 2; M14, CD14+ monocyte; M16, CD16+ monocyte; EC, Erythrocytes; MKI, MKI67+ proliferating cell; DC, Dendritic cell; MGK, Megakaryocyte.

Downregulation of RTEs expression in supercentenarians versus normal aged cases despite high age-associated signature scores in NK and T cells of supercentenarians

Supercentenarians who have reached 110 years of age are an excellent resource for investigating healthy aging. We hypothesized that supercentenarians should express RTEs less than normal-aged people because RTEs expression, particularly LINE and LTR expressions, can activate the inflammatory pathways (**Fig. 3**), which would be generally harmful to healthy aging. To understand whether there is any RTEs expression change in supercentenarians versus normal-aged people and identify the relationship to age-associated gene signatures, we compared the scRNA-seq data of the PBMCs of seven supercentenarians (over 110 years) to five non-centenarian (50 to 80 years) controls[16]. We collected the annotated cells of these twelve cases as a Seurat object for which the cells were annotated into ten different cell types. Compared with the control group, the supercentenarians demonstrate decreased RTEs expression in the natural killer (NK) cells, B-cells, T-cells, monocytes, and dendritic cells (**Fig. 5g** and **Supplementary Figs. 13**). Both T cell types show decreased LINE expression, while the noncytotoxic cluster, TC1, shows slightly decreased LTR expression, and the expanding cytotoxic T cells, TC2, show decreased SINE expression. On the other hand, the cytotoxic T cells also show an increased level of inflammatory cytokines and inflammaging gene signature scores. In addition, the NK cells show higher senescence and SASP scores in the supercentenarians while demonstrating decreased LINE and SINE expressions (**Fig. 5g** and **Supplementary Figs. 14-15**). Overall, this result reveals inflammatory activity in cytotoxic T cells and increased senescence in the NK cells that is not associated with the activation of RTEs in supercentenarians. By contrast, the expression of RTEs is significantly reduced in most of the immune cell types in supercentenarians.

Discussion

The role of RTEs in aging has been studied extensively but not in a systematic manner with large, non-cancerous human cohorts across RTE categories before this study. Current understanding of the relationship between RTEs and aging are largely conducted *in vitro* or in model organisms[7,9,33,35] that might not apply to human due to intraspecies differences[36]. Moreover, the link of RTEs to aging in non-cancerous human cohorts has not been studied systematically in large cohorts.

We combined bulk and single-cell transcriptomic data to examine the relationship between RTEs expression versus chronological and biological aging, demonstrating that chronological aging is not significantly linked with RTEs reactivation. In this process, we discovered that LTR and LINE expressions are positively correlated with the age-related gene signature scores. However, in contrast, the SINE expression demonstrates an inverse correlation with age-related scores in the PBMCs of osteoarthritis patients and no significant relationship in the whole blood of healthy individuals. We suspect the absence of a positive correlation between age-associated scores and SINE expression might be linked to the same discrepancy observed in the GSVA analysis, in which DNA repair pathways are upregulated in the groups of cases with high SINE and Alu expressions. This result suggests that reactivation of SINE, particularly Alu, might lead to DNA damage, a hallmark of aging. Although SINEs compose up to 13% of the human genome, and Alu elements make up the largest family of human mobile elements, Alu is not studied as extensively as L1 from the LINE class[1,37]. Therefore, this novel discovery requires further study to confirm the connection and elucidate the mechanism by which DNA repair might be linked to the derepression

of SINE and Alu expressions. In contrast, the groups of cases with high LINE and LTR expressions have upregulation of the pathways related to the inflammatory response, which is in line with the previous findings from human and model organisms[3,35].

The mechanisms of aging and age-related diseases (ARDs) converge on inflammaging[38]. Previous studies have shown RTE's pathogenetic role in ARDs including neurodegenerative diseases through elevation of DNA damage and genome instability[39–41]. SINE expression inversely correlated with the age-related signature scores in the PBMCs of osteoarthritis patients, while a positive correlation is observed in healthy monocytes. Moreover, a discrepant upregulation of DNA repair pathways is seen in the osteoarthritis cohorts expressing higher level L2, ERV1, Alu, and MIR. With osteoarthritis being a typical ARD, our analysis provides insight into the relationship between aging and RTEs in an ARD state in comparison with that of healthy individuals.

The relationship between DNA methylation and expression of RTEs with age was also investigated in our study. The methylation levels of all three RTE classes decrease with chronological age in our analysis, and the RTEs expression is in inverse correlation with the methylation level in LINE and LTR but not in SINE. DNA methylation at the CpG sites is an important mechanism for RTE repression; through the recruitment of DNA and histone methyltransferases, RTE sequences are repressed via DNA methylation and heterochromatinization[42]. In the aging process, surveillance of transcriptional regulators such as SIRT6 wanes, leading to hypomethylation and reduced heterochromatin repression[4]. L1 has been identified to be repressed through DNA methylation[43]. However, SINE has been suggested to be predominantly regulated by histone modification, and loss of DNA methylation has little effect on the transcription of SINEs[34], as reflected in our analysis.

Although expression microarrays have been extensively employed to study the expression of genes, they were not systematically used for studying the expression of RTEs. Here, we select the probes that cover the RTE regions to extract RTEs expression. The probe design of microarrays does not accommodate RTEs specifically, owing to its repetitive and interspersed nature[44]. To circumvent this issue, our analysis was conducted on the class and family levels but not subfamily levels of RTEs to retain enough probes (**Supplementary Fig. 1**) providing sufficient power for calculating the expression scores. We also supported our findings with scRNAseq, particularly when investigating the association between RTEs expression and chronological age, to circumvent the potential constraints posed by using microarray data. However, we are aware of the limitations imposed by using microarray in this study, particularly the low number of intergenic probes in the expression microarray data. Our study can be enriched with the advent of large RNA-seq cohorts for aging studies in the future. In summary, our analysis provides an overview of the relationship between the chronological and biological aging versus RTEs expression and methylation using multi-omics data from large human cohorts. Our analysis of the transcriptomics data showed no relation between RTEs expression and chronological aging. In contrast, the expressions of LINE and LTR are positively correlated with the age-related signature scores, showing a strong correlation with biological aging. The high expression of SINEs, on the other hand, is associated with an upregulation of DNA repair pathways in the PBMC cohort. Our exploration of multiple cell types based on a published PBMC scRNA-seq cohort unveiled plasma cell as the only cell type for which the expression of RTEs correlates with age-related signature scores. This matches a recent finding showing a late senescent-like phenotype marked by accumulation of TEs in plasma cells during pre-malignant stages[45]. Lastly, we explored a supercentenarian PBMC scRNA-seq cohort as a model for healthy aging and identified that RTEs expression is generally decreased in supercentenarians. However, the age-related signatures scores, particularly senescence and SASP scores, increased in the NK cells in supercentenarians versus normal aged group, highlighting immunosenescence as an important factor even in healthy aging.

Acknowledgements

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Code availability

Scripts and data used in this study are available on Github (*https://github.com/Karimi-Lab/TE_aging_manuscript*).

Methods

Illumina HT12-v4 probes

The probe lists of Illumina Human HT-12 V3 (29431 probes) and V4 (33963) share 29311 probes in common, which is 99.6% of the list of V3. Therefore, we proceeded with HT-12 V4 probes throughout the analysis to cover all studies that used either V3 or V4. To identify the Illumina probes covering RTE regions, we first filtered out the Illumina Human HT-12 v4 probes covering intergenic regions, obtaining a list of 924 unique probes. We then overlapped these probe locations with RepeatMasker[14] regions to acquire the probes covering RTE regions. In total, we could find 232 unique probes covering RTE regions.

Generating gene signature scores using *singscore*

For each of the curated gene sets, instead of looking at individual gene expressions, we used *singscore* (version 1.20.0)[30], a method that scores gene signatures in single samples using rank-based statistics on their gene expression profiles, to calculate the gene set enrichment scores. We first compiled the microarray expression matrix for average expression values from RPM values using *limma*[46] package in R, then we used the *rankGenes* function from the *singscore* package to rank each gene sample-wise. Eventually, the *multiScore* function was used to calculate signature scores for all six gene sets at once.

Statistical analyses

All statistical analyses were performed in R (version 4.3.0). Pearson correlation test was used to determine the r and *P* values in correlation analyses. For differential expression analysis, the Wilcoxon test is used to determine the significance. The threshold to determine significance is set at P value < 0.05.

Gene Set Variation Analysis (GSVA)

GSVA was performed using the *GSVA* package (version 1.48.3)[31] in R to compare the groups of low and high RTE expression groups. The gene lists were retrieved from the Molecular Signatures Database (MSigDB)[32] by searching for "inflammatory" and "dna_repair" as keywords. We further removed pathways that ended with "_DN" (as for downregulated) or "_UP" (as for

upregulated) to reduce repeat and confusion. Three repetitive gene sets were removed from our analysis (GOBP_POSITIVE_REGULATION_OF_ACUTE_INFLAMMATORY_RESPONSE, GOBP_POSITIVE_REGULATION_OF_CYTOKINE_PRODUCTION_INVOLVED_IN_INFLAMMATORY_RESPONSE, GOBP_POSITIVE_REGULATION_OF_ACUTE_INFLAMMATORY_RESPONSE_TO_ANTIGENIC_STIMULUS). The filtering process generated 50 gene sets for inflammatory response and 21 gene sets for DNA repair. To detect any alterations in gene expression, the log fold change threshold was kept low at $|\log FC| > 0.1$, and a significance threshold of $P < 0.05$ was set.

ScRNA-seq analysis workflow for RTEs

Two single-cell transcriptomic datasets of healthy human cohorts were adopted in this study (**Supplementary Table 6**). To obtain the RTEs expression in single-cell sequencing (scRNA-seq) data, the scRNA-seq bam files were processed through scTE[47] pipeline. More specifically, for each cell in each sample, the read counts for different classes and families of RTEs were generated using the scTE method. Subsequently, in each sample, we calculated the cumulative read counts for each RTE class per annotated cell type by adding the number of reads belonging to all the cells of each cell type. This resulted in pseudo-bulk read counts of RTE classes for different cell types in each sample.

In parallel, we also calculated the pseudo-bulk read counts for all genes per cell type in each sample using the genic read counts embedded in Seurat[48] objects collected for the two scRNA-seq datasets that we employed in our study (**Supplementary Table 6**). Concatenating pseudo-bulk RTE and gene read counts, we performed Reads Per Million (RPM) normalization per cell type per sample. For each cell type, the denominator for RPM normalization was the cumulative counts of genes and RTE classes for that cell type.

To calculate the age-associated gene signature scores, we converted the scRNA-seq read counts to bulk RNA-seq read counts by summing up the number of reads of all cells of the same type for each gene. This resulted in a bulk RNA-seq gene count matrix from which we calculated each sample's RPM values per gene. Next, the RPM matrix was provided to *singscore* to calculate gene signature scores. Lastly, we divided the samples into high vs low gene signature scores by using the median as the cut-off.

