AP-1 complex activation is a conserved signature of immune system aging and a potential regulator of inflammaging in human and mice

Emin Onur Karakaslar

Leiden University Medical Center

Muneer Hasham

Jackson Laboratory

Neerja Katiyar

Jackson Laboratory <https://orcid.org/0000-0001-7414-9674>

Ahrim Youn

Sanofi

Siddhartha Sharma

Jackson Laboratory

Cheng-han Chung

Jackson Laboratory

Radu Marches

Jackson Laboratory

Ron Korstanje

Jax

Jacques Banchereau

Jackson Laboratory, ImmunAI

Duygu Ucar (✉ Duygu.Ucar@jax.org)

Jackson Laboratories

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Abstract

Increased inflammation with age (i.e., inflammaging) is a hallmark of aging conserved in human and mice, but the underlying mechanisms are poorly understood, partially because a systematic comparative study of mouse and human immune system aging is lacking. We uncovered epigenomic/transcriptomic signatures of aging in spleen and peripheral blood lymphocytes from young (3 months) and old (18 months) mice in two strains: C57BL/6J (long-lived) and NZO/HILtJ (short-lived) and compared these to the aging signatures of human peripheral blood cells. The most predominant and conserved genomic signature of aging in human and mice tissues studied here was the epigenetic activation of several AP-1 complex members (Fos, Fosl2, Junb, Jund). Footprinting analyses showed that these transcription factors 'bind' more frequently with age and target pro-inflammatory and effector molecules, including the pro-inflammatory Il6. Analysis of single cell RNA-seq data from the mouse aging cell atlas (Tabula Muris Senis) revealed that AP-1 activation with age is a common feature across all immune cell types within spleens, yet macrophages express these molecules more often than other cells. Functional assays confirmed that spleen cells from older animals have increased c-JUN protein binding and increased IL6 production upon myeloid cell activation using poly(I:C) via TLR3. Western blot data revealed that c-JUN activation with age is not post-transcriptional since its phosphorylation levels were similar between young and old mice. Together, these data established that Jun and Fos families in the AP-1 complex are transcriptionally activated with age and target pro-inflammatory molecules and aging-related increases in the binding of these proteins likely modulate increased inflammation with age.

Introduction

Age-related changes in the immune system reduce older individuals' ability to generate protective responses to immunological threats and lead to increases in diseases and infections^(1, 2). Increased inflammation with age (i.e., inflammaging) is one of the hallmarks of immune system aging that is conserved across human and mice as well as across tissues and strains⁽³⁾, however the drivers of this aging signature is mostly unknown⁽⁴⁾. Human immune aging studies are mostly limited to blood, since blood is easy to access and gives an opportunity to study the status of the peripheral immune system with minimal invasion. These studies uncovered significant age-related changes in gene expression levels in whole blood, as well as in blood-derived peripheral mononuclear cells (PBMCs) and sorted immune cells⁽⁵⁻⁷⁾. Through genomic profiling, we and others, have uncovered that pro-inflammatory pathways and molecules are activated with age, whereas molecules/pathways related to T cell maintenance and signaling are inactivated^(4, 5, 7-10). Although these studies have described significant age-related changes in transcriptional regulatory programs of immune cells, including the activation of pro-inflammatory programs, they did not pinpoint potential upstream regulators of these genomic alterations.

Mouse models have been widely used in immune aging research; however, a comprehensive comparison of the aging mouse and human immune systems is missing, which would further advance the utility and

fidelity of mouse models for our understanding of aging mechanisms. Specifically, we know little about i) how transcriptomes and epigenomes of mouse immune cells are remodeled with age in blood or lymphoid organs; ii) whether these changes are shared between different mouse strains, *i.e.*, consistent across different genetic backgrounds; and iii) to what extent these changes are similar to age-related changes observed in human immune cells. To address these open questions, we completed comprehensive genomic characterization of the effects of age on the immune systems of two strains with different health- and life-spans: long-lived C57BL/6J (B6) and short-lived NZO/HILtJ (NZO) mice. B6 is the most frequently used mouse strain in research. Median lifespan is 901 days for B6 males and 866 days for females⁽¹¹⁾ - among the longest-lived lab strains. In contrast, NZO (*i.e.*, New Zealand obese) animals are among the shortest-living lab strains, where the median lifespan is 576 days for females and 423 days for males⁽¹¹⁾. Similar to humans and most animal species in nature, NZO females live longer than the males⁽¹²⁾. NZO mice develop severe obesity, and males are frequently used as models for Type 2 diabetes (T2D), insulin resistance, and obesity⁽¹³⁾.

In these two strains, we conducted a thorough investigation of mouse immune system aging by profiling peripheral blood leukocytes (PBL) and the spleen, the major lymphoid organ. We also profiled naive and memory CD8⁺ T cells sorted from the spleen, given the importance of CD8⁺ T cells in human aging^(5, 7). Epigenomic (ATAC-seq), transcriptomic (RNA-seq), and cell composition (flow cytometry) profiles of young (3 months old, mo) and old (18 mo) animals from these two tissues (spleen, PBL) uncovered multi-modal signatures of aging in both B6 and NZO mice. Comparison of these signatures with human aging signatures from the peripheral blood mononuclear cells (PBMCs) of 77 healthy young (22-40 years old, yo) and older (65+ yo) individuals⁽⁷⁾ revealed the epigenetic activation of Activator Protein 1 (AP-1) complex transcription factors (TFs) (*Jun*, *Fos*, and *Fosl2*)^(14, 15) as an evolutionarily conserved signature of immune aging. Computational analyses of AP-1 binding sites showed that these TFs bind around the promoters of pro-inflammatory genes including *Il6*. Analyses of single cell RNA-seq data from the Tabula Muris Senis⁽¹⁶⁾ (mouse aging cell atlas) consortium revealed that the transcriptional activation of AP-1 complex members *Jun* and *Fos* with age occurs across all immune cell subsets, yet was most significantly observed in macrophages. In alignment with these genomic findings, spleen cells from older animals produced more pro-inflammatory IL6 cytokine and had higher c-JUN protein expression level compared to cells from younger animals when activated *via* poly(I:C) (TLR3). Together, these data uncovered AP-1 complex activation with age as a conserved epigenetic biomarker of aging and a potential regulator of inflammaging. Data from the two mouse strains and human PBMCs can be queried at: <http://immune-aging.jax.org>