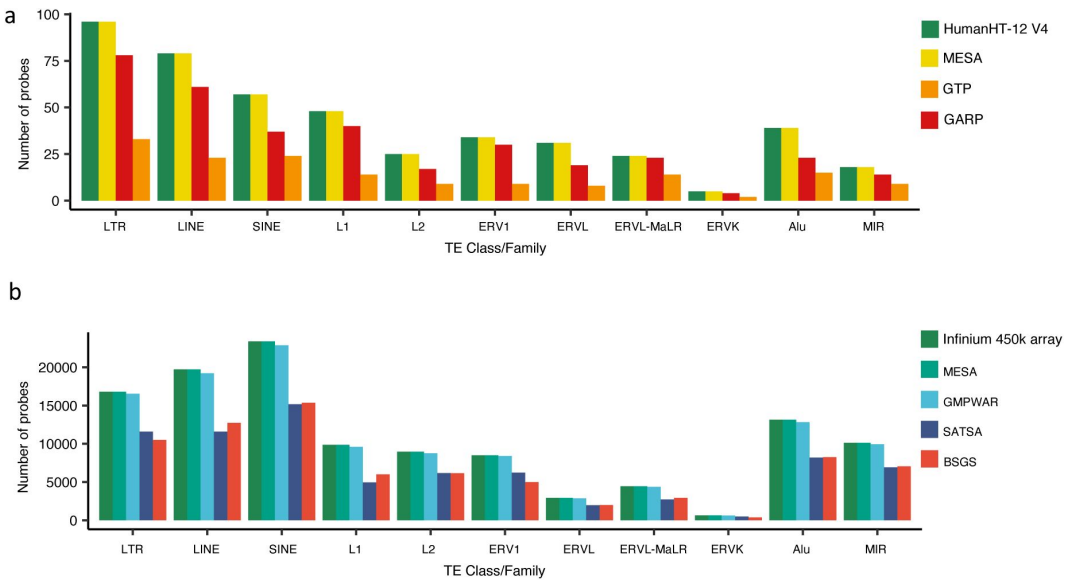
Supplementary Figures

Supplementary Notes

Descriptions of the study populations included in the analysis

Grady Trauma Project (GTP)

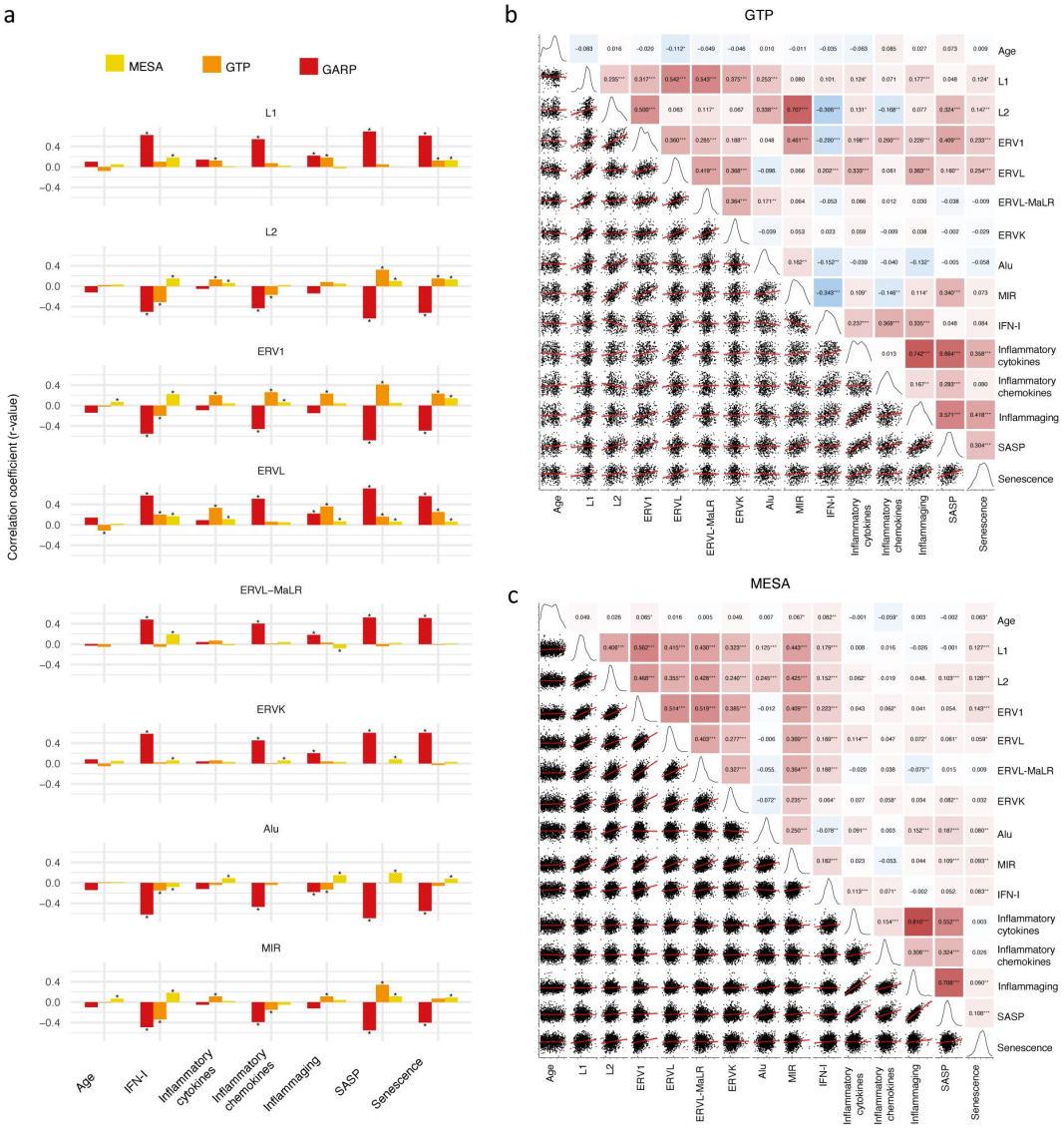
The research concentrated on African American individuals of low socioeconomic backgrounds who had undergone trauma, primarily recruited from Atlanta's Grady Memorial Hospital between 2005 and 2007 (Charles F. Gillespie et al., 2009). Exclusion criteria comprised mental retardation, active psychosis, or inability to provide informed consent. With over 7,000 participants, mostly urban African Americans with low socioeconomic status, the GTP specifically targeted a demographic with a history of significant trauma. The study involved whole blood collection between 8 to 9 a.m. following overnight fasting. Whole genome expression profiles were generated for 398 subjects at the Max-Planck Institute using Illumina HT-12 v3.0 or v4.0 arrays, focusing on 359 subjects of self-reported African American ancestry aged between 16-78 years. The expression



Supplementary Figure 1.

Number of RTE-covering probes.

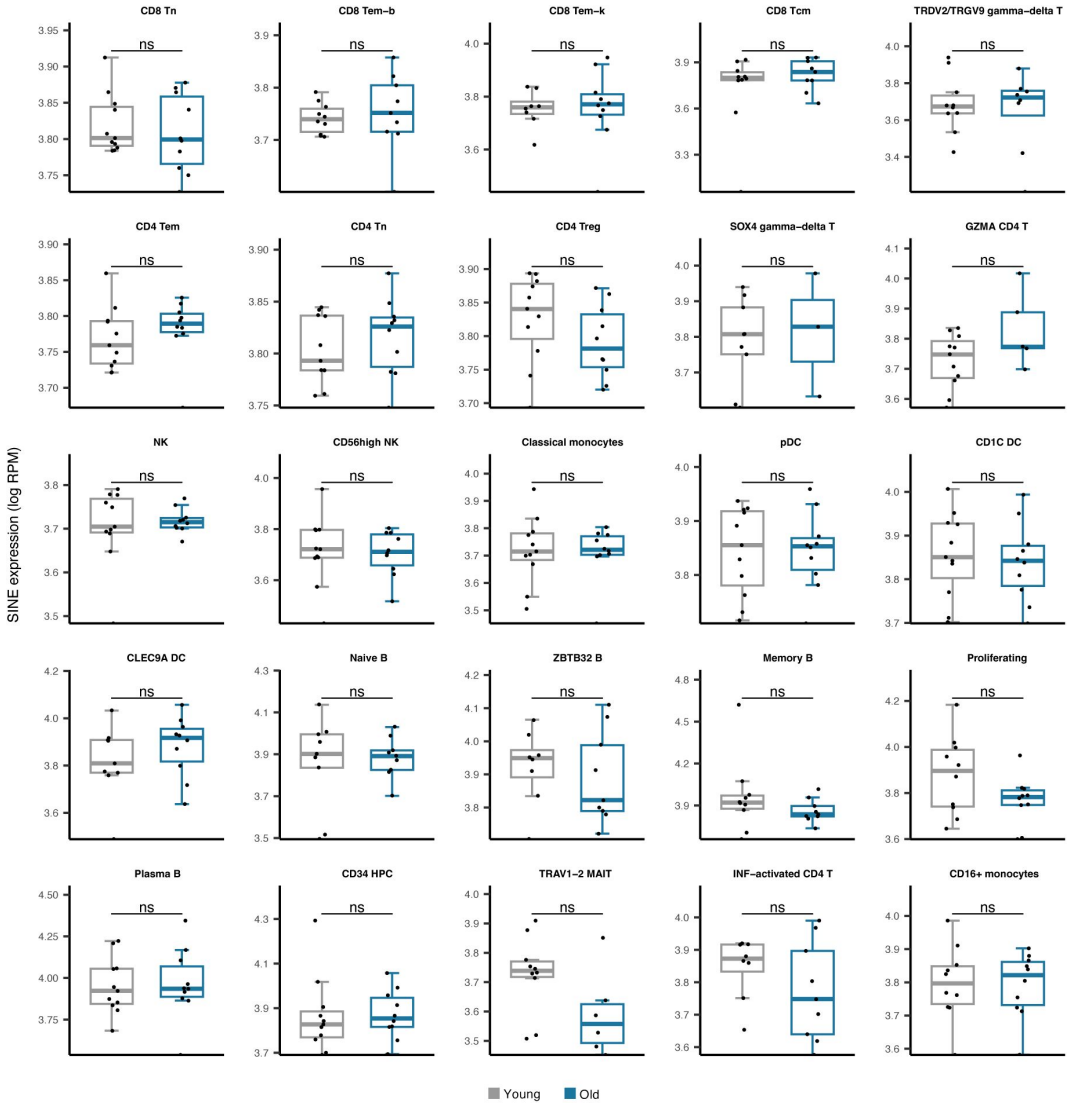
a, The number of microarray probes covering RTE regions in MESA, GTP, and GARP compared to the total number of RTE probes available in Illumina HumanHT-12 V4. **b**, The number of microarray probes covering RTE regions in MESA, GMPWAR, SATSA, BSGS compared to the total number of RTE probes available in Illumina Infinium 450k array.



Supplementary Figure 2.

Correlation analysis between age-associated gene signatures and RTE family expressions in human cohorts.

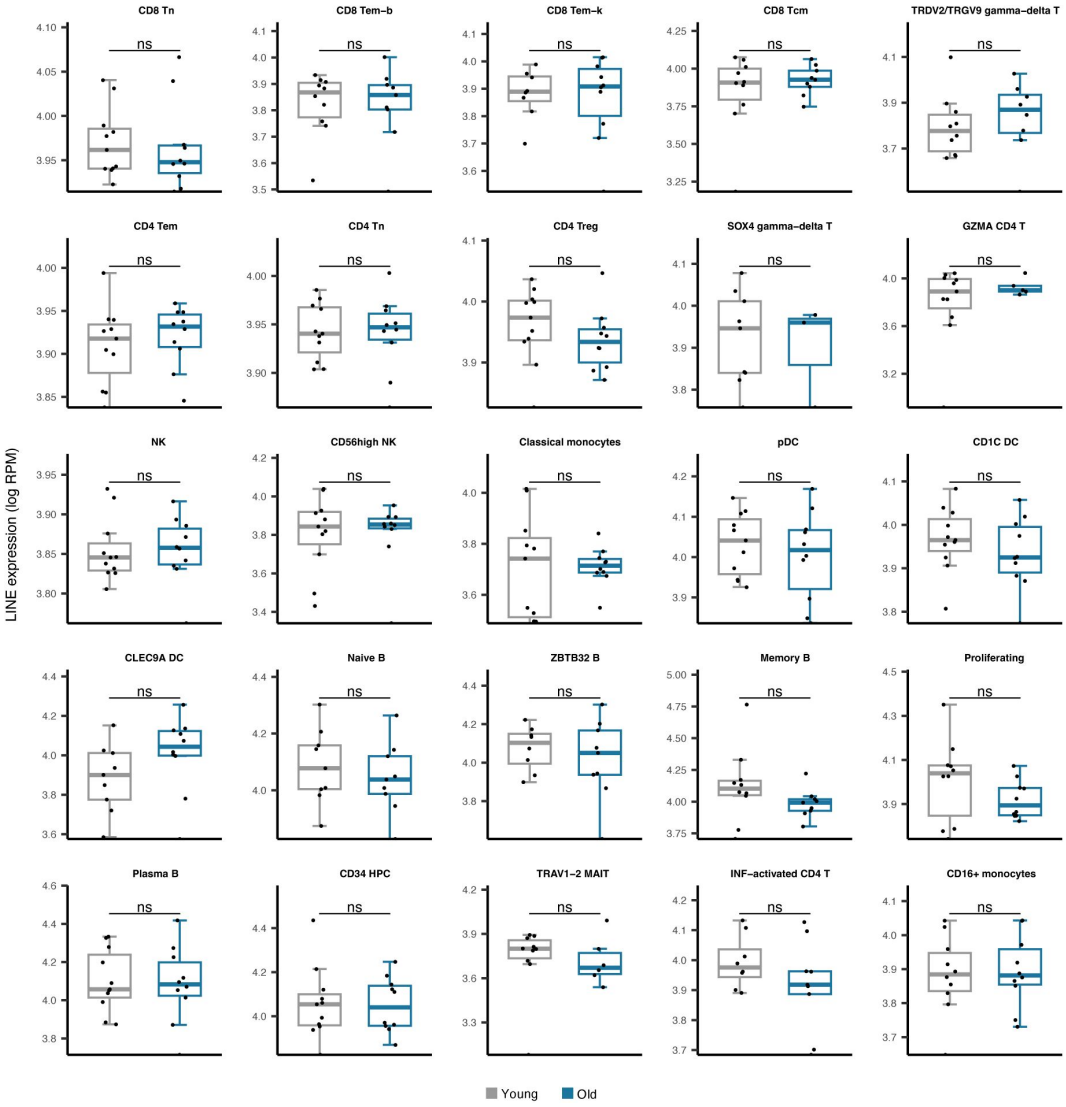
a, Weak correlation between a few RTE families and chronological age and strong positive correlation between L1, ERVL, ERVK, and MaLR with age-associated signature scores in PBMC samples (GARP cohort). **b, c**, Correlation matrix depicting all pair-wise combinations to identify the correlation between chronological age, RTE family expressions, and six age-associated gene expressions in monocytes (MESA) and the whole blood (GTP). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, Pearson's correlation.



Supplementary Figure 3.

Comparing SINE expression in 25 cell types obtained from young versus old PBMC human samples.

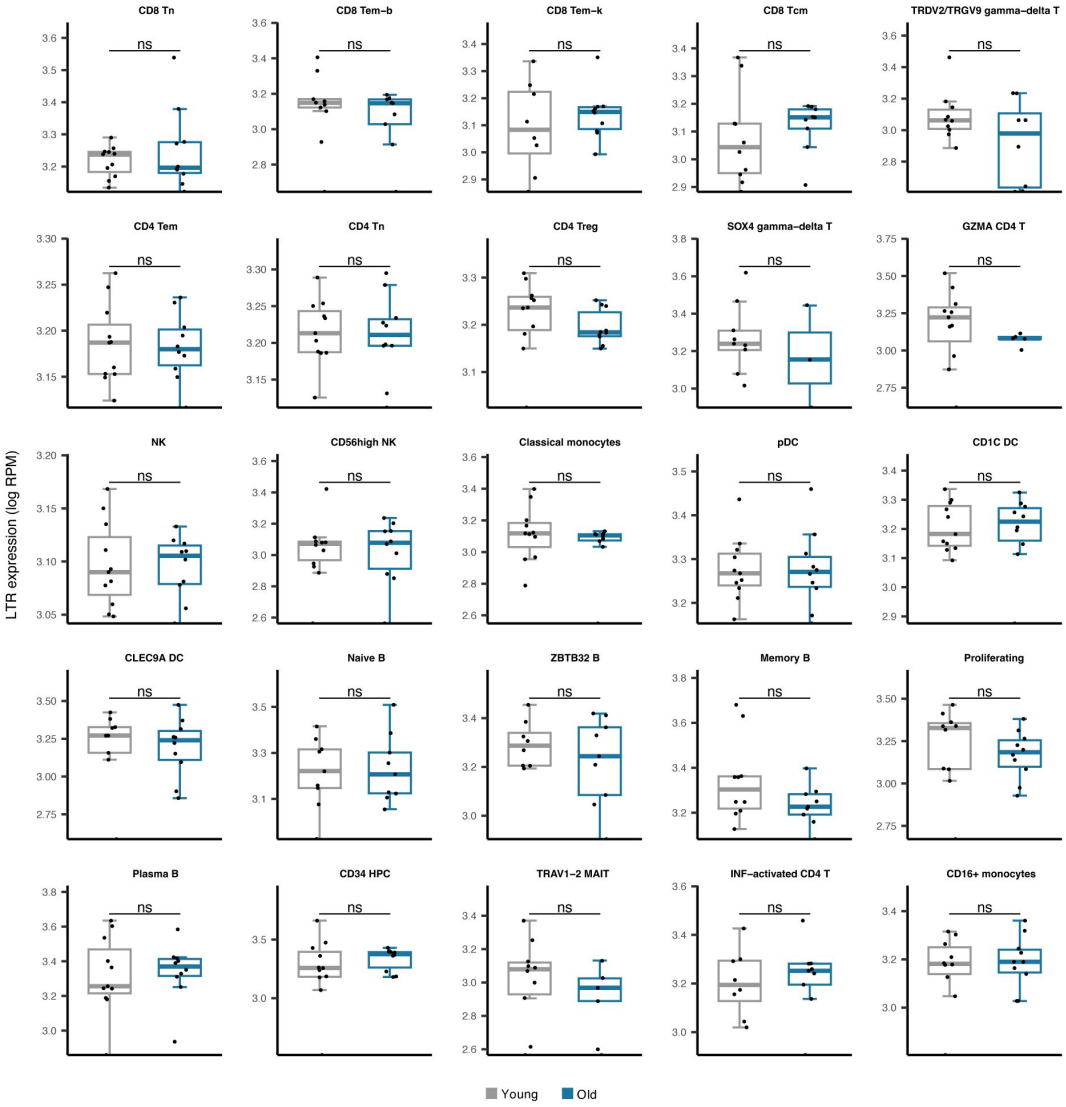
No significant difference was observed in any cell type. The young group comprises healthy male donors aged 25 to 29, n=11; the old groups are healthy male donors aged 62 to 70, n=10; Wilcoxon test; ns: insignificant.



Supplementary Figure 4.

Comparing LINE expression in 25 cell types obtained from young versus old PBMC human samples.

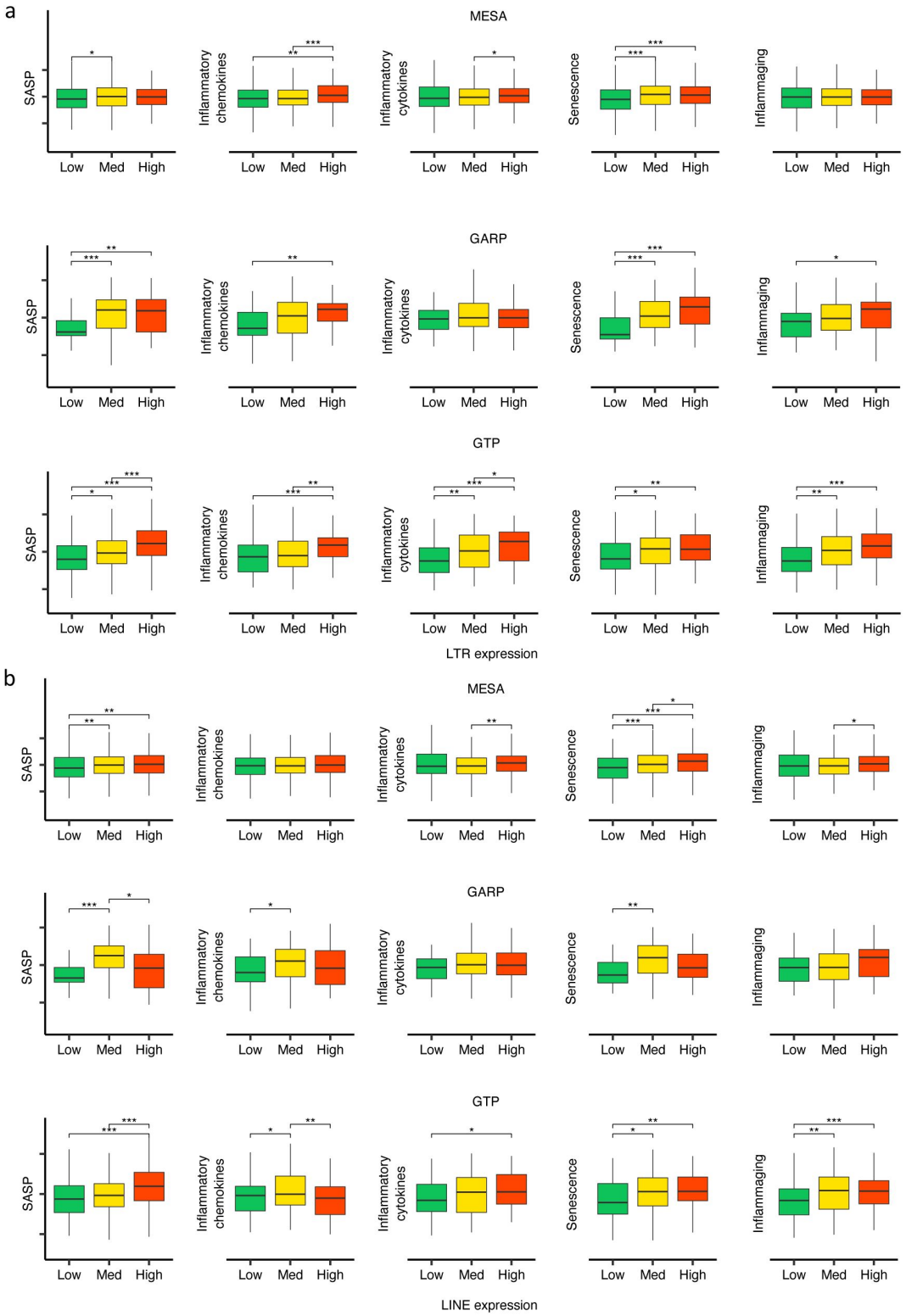
No significant difference was observed in any cell type. The young group comprises healthy male donors aged 25 to 29, n=11; the old groups are healthy male donors aged 62 to 70, n=10—Wilcoxon test; ns: insignificant.



Supplementary Figure 5.

Comparing LTR expressions in 25 cell types obtained from young versus old PBMC human samples.