Results

Profiling blood and spleen immune cells of young and old B6 and NZO mice

To understand how aging affects the mouse immune system, we generated flow cytometry, RNA-seq, and ATAC-seq profiles of circulating immune cells (PBLs) and the major lymphoid organ spleen in two mouse strains. We selected B6 and NZO strains as representatives of each end of the longevity spectrum of lab mice, to mimic the heterogeneity in individuals' lifespans and to uncover conserved immune aging signatures in long and short-living animals. PBL and spleen samples, including sorted CD8⁺ T cells, were collected from 3 months and 18 months mice to represent young and old mice, respectively, as well as to allow direct age comparison to existing single cell mouse transcriptomic atlases⁽¹⁷⁾ (**Figure 1A**). We also profiled spleens from middle-aged (12 mo) mice to study timing of age-related changes. Samples that passed quality control (QC) were used in downstream analyses (Methods): 74 flow cytometry samples (39 B6, 35 NZO), 103 RNA-seq samples (49 B6, 54 NZO), and 90 ATAC-seq samples (38 B6, 52 NZO) (summarized in **Figure 1B**, **Table S1**). Using flow cytometry, we characterized PBL and spleen cell compositions for a total of 36 cell types including total B (CD19⁺), CD8⁺ and CD4⁺ T cells (**Table S2**). After QC and filtering, 96,623 consensus ATAC-seq peaks from all cell/tissue types and 18,294 expressed genes from RNA-seq samples were used in downstream analyses.

Cell type, mouse strain, and age explain most of the variation in genomics data

Principal component analysis (PCA) revealed that in all three data modalities, and especially in RNA-seq data, samples were first separated by tissue/cell type and then by strain (**Figure 1C**). Sorted CD8⁺ T cells clearly separated from PBL and spleen samples in RNA-seq and ATAC-seq data, suggesting that cell type-specific patterns are the major contributor to the variation in genomics data. Within each cell/tissue type, we also detected strain- and age-specific variation, where samples separated with respect to strain first, followed by their age (**Figure S1A-C**). We quantified how much variation is attributable to meta-data in each modality using principal variance component analysis (PVCA)⁽¹⁸⁾. Tissue/cell type explained the majority of variation in both ATAC-seq (~41%) and RNA-seq (~55%), followed by strain differences (19% in ATAC-seq, 16% in RNA-seq) (**Figure 1D**). Age contributed the most to variation in the flow cytometry data (~20%), due to significant remodeling of PBL and spleen cell compositions with age. In contrast, age explained ~5% of the variation in ATAC-seq and RNA-seq data. Only 2% of variation in each modality were attributable to biological sex. Some variation (~10-20% across data modalities) could also be attributable to different pairwise interactions between the four tested explanatory variables (**Figure S1D**); for example, strain and age together explained ~7% of the variation in flow cytometry data. Together, these data and analyses suggest that cell type and strain are the main drivers of variation in mouse genomics data, followed by the age of the animals.

Naive T cell decline is the major age-related cell composition change in mouse and human

Using flow cytometry, we quantified the proportion of immune cell types within spleen and PBL samples (**Table S2**) by first profiling major populations: B (CD19), CD4⁺ and CD8⁺ T cells. The T cell subsets were further stratified into effector memory (EM, CD44^{high} CD62L⁻), effector (CD44^{low} CD62L⁻), central memory (CM, CD44^{high} CD62L⁺), and naive (CD44^{low} CD62L⁺) cells (see **Figure 2A** and **Figure S2A** for the gating strategy). Given its importance in T cell homeostasis and signaling^(10, 19) and in human immune system aging⁽⁷⁾, we also identified/gated IL7R⁺ cells among B and T cells. Similarly, we completed PD1⁺ gating of cells to identify exhausted T cells⁽²⁰⁾.

The spleen contains cells of both the lymphoid (B and T) and the myeloid compartment (granulocytes, monocytes, and dendritic cells - DCs) with discrete functions⁽²¹⁾. In both B6 and NZO, the proportion of lymphoid cells have decreased with age. The reduction in the production of the lymphoid compartment has been previously observed with aging, in alignment with our data⁽²²⁾. Cumulatively (for all ages and strains), spleen samples were on average composed of 45% B, 22% T, and 32% myeloid populations, whereas PBLs were composed of 37% B, 20% T, and 43% myeloid populations. Overall, B cell percentages were higher in spleen compared to PBL, whereas myeloid cell percentages were lower (**Figure 2B, Figure S3A**). The most striking difference between human PBMCs and mouse PBLs was the percentage of B cells. B (CD19⁺) cells typically constitute <10% of human PBMCs, whereas >30% of mouse PBLs were B cells (**Figure S3**). We noted that in both PBL and spleen samples, T cell percentages declined with age, which was a conserved trend between the strains as well as in human PBMCs (**Figures 2B, S3**).

To systematically study age-related changes in cell compositions, we built linear models that associate age (independent variable) with the percentage of each cell type (dependent variable) (**Figures 2C, S4**). These analyses revealed significant age-related changes that are conserved across strains and tissues including expected decreases in naive T cells (blue in **Figure 2C, Figures 2D-E** right panels) and increases in effector memory cells (red in **Figure 2C, Figures 2D-E** left panels) both for CD4⁺ and CD8⁺ T cell compartments. The decline reported in IL7R⁺ CD8⁺ T cells with age in human PBMCs⁽⁷⁾ was not observed in mice neither in blood nor in spleen samples; in contrast, there was a slight increase in these cells in mouse immune tissues with age (**Figure S4A**). As expected, percentages of exhausted PD1⁺ T cells mostly increased with age in both spleen and PBL (**Figure S4A**). The increases in PD1⁺ cell percentages were more significant in long-lived B6 compared to short-lived NZO at 18 months of age (**Figure S2A**), suggesting that the increases in exhausted T cell percentages might not directly relate to longevity of the organism.

We conducted similar association analyses between age and cell compositions using human PBMC flow cytometry data from our previous study⁽⁹⁾. These analyses revealed that in human PBMCs the decline in

total CD8⁺ T cells - stemming from the loss of naive CD8⁺ T cells - is the most significant cell-compositional change with age (**Figures 2F-G**). Although we detected some increases in memory T cell subsets in human PBMCs (e.g., CD8⁺ EMRA cells), these increases were not as significant as in mice, potentially due to individual-specific variation in cell compositions that might mask some of the overall aging effects in human. Trends in major cell populations in male and female subjects were consistent with each other in human (**Figure S4B**), however, some differences were observed in the magnitude of changes between sexes. For example, the percentage of naive CD8⁺ T cells in PBMCs declined from 16.2% in young men to 5.5% in older men, and from 14.5% to 9.7% with age in women, consistent with our previous observations⁽⁹⁾. Together, these data and analyses uncovered that PBL and spleen cell compositions significantly change with age and most of these changes are conserved between the two mouse strains. Declines in naive T cells were the most significant age-related changes in both human and mouse.

Age-related upregulation of pro-inflammatory genes are shared across tissues and mouse strains

We conducted differential gene expression/chromatin accessibility analyses between old (18 mo) and young (3 mo) animals for both spleen and PBL to identify age-related genomic changes (Methods). Using RNA-seq, we detected major age-related changes in the spleen for both strains; where 1960 genes were differentially expressed (DE) in B6 and 1795 in NZO, 675 of which were shared between strains (**Table S3, Figure 3A** top panel, FDR = 0.05 and $|\log FC| > 1$). PBL transcriptomes were also remodeled with age – albeit to a lesser extent - 455 DE genes in B6 and 862 DE genes in NZO. In addition, we identified differentially accessible (DA) ATAC-seq peaks with age, which were mapped to the closest transcription start site (TSS) (**Table S4, Figure 3A** bottom panel, FDR = 0.05 and $|\log FC| > 1$). Genes associated to opening peaks (more accessible with age) statistically significantly overlapped with genes upregulated with age in both tissues and strains (**Figure S5A**). Similarly, genes associated to closing peaks (less accessible with age) significantly overlapped with downregulated genes. Interestingly, changes in epigenomes and transcriptomes were more concordant for activated (opening/upregulated) signals compared to inactivated ones (closing/downregulated). Despite significant overlap, we also uncovered that some genes are associated to aging either in RNA-seq or ATAC-seq analyses (**Figure S5A**).

To functionally annotate genes and peaks remodeled with age, we performed gene set enrichment analysis (GSEA)⁽²³⁾ using immune modules⁽²⁴⁾ (**Figure 3B**) and gene sets derived from single cell RNA-seq PBMC data (**Figure S5B**)(see **Table S5** for GSEA results). Genes/peaks associated with inflammation and the myeloid lineage were activated with age in both epigenomes and transcriptomes (**Figures 3B, S5B**), whereas naïve T cell-related loci in particular were inactivated. This was consistent with age-related changes in human PBMCs (**Figure S5C**) with the exception of the upregulation of cytotoxicity in human data. Further investigation of pro-inflammatory molecules upregulated with age (i.e., genes in the inflammatory processes -Inflammation1- module) showed that most of these molecules were upregulated in both tissues: 19 were upregulated in both, whereas 21 others were up only in spleen (**Figure 3C**). Upregulation of these molecules was mostly conserved across the two strains with some exceptions,

e.g., *Cpd* was upregulated only in B6. Spleen samples from middle-aged animals (12 months) showed that most of these pro-inflammatory molecules were already upregulated at this timepoint (equivalent to 40-50 years old in human for B6⁽¹⁷⁾) (**Figure 3C**). Among these molecules, the pro-inflammatory cytokine *Il1b* was consistently upregulated in both tissues as well as both strains (**Figure 3D**, **Figure S5D** for ATAC-seq). Interestingly, in human PBMCs, we did not detect an activation of *IL1B* with age either in RNA-seq or in ATAC-seq data (**Figures S6A-C**). In alignment with this, other studies from healthy individuals reported no significant changes in IL1B serum levels with age⁽²⁵⁾. However, in both human and mouse, we observed an activation of the pro-inflammatory *Fos/2* with age both epigenetically and transcriptionally (**Figures 3E, S5E, S6B-D**). *Fos/2* is a member of the AP-1 complex and its overexpression has been associated with the regulation of inflammatory responses and dysregulation of both innate and adaptive immunity^(14, 26). Together, differential gene expression and chromatin accessibility analyses established the upregulation of myeloid genes, particularly pro-inflammatory genes (notably *Fos/2*), as a conserved signature of aging across mouse strains, blood and spleen samples, and between human and mice.

Signaling pathways and AP-1 complex members are up-regulated with age in CD8⁺ T cells

Differential gene expression analyses between young and old animals in naïve and memory CD8⁺ T cells sorted from splenic tissue uncovered significant age-related changes in both strains (FDR =0.05, |logFC|>1) (**Figure 4A, Table S3**). For example, 826 and 1718 genes in naïve cells were differentially expressed (DE) with age in B6 and NZO, respectively. In addition, we also identified ATAC-seq peaks that were differentially accessible (DA) between old and young animals (**Figure S7A, Table S4**), and associated these to nearest genes using distance to TSS (**Figure 4A** bottom panel). Comparisons of the genes associated to opening peaks with upregulated genes showed that there was significant overlap between these gene sets (**Figure S7B**); as in PBL and whole spleen, we found that epigenetic and transcriptional signatures of downregulated molecules overlapped less significantly than those of upregulated molecules.