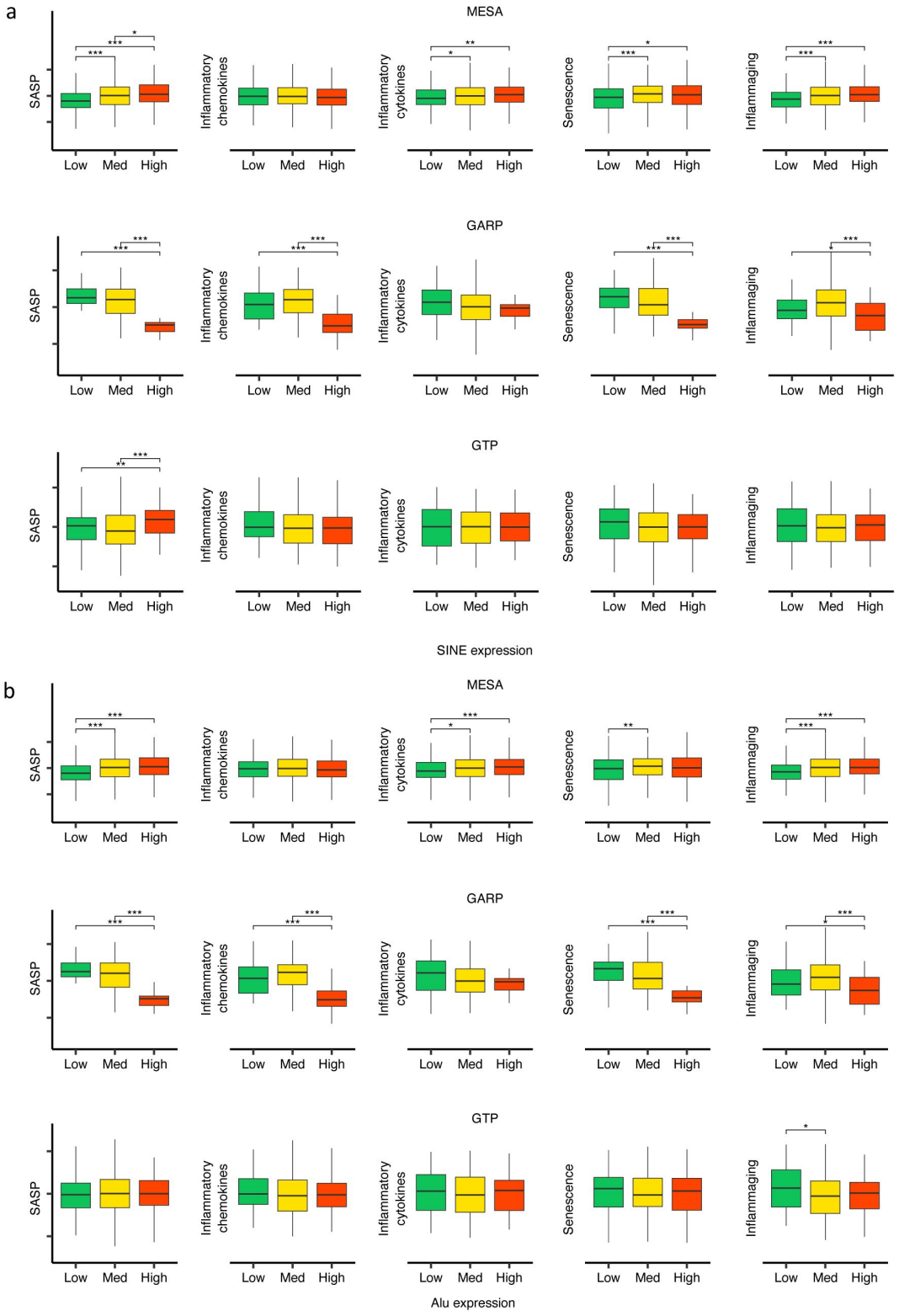
No significant difference was observed in any cell type. The young group comprises healthy male donors aged 25 to 29, n=11; the old groups are healthy male donors aged 62 to 70, n=10—Wilcoxon test; ns: insignificant.



Supplementary Figure 6.

Increasing trend of age-associated gene signature scores in high vs low LTR and LINE expression groups in the three human cohorts.

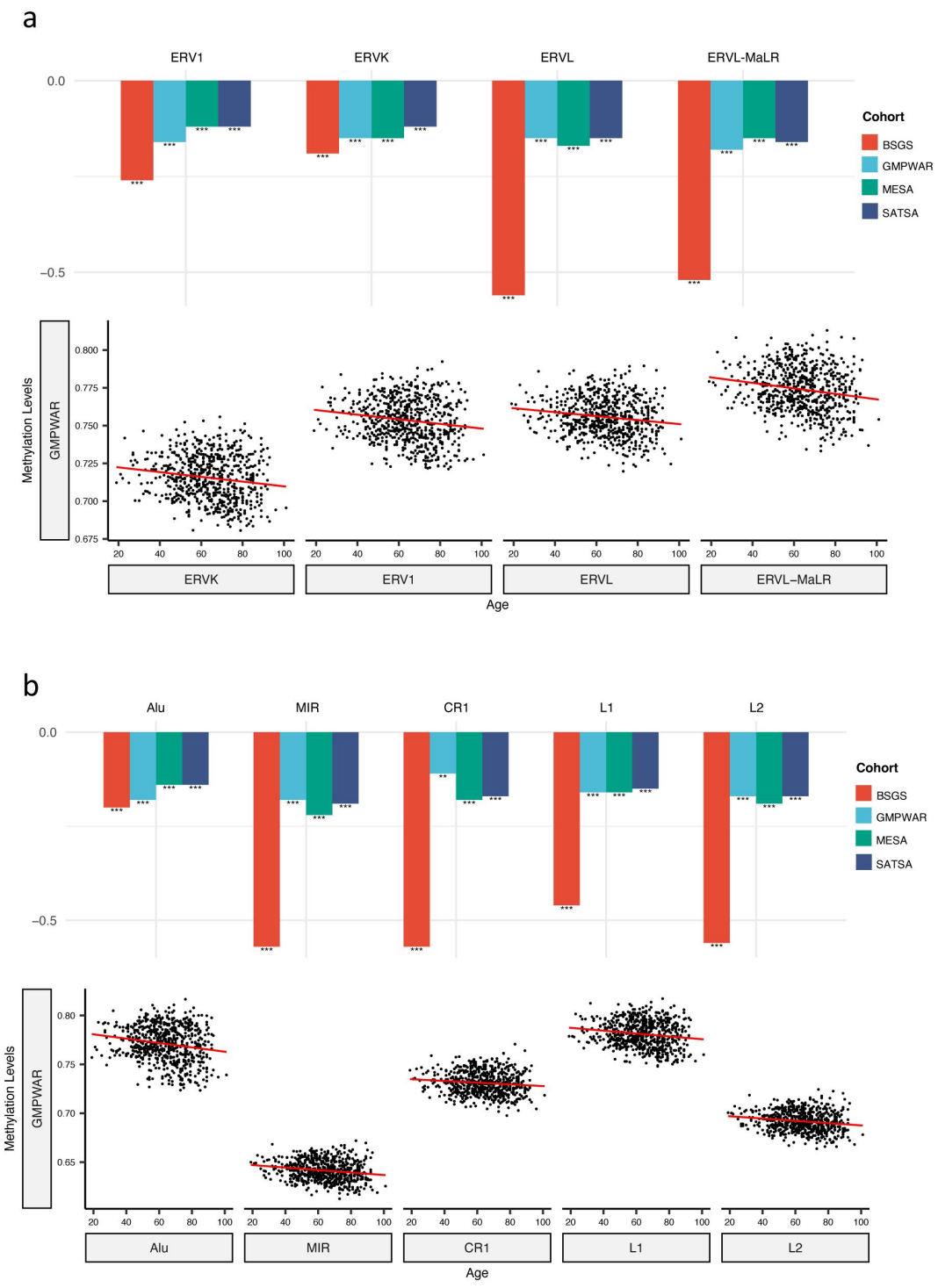
a,b The samples in each cohort were divided into **(**low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) LTR and LINE expression groups, respectively. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$,



Supplementary Figure 7.

Different patterns of age-associated signature scores in MESA vs GARP vs GTP for high vs low SINE and Alu expression groups in the three human cohorts.

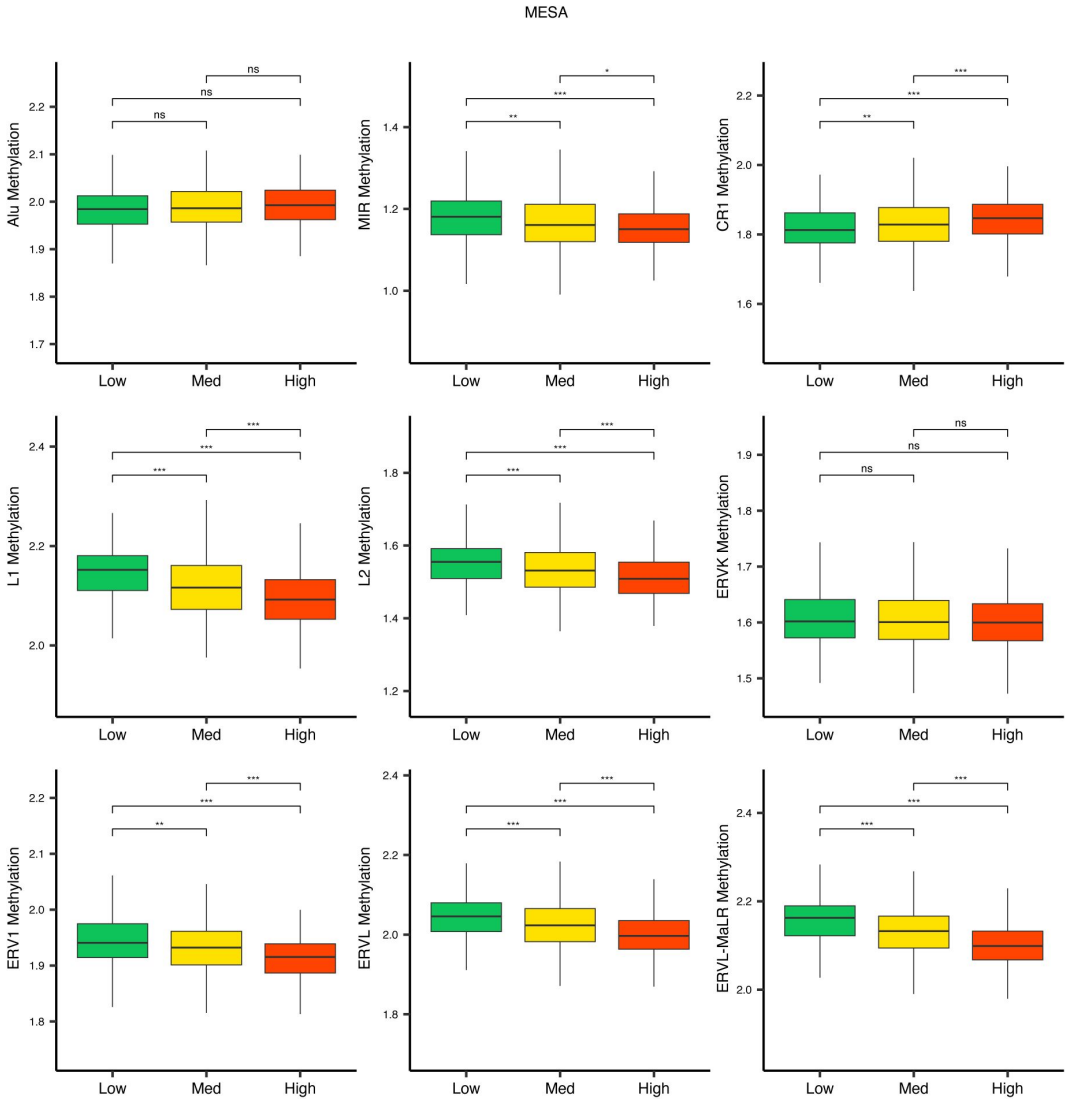
a, b, The samples in each cohort were divided into low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) SINE and Alu expression groups, respectively. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq$



Supplementary Figure 8.

Correlation of methylation levels of RTE families with chronological age in monocyte (MESA) and whole blood (BSGS, SATSA, and GMPWAR) samples.