Functional annotation of genes remodeled with age in CD8⁺ T cells in both modalities (epigenome, transcriptome) using WikiPathways (**Figure 4B; Table S5 (Methods)**) uncovered that genes involved in interleukin signaling pathways were frequently activated with age, most notably the IL-2 signaling pathway, which was activated consistently across strains and T cell subsets (**Figure 4B**). Further investigation of genes comprising the IL-2 signaling pathway revealed that a subset of these genes was transcriptionally activated with age in both naïve and memory cells (**Figure 4C**, *denotes statistically significantly different between ages). Among these, AP-1 complex members *Fos* and *Jun* were the most significantly activated genes both transcriptionally and epigenetically with age (**Figures 4C-D**). Similarly, AP-1 member *Fos/2* was also activated with age in naïve and memory T cells in both modalities (**Figures S7C-D**). One of the most prominent changes observed in human CD8⁺ T cells were the downregulation of *IL7R* and the IL7 signaling pathway with age⁽⁷⁾, which was not detected in mice CD8⁺ T cells (**Figures 4B, S7E**).

TF footprints for AP-1 complex members increase with age in both human and mouse

TF footprinting enables *in silico* inference of TF binding events by integrating chromatin accessibility patterns with the underlying TF sequence motifs⁽²⁷⁾. This also provides an opportunity to describe upstream regulators of age-related gene expression changes. We called TF footprints in mouse samples from pooled young and old animals in 4 different tissue/cell types using PIQ⁽²⁸⁾ and JASPAR⁽²⁹⁾ motifs, as well as in human PBMCs from young and old subjects. Samples are pooled to increase the depth of sequencing, which is an important factor for the quality and number of detected footprints⁽³⁰⁾. Next, we identified TF footprints enriched in peaks that were activated (i.e., opening) or inactivated (i.e., closing) with age in each cell type using BIFET⁽³⁰⁾ (Methods). TF footprints enriched in opening peaks were largely shared across 4 cell types in mice, whereas footprints enriched in closing peaks were cell type-specific (**Figure 5A**). Among the footprints that were enriched in opening peaks with age, six were also enriched in opening peaks from human PBMCs (**Figure 5B**). Remarkably, this included five AP-1 complex members (FOS, FOSL2, JDP2, JUNB, JUND) as well as NFE2L2 (i.e., NRF2), a protein that interacts with c-JUN and contributes to the regulation of the NLRP3 inflammasome⁽³¹⁾. Chromatin accessibility around the promoters of these molecules, as well as their gene expression levels, also increased with age in mice across strains and tissues (**Figures 5C, S8A**). Most notably, the expression of *Fos* increased significantly across all 4 mouse sample types and in both strains, for example, we detected ~7-fold and ~5-fold increases in NZO and B6 spleen respectively (**Figure S8A**). These data suggest that transcriptional activation of the AP-1 complex is a signature of immune aging conserved across mouse strains and tissues, confirmed by the clear separation of age groups in all tissues based on only the expression levels of 25 genes in the AP-1 complex (**Figures 5D, S8B, Table S6**). In human PBMCs, the chromatin accessibility levels around these TFs also increased with age – more significantly in men (**Figures 5C, S8A**). Motif enrichment analyses revealed that opening peaks across all cell types were enriched among the motifs of these TFs, where enrichments were more significant for the shorter living NZO strain (**Figure S8C**). We also detected similar enrichments for these TFs in human PBMC and naïve CD8⁺ T cells, suggesting again increased binding activity for these TFs with age (**Figure S8D**).

In addition to the enrichment of these 6 TFs among opening peaks, footprints for these TFs made up a larger proportion of all detected footprints in older samples across the 4 tissues (**Figure 5E, Table S7**), which suggests that these TFs were actually bound and occupy the DNA where chromatin is opening with age. To understand which cellular functions are modulated by the increased ‘binding’ of these TFs, we identified their gene targets (Methods) and conducted functional enrichment analyses. Footprints of these TFs detected from older samples were enriched around the molecules involved in pro-inflammatory pathways (e.g., Nf-KB activation) across tissues and strains (**Figures 5F,G, Table S8**). Interestingly, enrichments for pro-inflammatory pathways were more significant for the shorter-living NZO strain, which might hint to elevated inflammation signatures for this strain compared to B6 (**Figure 5G**). Gene targets of these TFs include members of the Nf-KB pathway (*Rel*, *Rela*, *Nfkbiz*), pro-inflammatory cytokines and chemokines (*Il1b*, *Il6*, *Il15*, *Cxcl10*), genes expressed by activated myeloid (*Cd86*, *Cd44*, *Il7r*, *S100a11*) and lymphoid cells (*Cd44*, *Cd28*)⁽³²⁾, cytotoxic molecules (*Gzmk*, *Gzmb*, *Klrg1*), plasma cell marker *Cd38* (**Table S8**), and *Fosl2* itself in mice PBL and spleen. Similarly, in human PBMCs, footprints of these TFs targeted pro-inflammatory (*FOSL2*, *LMNA*, *CASP8*, *NFKBIZ*), cytotoxic (*GNLY*, *PRF1*, *GZMB*), and activated

myeloid cell markers (*CD44*, *IL7R*)⁽³²⁾ and *FOSL2* itself (**Table S9**). These enrichments support the previously reported cross-regulation of AP-1 complex and NF- κ B pathways in myeloid cells^(33, 34) and provide insights into which other pathways/functions are potentially activated by these TFs in both myeloid and lymphoid cells. Together our findings nominate AP-1 complex members as important biomarkers of immune system aging, which are involved in regulating pro-inflammatory pathways and potentially contributing to the age-related increases in inflammation – one of the most conserved hallmarks of immune aging.

c-JUN protein production increases with age upon stimulation *via* Toll-like receptors

To uncover whether AP-1 activation with age stems from certain cell types, we reanalyzed single cell RNA-seq data from the Tabula Muris Senis consortium⁽¹⁶⁾, from spleen cells of young (3-month) and old (18-month) B6 mice. We annotated spleen cells as B, T, NK cells and monocytes/macrophages using known marker genes (**Figure 6A**). In alignment with our flow cytometry data majority of spleen cells were composed of B cells, followed by T cells, and by innate cells (**Figures S9A-B**). Next, we studied the activation of Jun/Fos members in these single cells. Expression of these molecules (particularly *Jun* and *Fos*) increased with age across all immune cell types (**Figures 6B-C**). Further analyses showed that increases are due to both i) increased expression at the single cell level, and ii) increased number of cells expression this molecule (**Figure S9C**). Despite ubiquitous activation across all immune cell types, macrophages expressed these molecules more frequently than other cell types both in young and old animals among all subsets (**Figures 6B-C**). For example, almost half of all macrophages expressed *Jun* and *Fos* in younger animals, which increased to 78% of macrophages with age for *Fos* and 58% for *Jun*.

An increase in the transcriptional activity of a gene may or may not indicate increased function. This is especially true for the AP-1 protein complex, which is a heterodimer composed of c-FOS, c-JUN, ATF and JDP protein families and its activity would depend on the formation of the complex and post translational modifications such as phosphorylation⁽³⁵⁾. To understand whether these expression changes affect the function of cells, we used a c-JUN transcription factor functional assay kit (Abcam) to quantify c-JUN binding activity in nuclear extracts from B6 spleen (Methods) upon stimulation using three different methods: 1) anti CD3-anti CD28 to stimulate T cells; 2) LPS to stimulate B cells and monocytes *via* TLR4, and 3) poly I:C to stimulate monocytes *via* TLR3. The c-JUN binding activity level did not significantly change between age groups upon T cell stimulation, however, it increased with age upon TLR-mediated stimulation (**Figure 6D**), particularly upon poly I:C stimulation ($p=0.0063$). TLR3 activation by poly(I:C) has been shown to regulate inflammatory responses in tissues⁽³⁶⁾. Functional assays of binding from lysed cells cannot differentiate between increased phosphorylation and elevated transcription. Both outcomes would result in increased binding, to determine the mechanism of the observed increased binding, the poly(I:C) activated cells were lysed, fractionated into nuclear and cytosolic fractions, and western blotted for phospho-c-JUN (P-c-JUN), c-JUN and loading markers LamininB1 for nuclear extracts and GAPDH for cytosolic extracts (**Figure 6E**). Ex vivo Poly (I:C) activated splenocytes from older animals expressed more nuclear c-JUN protein at baseline compared to those

from younger animals. Moreover, nuclear and cytosolic c-JUN protein levels increased more significantly in older animals upon poly(I:C) stimulation (**Figure 6E**). Interestingly, no significant age-related changes are detected in phosphorylated c-JUN protein levels, confirming that there is more protein production with age and this mechanism is regulated at the transcriptional level and not post-transcriptional.

Footprinting analyses showed that there is increased number of footprints around the *Il6* promoter with age, a predominant pro-inflammatory cytokine and a biomarker of inflammaging⁽³⁷⁾ (**Figure 6F**). To complete our functional studies, we also measured the activity of IL6 protein as a product of increased c-JUN binding. For this, splenocytes were stimulated *ex vivo* with poly(I:C) for 1 day and the supernatant was measured for IL6 cytokine. As expected, poly(I:C) stimulation led to increases in IL6 levels both in young and old splenocytes, however this inflammatory response was significantly elevated with age (**Figure 6G**): a ~5-fold increase in IL6 production in female mice and ~3-fold in male mice. Expression levels of Toll-like receptor (TLR) genes did not change significantly with age in spleen-derived immune cells (**Figures S9D**), suggesting that the increased inflammatory responses upon stimulation are modulated by downstream regulators of TLR signaling pathways. Together these functional data showed that there is increased binding activity of c-JUN protein in mice splenocytes with age especially when monocytes are stimulated, this increase is modulated at the transcriptional level and is not regulated post-transcriptionally.

Discussion

Through comprehensive genomic profiling of diverse immune cells and tissues, transcriptional activation of the Activating Protein-1 (AP-1) complex members - particularly *Jun*, *Fos*, *Fosl2* - with age emerged as a conserved biomarker of immune system aging across two mouse strains, diverse immune tissues and cell types, and between human and mice. AP-1 is a transcription factor complex that regulates gene expression programs in response to diverse stimuli, such as stress and viral infections⁽³⁸⁾ and have been shown to be involved in regulating inflammatory responses and in cross-regulating the pro-inflammatory NF-KB pathway^(14, 33, 34). In addition to epigenomic and transcriptomic activation of these genes, western blot data for c-JUN protein and computational TF footprinting analyses established that, with age, these proteins are expressed and bind at higher levels, especially when immune cells are stimulated *via* TLRs. A recent human study showed that chromatin accessibility around AP-1 members decreased upon vaccination with trivalent influenza vaccine (TIV) and H5N1+AS03 in young adults⁽³⁹⁾ - a potential mechanism to suppress excess inflammation. Interestingly, this epigenetic remodeling around the AP-1 complex members boosted responsiveness to other viruses – Zika and Dengue⁽³⁹⁾. Here we show chronic activation of these complex members with age both in human and mice. Future vaccination studies in older adults will be important to uncover whether age-related activation of AP-1 complex contributes to reduced vaccine responsiveness in this population and whether the activation of these TFs is an epigenetic scar of previous pro-inflammatory responses⁽⁴⁰⁾.