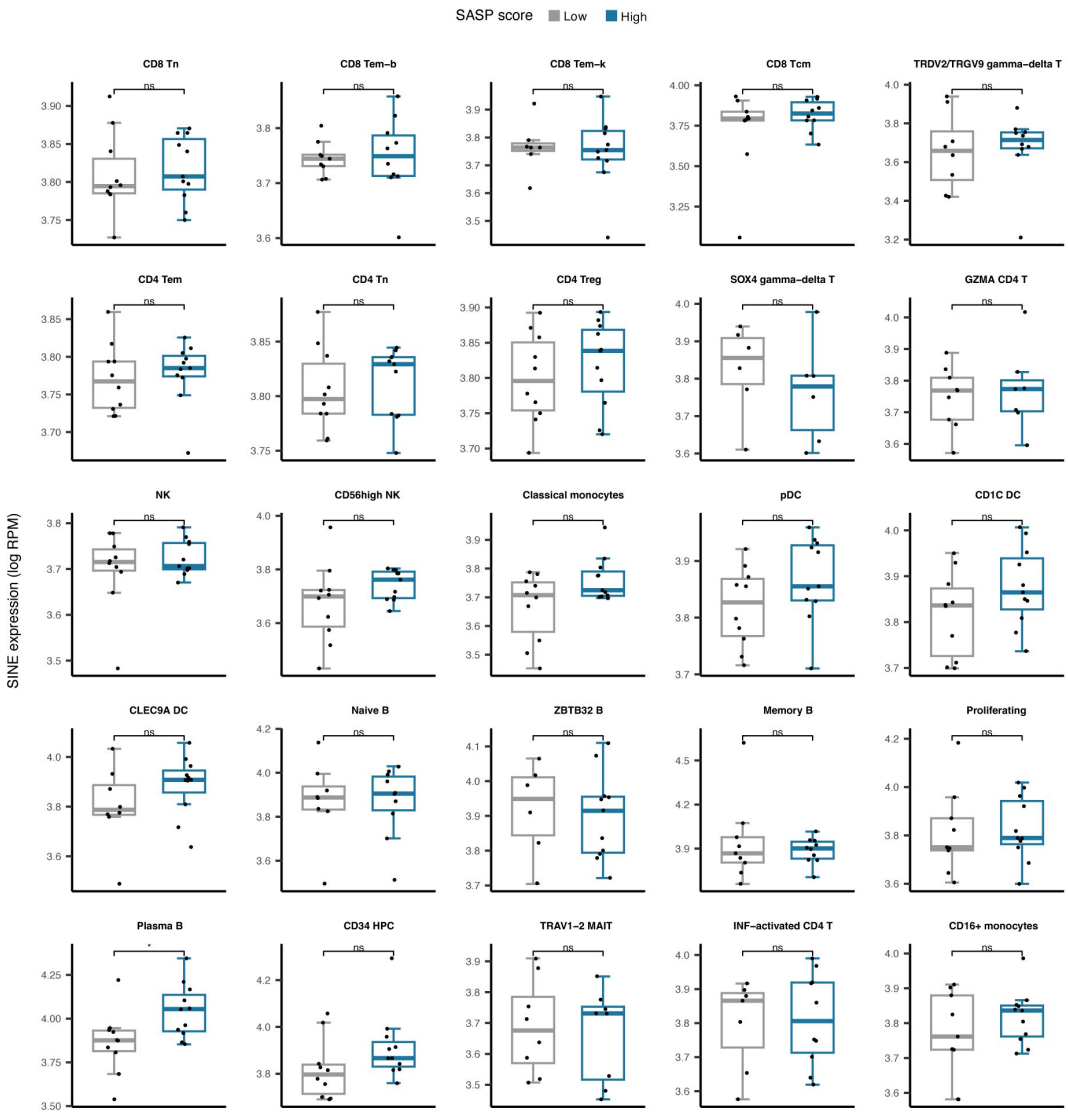
a, b, Methylation levels of LTR and LINE/SINE families negatively correlate with chronological age in monocytes (MESA) and the whole blood (BSGS, SATSA, and GMPWAR). *** $P \leq 0.001$, Wilcoxon test.



Supplementary Figure 9.

Methylation levels versus low (1st quartile), medium (2nd and 3rd quartile), and high (4th quartile) expressions of RTE families in monocytes.

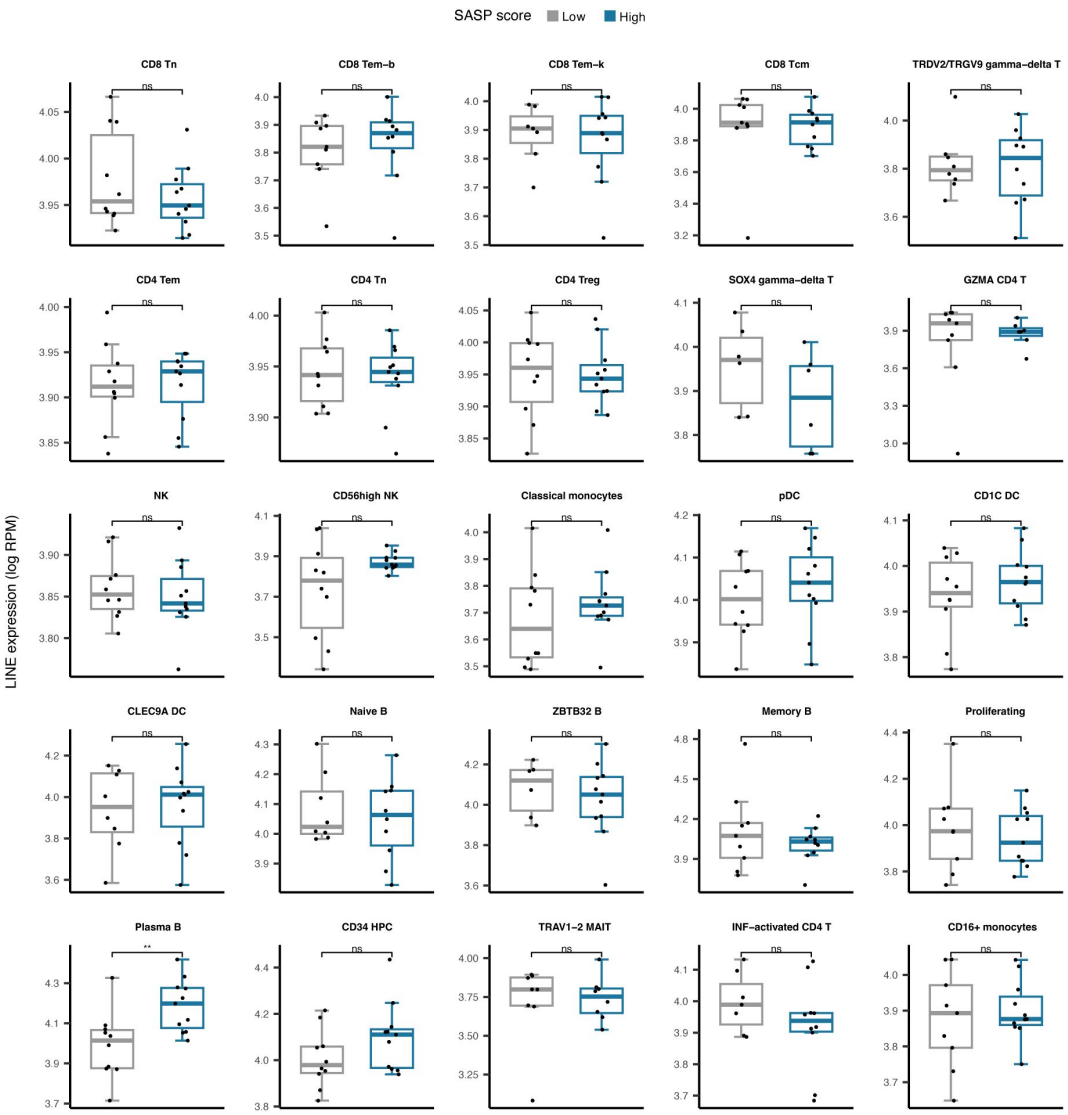
While LINE families, MIR, and LTR families except ERVK show lower levels of methylation in higher expression groups, this pattern is not seen in Alu, CR1, and ERVl. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns: not significant, Wilcoxon test.



Supplementary Figure 10.

Cell type-specific SINE expression versus low and high SASP score groups among 25 cell types in PBMC.

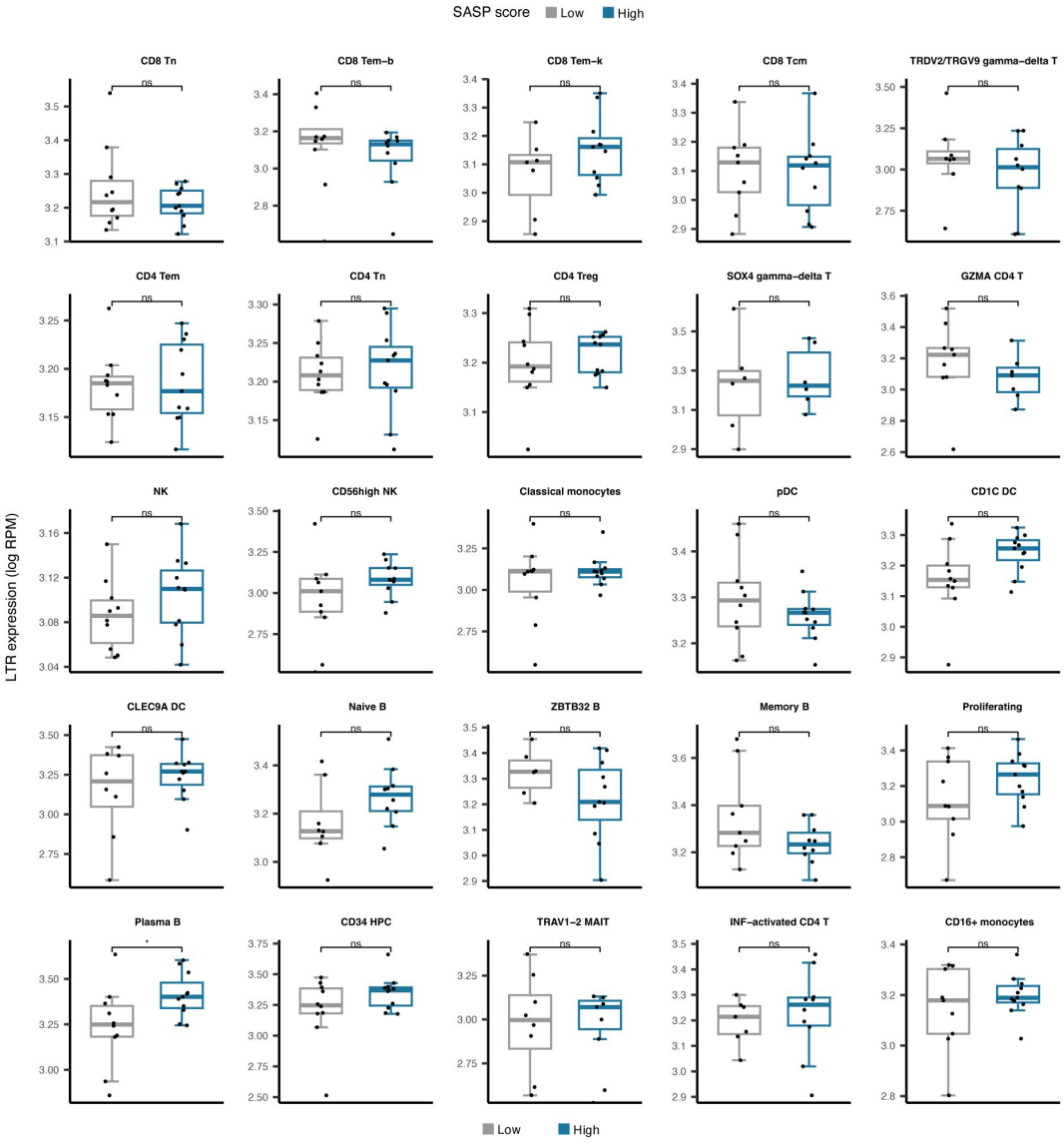
Plasma B cells demonstrate significantly elevated SINE expression in samples with high SASP scores. * $P \leq 0.05$, ns: not significant, Pearson's correlation.



Supplementary Figure 11.

Cell type-specific LINE expression versus low and high SASP score groups among 25 cell types in PBMC.

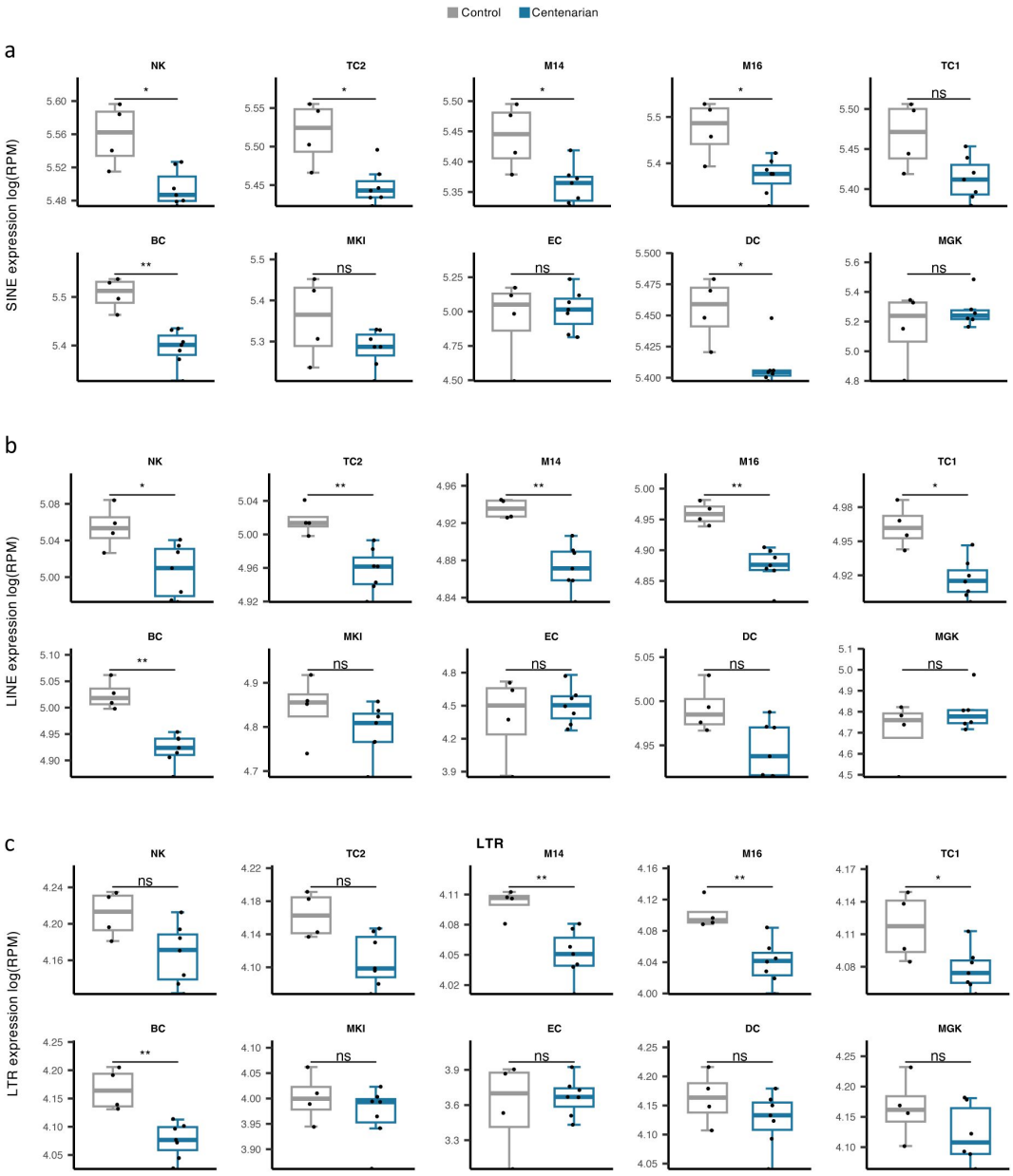
Plasma B cells demonstrate significantly elevated LINE expression in samples with high SASP scores. * $P \leq 0.05$, ns: not significant, Pearson's correlation.



Supplementary Figure 12.

Cell type-specific LTR expression versus low and high SASP score groups among 25 cell types in PBMC.

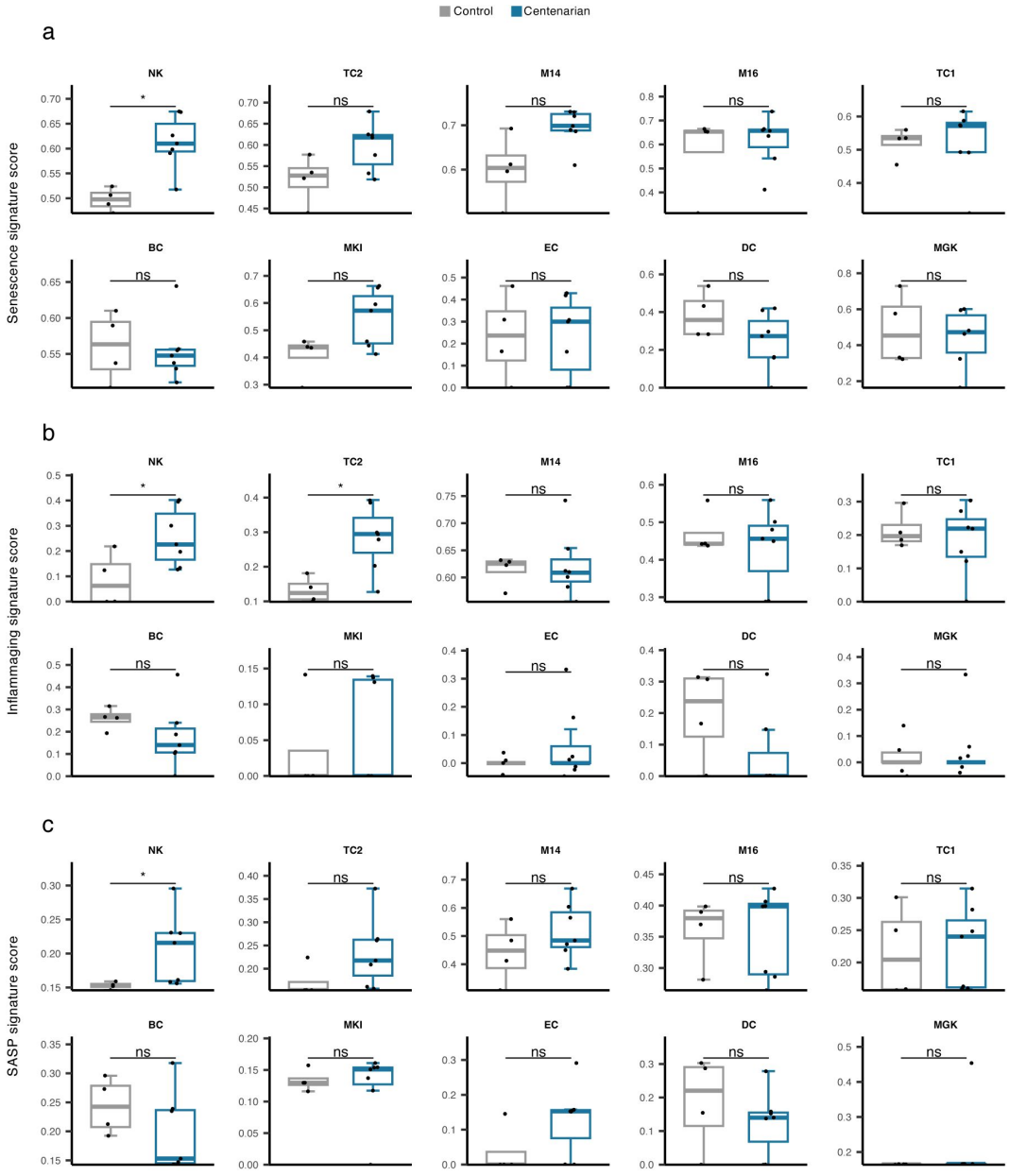
Plasma B cells demonstrate significantly elevated LTR expression in samples with high SASP scores. * $P \leq 0.05$, ns: not significant, Pearson's correlation.



Supplementary Figure 13.

Comparison of cell type-specific RTEs expression in supercentenarians versus normal aged cases.

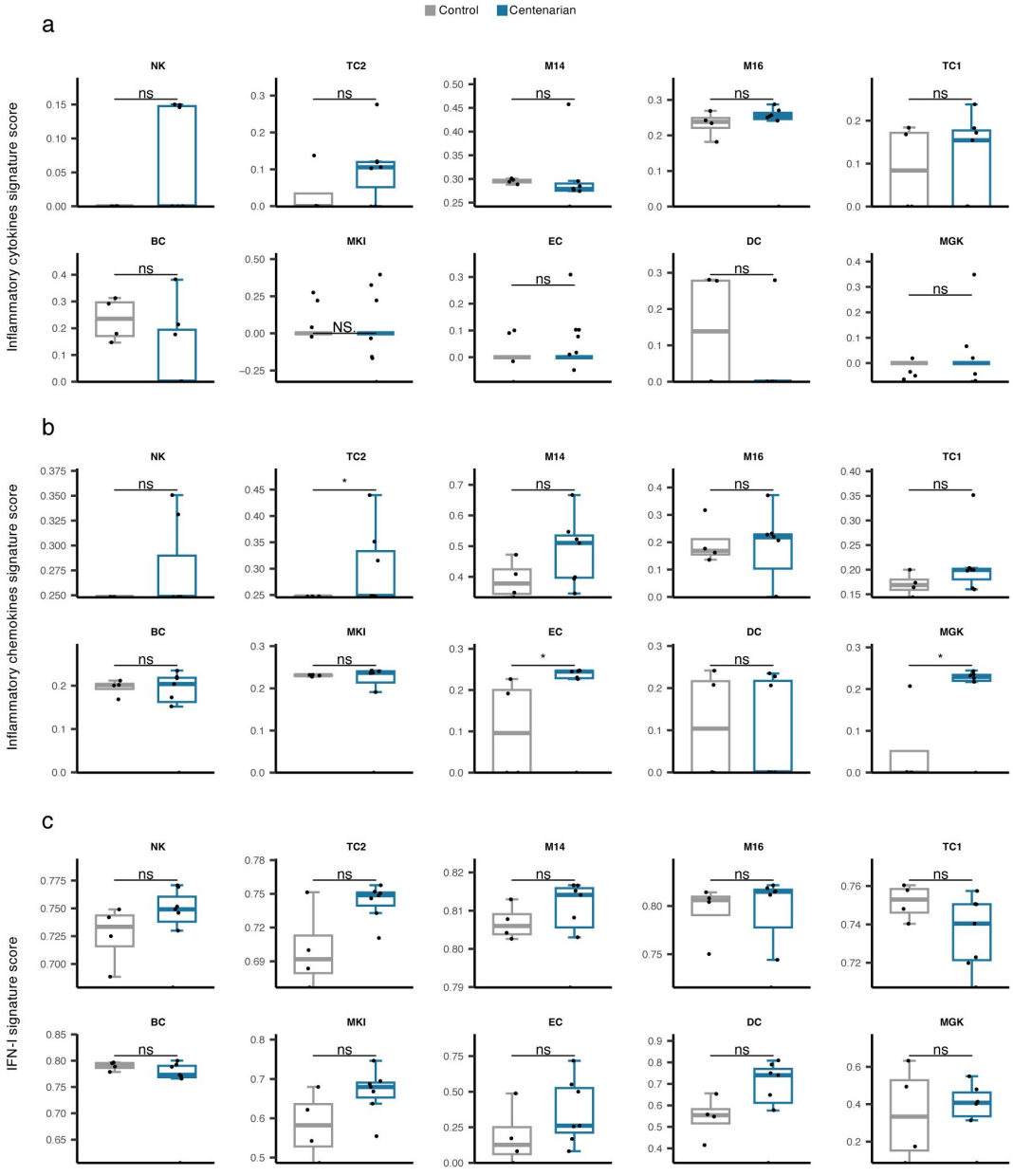
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns: not significant, Wilcoxon test.



Supplementary Figure 14.

Comparison of cell type-specific Senescence, inflammaging, and SASP gene signature scores in supercentenarians versus normal aged cases.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns: not significant, Wilcoxon test.