Single cell RNA-seq data from Tabula Muris Senis⁽¹⁶⁾ showed that, although the gene expression levels of AP-1 complex members increase significantly across all immune cell types with age, older macrophages express these molecules at higher percentages compared to other immune cell types. Our footprinting analyses suggest that these TFs target and potentially activate important molecules across distinct immune cell types: pro-inflammatory molecules in both myeloid and lymphoid cells, cytotoxic molecules in T/NK cells, and *Cd38* in B cells. We observed increased c-JUN binding activity with age in spleen cells upon *Tlr3* stimulation using poly(I:C). *Tlr3* is a highly conserved molecule that recognizes double stranded (ds) RNA associated with viral infection and induces the activity of interferon response and proinflammatory molecules in myeloid cells⁽⁴¹⁾. One of the gene targets of JUN/FOS proteins is the pro-inflammatory cytokine *Il6* and we detected more footprints for these proteins in the vicinity of this cytokine in older immune cells. In alignment with this, we observed increased IL6 production from the spleens of older animals upon monocyte stimulation. Together, these results suggest that age-related activation of AP-1 members is a conserved epigenetic biomarker of immune system aging and activation of these TFs with age is a potential upstream regulator of increased inflammatory responses with age.

Our data and analyses revealed that age-related changes in the myeloid compartment (i.e., increased inflammation and its regulators) is more conserved between human and mice when compared to the changes in the lymphoid compartment. For example, in human PBMCs, a prominent change we detected was the chromatin closing and reduced expression of *IL7R* and its downstream molecules in the IL7 signaling pathway⁽⁷⁾ that stem from CD8⁺ T cells, which was accompanied by a decline in IL7R⁺ CD8⁺ cells⁽⁷⁾. In mouse tissues and strains studied here, we did not detect either a decline in the expression of the *Il7r* gene with age or a decline in the percentages of IL7R⁺ CD8⁺ cells. This discordance between human and mice CD8⁺ T cell aging patterns likely stems from differences in their antigenic challenges; lab mice live in a highly controlled environment (strict diet, un-challenged immune system) unlike humans. Despite this, recent single cell studies from human and mice immune cells uncovered other T cell changes that are conserved between human and mice⁽⁴⁾. Myeloid compartment activation stems mostly from intrinsic signals that accumulate with age; e.g., cellular damage that increases with age that activates myeloid cells *via* damage-associated molecular patterns (DAMPs). Here, we used long- and short-living strains to mimic both healthy and unhealthy aging in humans and to uncover the most conserved signatures. Despite the fact that we observed some differences between strains (e.g., further activation of AP-1 members in NZO), majority of the aging signatures were conserved between the strains. Bigger cohorts are needed to further delve into differences between strains including the differences between female and male mice.

Declarations

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Author contributions: DU designed the study with help from JB, MH, RK. OK, NK, AY, analyzed the data. RK and MH collected samples. MH and CC conducted functional experiments. DU, OK, MH wrote the paper. SS developed the R Shiny app. All authors read and edited the manuscript.

Competing interests: The authors declare no competing financial interests.

While this work was performed and the manuscript was being prepared, J.B. served on the Board of Directors (BOD) for Neovacs; served on the Scientific Advisory Board (SAB) for Georgiamune LLC; BOD member and a stock holder for Ascend Biopharmaceuticals; Scientific Advisory Board (SAB) member and a stock holder for Cue Biopharma; and a stock holder for Sanofi.

JAX (J.B.) and Sanofi also entered into a collaborative research agreement to work on a long-read sequencing project (ended in Jul 2021). Since Aug 2021, J.B. joined Immunai in New York as their new Chief Scientific Officer (CSO) and is also continuing a limited affiliation with JAX until end of Feb 2022.

Data availability: Raw fastq and processed read count files for all samples are deposited to GEO accession code GSE159798.

Methods

Animals and Housing

C57BL/6J (stock 000664) and NZO/HILtJ (stock 002105) animals were obtained from The Jackson Laboratory and kept in individually ventilated cages with free access to food (5KOG, LabDiet) and water. The pathogen-free room (health status report attached) was kept between 20°C and 22°C, with a 12-hour

light:dark cycle. Spleen and blood samples were obtained immediately after euthanasia through cervical dislocation. The mouse study was approved by The Jackson Laboratory's Institutional Animal Care and Use Committee.

Flow Cytometry data generation and analyses

Data is obtained from spleen and peripheral blood lymphocytes (PBLs) of C57BL/6J and NZO/HILtJ mouse strains, hereafter B6 and NZO, respectively, at ages 3, 12, and 18 months. Cells were stained with i) CD8 FITC, Clone 53-6.7 BD Bioscience Cat# 553031, used at 1:240 final concentration, ii) CD3e PE, Clone 145-2C11 eBioscience (Now ThermoFisher) cat# 12-0031-85, used at 1:240 final concentration, iii) CD62L PE-Cy7, Clone MEL-14 Tonbo Biosciences Cat# 60-0621-U100, used at 1:480 final concentration, iv) CD44 APC-Cy7, Clone IM7 Tonbo Biosciences Cat# 25-0441-U100, used at 1:240 final concentration and for 30 minutes at 4 degrees C. Propidium Iodide used for cell viability at 0.5ug/ml.

Then cells were sorted on a FACSARIA II (BD Biosciences). Briefly, doublets were gated out, Viable PI- cells were gated, CD3e⁺, CD8⁺ cells were gated and subdivided into CD44^{Low}, CD62L^{High} Naïve cells and CD44^{High}, CD62L^{+/-} Memory cells. Up to 50,000 cells were sorted for ATAC-seq, remaining cells collected for other RNA preparations. Cells were collected in tubes coated with Fetal Bovine Serum (FBS). The percentages of B, CD4, CD8, Naïve CD4, Central memory CD4, Effector memory CD4, Effector memory RA CD4, Naïve CD8, Central memory CD8, Effector memory CD8, Effector memory RA CD8 were measured. In addition, within each of these cell types, the percentages of IL7R⁺ and PD1⁺ cells were measured. After summing up B, CD4⁺ and CD8⁺ T cells, we labelled the rest of the percentages as monocytes since NK cells, neutrophils and other cell types compose only ~5% of spleen and PBL. Naïve and Memory CD8 T cells were sorted from spleens as follows: Spleens were removed from mice and teased apart in nylon mesh bags in PBS with 2% FBS, 5mM EDTA, and 0.02% Sodium Azide (FACS Buffer). The cells were lysed with "Gey's Buffer" (a modification of GBSS as described in the attached paper where the Sodium Chloride was exchanged with Ammonium Chloride at the same molar concentration) for 5 minutes and then washed with FACSBuffer and counted to determine concentration. Cells were stained at approximately 10⁸/ml with CD8 FITC, CD3e PE, CD62L PE-Cy7, and CD44 APC for 30 minutes at 4 degrees. Cells were washed and resuspended for sorting in FACS Buffer. I sorted Naïve (CD62L⁺, CD44^{Low}) and Memory (CD62L^{+/-}, CD44^{High}) CD8⁺, CD3e⁺ cells. To quantify cell compositional changes with age, we built linear models where age is the independent variable and cell type percentage is the dependent variable. For each model, we computed the slope of change per increase in age by 1 unit and a corresponding p-value, which is later corrected using Benjamini-Hochberg procedure.