Supplementary Fig. 15.

Comparison of cell type-specific Inflammatory cytokines, Inflammatory chemokines, and IFN-I gene signature scores in supercentenarians versus normal aged cases.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns: not significant, Wilcoxon test.

data is available under GSE58137 at the GEO public repository. The Institutional Review Boards of Emory University School of Medicine and Grady Memorial Hospital granted approval for all procedures in this study. Funding primarily came from the National Institutes of Mental Health (MH071537).

Genetics, Arthrosis and Progression study (GARP)

The GARP study focused on Dutch Caucasian siblings diagnosed with symptomatic osteoarthritis across multiple joint sites. It comprised 191 sibling pairs (n=382 individuals) of white Dutch ancestry, aged between 40 and 70, diagnosed with primary symptomatic osteoarthritis in multiple joints including hand, spine, knee, or hip (N Riyazi et al., 2005). Whole genome expression profiles were analyzed from 108 participants (68 unrelated families) of the GARP study and 26 age-matched healthy controls. The blood of participants was collected, and mononuclear blood cells were separated prior to RNA isolation. Sample libraries were hybridized onto the microarrays (Illumina Human HT-12_v3_BeadChip's; Illumina). For the methylation analysis, genomic DNA was extracted, and the DNA methylation was assayed at over 450,000 sites on the Illumina Infinium HumanMethylation 450K BeadChips. The data is available at GEO public repository under the accession GSE48556.

Multi-Ethnic Study of Atherosclerosis (MESA)

MESA is a study of subclinical cardiovascular disease and the factors that forecast its progression to clinically evident forms initiated in 2000. This comprehensive investigation involves 6,814 asymptomatic individuals aged 45-84, presenting a diverse demographic: 38% Caucasian, 28% African American, 22 percent Hispanic, and 12 percent Asian (primarily of Chinese descent). Enrolling 6,500 individuals evenly distributed across genders, the study targets those without clinical cardiovascular disease at the outset, representing four racial/ethnic groups from six US communities (Forsyth County, North Carolina; St. Paul, Minnesota; Chicago, Illinois; New York, New York; Baltimore, Maryland; Los Angeles County, California). The study protocol has been approved by the institutional review boards of the six field centers: Wake Forest University, Columbia University, Johns Hopkins University, University of Minnesota, Northwestern University, and University of California - Los Angeles. Detailed information about the design of MESA can be found at *https://internal.mesa-nhlbi.org*, and the data is accessible under GSE56045 at GEO.

Blood samples were gathered to isolate peripheral blood mononuclear cells, from which monocytes were separated using anti-CD14 monoclonal antibody-coated magnetic beads. Flow cytometry analysis consistently demonstrated monocyte samples of over 90% purity across 18 specimens. The DNA and RNA were extracted simultaneously. RNA with integrity scores above 9.0 was chosen for global expression microarrays. Genome-wide expression analysis employed the Illumina HumanHT-12 v4 Expression BeadChip and Illumina Bead Array Reader, following the Illumina expression protocol. Additionally, the Illumina HumanMethylation450 BeadChip and HiScan reader were utilized for epigenome-wide methylation analysis, assigning individual samples to the BeadChips and chip positions using the same sampling scheme as for the expression BeadChips. Details for sample preparation, data processing and quality control were processed as demonstrated in the design paper (Diane Bild et al., 2002). The expression and methylation data are available at GEO public repository under the accession GSE56045 and GSE56046, respectively.

Swedish Adoption/Twin Study of Aging (SATSA)

SATSA is a comprehensive exploration of aging variations using twins raised in different environments influenced by genetic and environmental factors. The study draws its data from the Swedish Twin Registry, a population-based national register encompassing twins born from 1886 to 2000. Additionally, the comprehensive assessment across biological, psychological, and social domains allows for the examination of patterns, predicting the onset of age-related health issues.

Started in 1984, the study employed rigorous questionnaires every three years until 2010, engaging over 2,000 twins. In parallel, a subset of 861 individuals participated in eight in-person testing (IPT) sessions, encompassing health, cognition, and memory assessments. Notably, IPT9 and IPT10 introduced daily tracking of memory, emotions, and social interactions, providing valuable insights into short-term fluctuations and early indicators of declining health.

A total of 1,122 blood samples were collected at five time points spanning from 1992 to 2012. Across these five longitudinal waves, participant counts with 1 to 5 measurements were 99, 86, 90, 80, and 30, respectively. Phenotype data, encompassing chronological age, sex, and zygosity, were gathered through comprehensive questionnaires and physical testing at each sampling wave. Following the manufacturer's protocol optimized for Illumina's Infinium 450K assay, 200 ng of DNA was prepared for each sample. The bisulfite-converted DNA samples were then hybridized to the Infinium HumanMethylation450 BeadChips using Illumina's Infinium HD protocol. This process allowed the measurement of DNA methylation levels for 485,512 CpGs in each sample. Following the quality control procedures described by Peters et al., 1,011 samples from 385 twins were retained after processing and quality control on the methylation data (Marjolein Peters et al., 2015). The data is available at ArrayExpress public repository under the accession E-MTAB-7309.

Brisbane Systems Genetics Study (BSGS)

Participants BSGS were enrolled in the Brisbane Twin Nevus (BTN) and MAPS (Nevus and cognition studies) projects. The study was approved by the Queensland Institute for Medical Research-Human Research Ethics Committee. Over the span of 16 years, adolescent MZ and DZ twins, alongside their siblings and parents, were recruited for an ongoing investigation into genetic and environmental influences on pigmented nevi and the associated risk of skin cancer and cognition. The study comprises individuals of mainly Anglo-Celtic descent with northern European origins (Joseph Powell et al., 2012).

Whole blood samples were collected for total RNA extraction, following the sample preparation process detailed by Powell et al. Gene expression profiles were generated by hybridizing 750 ng of cDNA to the Illumina HumanHT-12 v4.0. This process yielded gene expression data for 962 individuals from 314 families. In parallel, DNA methylation analysis was conducted on 614 individuals from 117 European descent families recruited through BSGS5. Bisulfite converted DNA samples were hybridized to 12-sample Illumina HumanMethylation450 BeadChips using the Infinium HD Methylation protocol and Tecan robotics, assessing methylation status across 485,577 CpG sites, covering 99% of RefSeq genes. Post-methylation probe filtration by Powell et al. resulted in 417,069 probes. Both expression and methylation data are accessible in the GEO public repository under the accessions GSE33321 and GSE56105.

Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates (GMPWAR)

This study aims to construct an aging model that gauges one's methylome age acceleration. Approved by the University of California, San Diego, the University of Southern California, and West China Hospital, the research involved 426 Caucasian and 230 Hispanic individuals, spanning ages 19 to 101, providing extensive methylome-wide profiles. Whole blood samples were collected, and genomic DNA underwent analysis using the Illumina Infinium HumanMethylation450 BeadChip assay, assessing methylation states across 485,577 CpG markers. Stringent quality controls by Hannum et al. ensured the exclusion of unreliable markers and samples (Gregory Hannum et al., 2013). Access to the complete methylation profiles is available at the GEO repository (GSE40279).

ScRNA-seq of PMBCs of healthy aging populations

Approval for all human studies was granted by the Washington University in St. Louis School of Medicine Institutional Review Board (IRB-201804084). The study enrolled healthy Caucasian individuals, both males and females, who were non-obese with a BMI below 30, between 2018 and 2019. The research involved specific age and BMI criteria: young males aged 25-29 years (BMI 21.2-28.9 kg/m2, n = 14), and older non-frail males aged 62-70 years (BMI 17.8-29.8 kg/m2, n = 14). Exclusion criteria comprised individuals with a history of cancer, inflammatory conditions, bloodborne diseases, smokers, or recent illness (Denis Mogilenko et al., 2021).

Blood samples (~100 ml) were obtained via venous puncture in the morning (7–10 a.m.) after an overnight fast. Single-cell suspensions were isolated and underwent droplet-based massively parallel single-cell RNA sequencing using the Chromium Single Cell 5 Reagent Kit according to the manufacturer's instructions (10x Genomics). Processing involved sample demultiplexing, barcode handling, and cell counting using the Cell Ranger Single-Cell Software Suite (10x Genomics). Subsequent analysis utilized the Seurat R package (Butler et al., 2018), which identified 25 clusters representing primary immune cell populations. Notably, one sample (D18) was excluded from analysis due to its outlier status, identified by distinct clustering in the single-cell RNA sequencing analysis. Raw and processed scATAC-seq data are available at *https://www.synapse.org* repository (syn22255433)

ScRNA-seq of PBMCs of supercentenarians

All experiments using human samples in this study were approved by the Keio University School of Medicine Ethics Committee (approval no. 20021020) and the ethical review committee of RIKEN (approval no. H28-6). PBMCs derived from 7 supercentenarians (SC1–SC7) and 5 controls (CT1–CT5, aged in their 50s to 80s) were profiled using droplet-based single-cell RNA sequencing technology (10× Genomics). Fresh whole blood from supercentenarians, their offspring residing with them, and unrelated donors was collected, and PBMCs were isolated from whole blood. Single-cell libraries were prepared from freshly isolated PBMCs by using Chromium Single Cell 3′v2 Reagent Kits. The analysis pipelines in Cell Ranger version 2.1.0 and Cell Ranger R Kit (version 2.0.0) were used for sequencing data processing (Kosuke Hashimoto et al., 2019). Raw UMI counts and normalized expression values for scRNA-Seq are publicly available at *http://gerg.gsc.riken.jp/SC2018/*.

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Reviewer #1 (Public Review):

Summary:

Tsai and Seymen et al. investigate associations between RTE expression and methylation and age and inflammation, using multiple public datasets. The concept of the study is in principle interesting, as a systematic analysis of RTE expression during human aging is lacking. Unfortunately, the reliance on expression microarray data, used to perform the core analysis of the paper places much of the study on shaky ground. The findings of the study would not be sufficiently supported until the authors validate them with more suitable methods.

Strengths:

This is a very important biological problem.

Weaknesses:

RNA microarray probes are obviously biased to genes, and thus quantifying transposon analysis based on them seems dubious. Based on how arrays are designed there should at least be partial (perhaps outdated evidence) that the probe sites overlap a protein-coding or non-coding RNA. The authors state they only used intergenic probes, but based on supplementary files, almost half of RTE probes are not intergenic but intronic (n=106 out of 264). This is further complicated by the fact that not all this small subset of probes is available in all analyzed datasets. For example, 232 probes were used for the MESA dataset but only 80 for the GTP dataset. Thus, RTE expression is quantified with a set of probes which is extremely likely to be highly affected by non-RTE transcripts and that is also different across the studied datasets. Differences in the subsets of probes could very well explain the large differences between datasets in multiple of the analyses performed by the authors, such as in Figure 2a, or 3a. It is nonetheless possible that the quantification of RTE expression performed by the authors is truly interpretable as RTE expression, but this must be validated with more data from RNA-seq. Above all, microarray data should not be the main type of data used in the type of analysis performed by the authors.

https://doi.org/10.7554/eLife.96575.1.sa1

Reviewer #2 (Public Review):

Summary:

Yi-Ting Tsai and colleagues conducted a systematic analysis of the correlation between the expression of retrotransposable elements (RTEs) and aging, using publicly available transcriptional and methylome microarray datasets of blood cells from large human cohorts, as well as single-cell transcriptomics. Although DNA hypomethylation was associated with chronological age across all RTE biotypes, the authors did not find a correlation between the levels of RTE expression and chronological age. However, expression levels of LINEs and LTRs positively correlated with DNA demethylation, and inflammatory and senescence gene signatures, indicative of "biological age". Gene set variation analysis showed that the inflammatory response is enriched in the samples expressing high levels of LINEs and LTRs. In summary, the study demonstrates that RTE expression correlates with "biological" rather than "chronological" aging.

Strengths:

The question the authors address is both relevant and important to the fields of aging and transposon biology.