RNA Isolation and QC

Total RNA was isolated from 1 million cells using the RNeasy Mini kit (Qiagen), according to the manufacturers' protocols, including the optional DNase digest step. For samples with fewer than 1 million cells, RNA was isolated using the RNeasy Micro kit (Qiagen). Sample concentration and quality were assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific) and the RNA 6000 Nano and Pico LabChip assays (Agilent Technologies).

RNA Library generation

30 ng of total RNA, with the addition of 6 µl ERCC Spike-In Control Mix 1 (Ambion Thermo Fisher) diluted 1:10,000, was used for library construction. Libraries were prepared by the Genome Technologies core facility at The Jackson Laboratory using the KAPA mRNA HyperPrep Kit (KAPA Biosystems), according to the manufacturer's instructions. Briefly, the protocol entails isolation of polyA containing mRNA using oligo-dT magnetic beads, RNA fragmentation, first and second strand cDNA synthesis, ligation of Illumina-specific adapters containing a unique barcode sequence for each library, and PCR amplification. Libraries were checked for quality and concentration using the D5000 assay on the TapeStation (Agilent Technologies) and quantitative PCR (KAPA Biosystems), according to the manufacturer's instructions.

RNA Sequencing

Libraries were pooled and sequenced 75 bp single-end on the HiSeq 4000 (Illumina) using HiSeq 3000/4000 SBS Kit reagents (Illumina), targeting 40 million reads per sample. We obtained RNA-seq data from spleen, PBL and sorted T cells (derived from spleen) of B6 and NZO mouse strains at age 3, 12, and 18 months. Single-end RNA-seq reads were aligned to the mouse genome (mm10) with Bowtie 2⁽⁴²⁾ and counts were generated with *RSEM*⁽⁴³⁾. To normalize the raw counts count-per-million (*cpm*) function from edgeR package is used and the genes that are $\log(\text{cpm}) < 1$ and expressed less than 2 samples were excluded from rest of the analyses. For differential analysis pipeline, however, raw counts are used with the default options of edgeR package⁽⁴⁴⁾, and via TMM normalization.

ATAC-seq library generation

ATAC-seq libraries were prepared using 50,000 cells, as previously described⁽⁴⁵⁾, with the following modifications: digitonin was added to the transposition reaction at a final concentration of 0.01%; the transposition reaction was purified using the Genomic DNA Clean & Concentrator-10 kit (Zymo Research Corporation); PCR amplification was carried out using the Nextera DNA Library Prep (Illumina) Index Adapters, Nextera PCR Master Mix, and PCR Primer Cocktail for 10 cycles of PCR; PCR reaction was purified using 1.7x SPRI beads (Agencourt AMPure XP, Beckman Coulter). Libraries were checked for quality and concentration using the DNA High-Sensitivity LabChip assay (Agilent Technologies) and

quantitative PCR (KAPA Biosystems), according to the manufacturer's instructions. Libraries were pooled and sequenced 75 bp paired-end on the NextSeq 500 (Illumina) using NextSeq High Output Kit v2 reagents (Illumina).

ATAC-seq data analyses

We obtained ATAC-seq data from spleen, PBL and sorted T cells from spleen of B6 and NZO mouse strains at age 3, 12, and 18 months. Paired-end ATAC-seq reads were quality trimmed using *Trimmomatic*⁽⁴⁶⁾ and trimmed reads were aligned to mouse genome (mm10) using *BWA*⁽⁴⁷⁾. After preprocessing and quality filtering, peaks were called on alignments with *MACS2* using the BAMPE option⁽⁴⁸⁾. The consensus peakset for PCA and PVCA plots were generated gathering all peaks from all tissue/cell types, whereas for differential accessibility analyses the samples of the same tissue were merged to generate one consensus peak set by using R package *DiffBind*⁽⁴⁹⁾. Peaks only present in at least two samples were included in the analysis. Raw read counts were normalized using the *cpm* function via the log option turned on from edgeR package⁽⁴⁴⁾.

Statistical Methods

Principal Variance Component Analysis (PVCA) was used in order to determine the sources of variability in flow cytometry data⁽¹⁸⁾, which combines the strengths of Principal Component Analysis (PCA) and Variance Component Analyses (VCA). So, using PVCA the proportions variances were attributed to each factor. To compare the normalized gene expressions and peak counts across different tissues and cell types, Wilcoxon rank sum test was used.

Differential Analyses

To identify differentially expressed genes and differentially accessible peaks between age groups, we used the R package *edgeR*⁽⁴⁴⁾ was used. It fits a generalized linear model (GLM) that includes age as a continuous independent variable and read counts from either ATAC-seq or RNA-seq as dependent variables to test for the effect of age on read counts. We stratified data by tissue and strain and fit GLM within strata. In addition to the age, we included sex as a covariate, which did not yield any statistically significant results. P-values for the age effect were adjusted using the Benjamini-Hochberg procedure, and genes or peaks with FDR-adjusted p value < 0.05 were considered differential.

Enrichment Analyses

Immune modules were obtained from human PBMCs⁽²⁴⁾. Human and mouse orthologs were identified using R package *biomaRt*⁽⁵⁰⁾. Consensus peaks were annotated using *HOMER*⁽⁵¹⁾ and gene-based analyses were restricted to promoter peaks annotated to the nearest transcription start sites (TSS) of expressed genes. Hypergeometric p-value is calculated for each module of the inflammation genesets.