Weaknesses:

The choice of methodology does not fully support the primary claims. Although microarrays can detect certain intergenic transposon sequences, the authors themselves acknowledge in the Discussion section that this method's resolution is limited. More critical considerations, however, should be addressed when interpreting the results. The coverage of transposon sequences by microarrays is not only very limited (232 unique probes) but also predetermined. This implies that any potential age-related overexpression of RTEs located outside of the microarray-associated regions, or of polymorphic intact transposons, may go undetected. Therefore, the authors should be more careful while generalising their conclusions.

Additionally, for some analyses, the authors pool signals from RTEs by class or family, despite the fact that these groups include subfamilies and members with very different properties and harmful potentials. For example, while sequences of older subfamilies might be passively expressed through readthrough transcription, intact members of younger groups could be autonomously reactivated and cause inflammation. The aggregation of signals by the largest group may obscure the potential reactivation of smaller subgroups. I recommend grouping by subfamily or, if not possible due to the low expression scores, by subgroup. For example, all HERV subfamilies are from the ERVL family.

Next, Illumina arrays might not accurately represent the true abundance of TEs due to non-specific hybridization of genomic transposons. Standard RNA preparations always contain traces of abundant genomic SINEs unless DNA elimination is specifically thorough. The problem of such noise should be addressed.

Lastly, scRNAseq was conducted using 10x Genomics technology. However, quantifying transposons in 10x sequencing datasets presents major challenges due to sparse signals. Smart-seq single-cell technology is better suited to this particular purpose. Anyway, it would be more convincing if the authors demonstrated TE expression across different clusters of immune cells using standard scRNAseq UMAP plots instead of boxplots.

I recommend validating the data by RNAseq, even on small cohorts. Given that the connection between RTE overexpression and inflammation has been previously established, the authors should consider better integrating their observations into the existing knowledge.

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Author response:

> *eLife assessment*
>
> *This study investigates associations between retrotransposon element expression and methylation with age and inflammation, using multiple public datasets. The study is valuable because a systematic analysis of retrotransposon element expression during human aging has been lacking. However, the data provided are incomplete due to the sole reliance on microarray expression data for the core analysis of the paper.*

Both reviewers found this study to be important. We have selected the microarray datasets of human blood adopted by a comprehensive study of ageing published in Nature Communications (DOI: doi: 10.1038/ncomms9570). We only included the datasets specifically collected for ageing studies. Therefore, the large RNA-seq cohorts for cancer, cardiovascular, and neurological diseases were not relevant to this study and cannot be included.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Tsai and Seymen et al. investigate associations between RTE expression and methylation and age and inflammation, using multiple public datasets. The concept of the study is in principle interesting, as a systematic analysis of RTE expression during human aging is lacking.*

We thank the reviewer for the positive comment.

> *Unfortunately, the reliance on expression microarray data, used to perform the core analysis of the paper places much of the study on shaky ground. The findings of the study would not be sufficiently supported until the authors validate them with more suitable methods.*

In our discussion section in the manuscript, we have clarified that “we are aware of the limitations imposed by using microarray in this study, particularly the low number of intergenic probes in the expression microarray data. Our study can be enriched with the advent of large RNA-seq cohorts for aging studies in the future.” However, the application of microarray for RTE expression analysis was introduced previously. In fact, in a manuscript published by Reichmann et al. (DOI: 10.1371/journal.pcbi.1002486) which was cited 76 times, the authors showed and experimentally verified that cryptic repetitive element probes present in Illumina and Affymetrix gene expression microarray platforms can accurately and sensitively monitor repetitive element expression data. Inspired by this methodological manuscript with reasonable acceptance by other researchers, we trusted that the RTE microarray probes could accurately quantify RTE expression at class and family levels.

> *Strengths:*
>
> *This is a very important biological problem.*
>
> *Weaknesses:*

> *RNA microarray probes are obviously biased to genes, and thus quantifying transposon analysis based on them seems dubious. Based on how arrays are designed there should at least be partial (perhaps outdated evidence) that the probe sites overlap a protein-coding or non-coding RNA.*

We disagree with the reviewer that quantifying transposon analysis based on microarray data is dubious. As previously shown by Reichmann et al., the quantification is reliable as long as the probes do not overlap with annotated genes and they are in the correct orientation to detect sense repetitive element transcripts. Reichman et al. identified 1,400 repetitive element probes in version 1.0, version 1.1 and version 2.0 of the Illumina Mouse WG-6 Beadchips by comparing the genomic locations of the probes with the Repeatmasked regions of the mouse genome. We applied the same criteria for Illumina Human HT-12 V3 (29431 probes) and V4 (33963) to identify the RTE-specific probes.

> *The authors state they only used intergenic probes, but based on supplementary files, almost half of RTE probes are not intergenic but intronic (n=106 out of 264).*

All our identified RTE probes overlap with intergenic regions. However, due to their repetitive natures, some probes overlap with intronic regions, too. We can replace "intergenic" with "noncoding" in our revision to show that they do not overlap with the exons of protein-coding genes. However, we do not rule out the possibility that some of our detected RTE probes might overlap noncoding RNAs. In fact, the border between coding and non-coding genomes has recently become very fuzzy with new annotations of the genome. RTE RNAs can be easily considered as non-coding RNAs if we challenge our junk DNA view.

> *This is further complicated by the fact that not all this small subset of probes is available in all analyzed datasets. For example, 232 probes were used for the MESA dataset but only 80 for the GTP dataset. Thus, RTE expression is quantified with a set of probes which is extremely likely to be highly affected by non-RTE transcripts and that is also different across the studied datasets. Differences in the subsets of probes could very well explain the large differences between datasets in multiple of the analyses performed by the authors, such as in Figure 2a, or 3a. It is nonetheless possible that the quantification of RTE expression performed by the authors is truly interpretable as RTE expression, but this must be validated with more data from RNA-seq. Above all, microarray data should not be the main type of data used in the type of analysis performed by the authors.*

In this study, we did not compare MESA with GTP etc. We have analysed each dataset separately based on the available data for that dataset. Therefore, sacrificing one analysis because of the lack of information from the other does not make sense. We would do that if we were after comparing different datasets. Moreover, the datasets are not comparable because they were produced from different blood cell types.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Yi-Ting Tsai and colleagues conducted a systematic analysis of the correlation between the expression of retrotransposable elements (RTEs) and aging, using publicly available transcriptional and methylome microarray datasets of blood cells from large human cohorts, as well as single-cell transcriptomics. Although DNA hypomethylation was associated with chronological age across all RTE biotypes, the authors did not find a correlation between the levels of RTE expression and chronological age. However, expression levels of LINEs and LTRs positively correlated with DNA demethylation, and inflammatory and senescence gene signatures, indicative of "biological age". Gene set*

> *variation analysis showed that the inflammatory response is enriched in the samples expressing high levels of LINEs and LTRs. In summary, the study demonstrates that RTE expression correlates with "biological" rather than "chronological" aging.*
>
> *Strengths:*
>
> *The question the authors address is both relevant and important to the fields of aging and transposon biology.*

We thank the reviewer for finding this study relevant and important.

> *Weaknesses:*
>
> *The choice of methodology does not fully support the primary claims. Although microarrays can detect certain intergenic transposon sequences, the authors themselves acknowledge in the Discussion section that this method's resolution is limited. More critical considerations, however, should be addressed when interpreting the results. The coverage of transposon sequences by microarrays is not only very limited (232 unique probes) but also predetermined. This implies that any potential agerelated overexpression of RTEs located outside of the microarray-associated regions, or of polymorphic intact transposons, may go undetected. Therefore, the authors should be more careful while generalising their conclusions.*

This is a bioinformatics study, and we have already admitted and discussed the limitations in the discussion section of this manuscript. All technologies have their own limitations, and this should not stop us from shedding light on scientific facts because of inadequate information. In the manuscript, we have discussed that all large and proper ageing studies were performed using microarray technology. Peters et al. (DOI: doi: 10.1038/ncomms9570) adopted all these microarray data in their transcriptional landscape of ageing manuscript. Our study essentially applies the Reichmann et al. method to the peripheral blood-related data from the Peters et al. manuscript. Since hypomethylation due to ageing is a well-established and broad epigenetic reprogramming, it is unlikely that only a fraction of RTEs is affected by this phenomenon. Therefore, the subsampling of RTEs should not affect the result so much. Indeed, this is supported in our study by the inverse correlation between DNA methylation and RTE expression for LINE and SINE classes despite having limited numbers of probes for LINE and SINE expressions.

> *Additionally, for some analyses, the authors pool signals from RTEs by class or family, despite the fact that these groups include subfamilies and members with very different properties and harmful potentials. For example, while sequences of older subfamilies might be passively expressed through readthrough transcription, intact members of younger groups could be autonomously reactivated and cause inflammation. The aggregation of signals by the largest group may obscure the potential reactivation of smaller subgroups. I recommend grouping by subfamily or, if not possible due to the low expression scores, by subgroup. For example, all HERV subfamilies are from the ERVL family.*

We agree with the reviewer that different subfamilies of RTEs play different roles through their activation. However, we will lose our statistical power if we study RTE subfamilies with a few probes. Global epigenetic alteration and derepression of RTEs by ageing have been observed to be genome-wide. While our systematic analysis across RTE classes and families cannot capture alterations in subfamilies due to statistical power, it is still relevant to the research question we are addressing.

> *Next, Illumina arrays might not accurately represent the true abundance of TEs due to non-specific hybridization of genomic transposons. Standard RNA preparations always contain traces of abundant genomic SINEs unless DNA elimination is specifically thorough. The problem of such noise should be addressed.*

We have checked the RNA isolation step from MESA, GTP, and GARP manuscripts. The total RNA was isolated using the Qiagen mini kit following the manufacturer's recommendations. The authors of these manuscripts did not mention whether they eliminated genomics DNA, but we assumed they were aware of the DNA contamination and eliminated it based on the manufacturer's recommendations. We have looked up the literature about non-specific hybridization of RTEs but could not find any evidence to support this observation. We would appreciate the reviewers providing more evidence about such RTE contaminations.

> *Lastly, scRNAseq was conducted using 10x Genomics technology. However, quantifying transposons in 10x sequencing datasets presents major challenges due to sparse signals.*

Applying the scTE pipeline (https://www.nature.com/articles/s41467-021-21808-x), we have found that the statical power of quantifying RTE classes (LINE, SINE, and LTR) or RTE families (L1, L2, All, ERVK, etc.) are as good as each individual gene. However, our proposed method cannot analyse RTE subfamilies, and we did not do that.

> *Smart-seq single-cell technology is better suited to this particular purpose.*

We agree with the reviewer that Smart-seq provides higher yield than 10x, but there is no Smart-seq data available for ageing study.

> *Anyway, it would be more convincing if the authors demonstrated TE expression across different clusters of immune cells using standard scRNAseq UMAP plots instead of boxplots.*

Since the number of RTE reads per cell is low, showing the expression of RTEs per cell in UMAP may not be the best statistical approach to show the difference between the aged and young groups. This is why we chose to analyse with pseudobulk and displayed differential expression using boxplot rather than UMAP for each immune cell type.

> *I recommend validating the data by RNAseq, even on small cohorts. Given that the connection between RTE overexpression and inflammation has been previously established, the authors should consider better integrating their observations into the existing knowledge.*

Until recently, there were no publicly-available, non-cancerous, large cohort of RNA-seq data for ageing studies. We tried to gain access to the two RNA-seq datasets suggested by reviewer 2: Marquez et al. 2020 (phs001934.v1.p1, controlled access) and Morandini et al. 2023 (GSE193141, public access).

Unfortunately, Marquez et al. 2020 data is not accessible because the authors only provide the data for projects related to cardiovascular diseases. However, we did analyse Morandini et al. 2023 data, and we can confirm that no association was observed between any class and family of RTEs with chronological ageing, which is the second strong piece of evidence supporting the statement in the manuscript. However, as expected, we found a positive correlation between RTE expression and IFNI signature score.