Then, p-values are adjusted for multiple hypothesis testing using Benjamini-Hochberg correction. For all analyses, modules that have FDR-adjusted p value < 0.05 considered as enriched.

Single cell analyses

We have downloaded 10x single-cell RNAseq spleen data of Tabula Muris Senis⁽¹⁶⁾ from UCSC browser (<https://cells.ucsc.edu/?ds=tabula-muris-senis+droplet+spleen>) and transferred it into R environment (v4.0.5). Next, we selected 3- and 18-months samples (2 samples per age point) and ran the standard Seurat (v4.0.2) pipeline with the default parameters (log normalization, 10 PCs, and UMAP for dimensionality reduction). Then, we removed the cell types which had less than 100 cells in total along with the doublet cluster, remaining cells from four major cell types (NK, Macrophage, B, and T cells) were used in all single-cell related analyses. We detected the cluster cell types based on their respective marker genes here; B cell (*Blnk*, *Cd79a*, *Cd79b*), T cell (*Cd3d*, *Cd3e*, *Cd3g*), NK (*Ncr1*, *Gzma*), Macrophage (*Itgax*). We have used t-test to quantify the differences between the expression of cells in young (3 mo) vs. old (18 mo) mice samples for *Jun* and *Fos*.

Footprinting Analysis

ATAC-seq data from spleen and PBL were scanned for TF footprints using the PIQ algorithm⁽²⁸⁾. This method integrates genome-wide TF motifs (i.e., position weight matrices) with chromatin accessibility profiles to generate a list of potential TF binding sites that are bound by a TF. The method also produces a quality score for each footprint (positive predictive value). Only the TF footprints with positive predictive values > 0.9 are used in downstream enrichment analyses.

Before footprint calling, we merged samples of the same sex, strain, tissue and age group to increase read depth, which improved the quality and detection power of PIQ. In addition to that we used *SAMtools*⁽⁵²⁾ to randomly downsample aligned reads from each merged data set to 50 M reads to minimize the impact of the high correlation between library depth and footprint positive predictive values. We used a set of motifs available in the JASPAR 2016 database (n= 454 for human and n=189 for mouse)⁽⁵³⁾. Finally, footprint calls were further filtered to include in analyses only those associated to TFs that are expressed in the PBL or spleen. For each cell type, we applied *BiFET*⁽³⁰⁾ to identify TFs whose footprints are significantly more detected in opening/closing peaks compared to background peaks (peaks whose chromatin accessibility do not change with age). In each tissue, we selected TFs whose *BiFET* q-values are less than 0.05 for at least two samples. We followed the same protocols for our previously published human chromatin accessibility data⁽⁹⁾ and merged PIQ calls of young (<40 years) and older individuals (>65 years). For AP-1 complex related TF analyses, we selected each subunit from

JASPAR annotated files. Then, the locations of these TFs were annotated to their closest TSS using *ChIPseeker* package⁽⁵⁴⁾ (v1.27.3) to uncover the most effected sites. Finally, we calculated enrichment scores of these sites using hypergeometric p-value test and our immune signature gene sets.

c-JUN assays and immunoblots

Lysates were extracted from splenocytes, both activated and unactivated, according to the manufacturer's instruction of Nuclear Extraction Kit (Abcam, ab113474), and quantified by Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo-Fisher, 23200). The c-Jun transcription factor was assayed by c-Jun Transcription Factor Assay Kit (Colorimetric) according to manufacturer's instruction (Abcam, ab207195). Activation of the splenocytes were done with anti-CD3 and anti-CD28 (kind gift of Dr. Dave Serezze's lab at The Jackson Laboratory) and Mouse TLR1-9 Agonist kit (InvivoGen, tlr1-kit1mw) according to manufacturer's recommendations in DMEM (Gibco/Thermo-Fisher, 12430112) with 10% FBS (Hyclone/Thermo-Fisher, SH3007003), HEPES (Thermo-Fisher, 15630080) and Pen/Step/Glutamine (Thermo-Fisher, 10378016). IL6 in the supernatants was measured by Mouse IL-6 ELISA kit (Abcam, ab222503) according to supplier's protocol.

The capillary immunoblotting analysis was performed, using Wes (ProteinSimple, Santa Clara, CA, USA), according to the ProteinSimple user manual. The lysates of the primary male mice splenocytes were mixed with a master mix (ProteinSimple) to a final concentration of 1 × sample buffer, 1 × fluorescent molecular weight marker, and 40 mM dithiothreitol and then heated at 95 °C for 5 min. The samples, blocking reagents, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate (ProteinSimple) and separation and stacking matrices were also dispensed to the designated wells in a 25 well plate. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. A capillary immunoblotting analysis 10378016 is was carried out at room temperature, and the instrument's default settings were used. Capillaries were first filled with a separation matrix followed by a stacking matrix, with about 40 nL of the sample used for loading. During electrophoresis, the proteins were separated by molecular weight through the stacking and separation matrices at 250 volts for 40–50 min and then immobilized on the capillary wall, using proprietary photo-activated capture chemistry. The matrices were then washed out. The capillaries were next incubated with a blocking reagent for 15 min, and the target proteins were immunoprobed with primary antibodies followed by HRP-conjugated secondary antibodies The antibodies of GAPDH (sc-25778, 1:200, Santa Cruz Biotechnology), Lamin B1 (12586S, 1:100, Cell Signaling Technology), Phospho-c-Jun (9261S, 1:50, R Cell Signaling Technology), c-Jun (9165S, 1:50, Cell Signaling Technology), were diluted in an antibody diluent (ProteinSimple).

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Figures

Figure 1

Figure 1

A Schematic of study design. PBL and spleen were collected from 3 and 18 month old mice, and CD8+ T-cells were sorted from spleen. Spleen was collected from 12 month old mice. From these samples, ATAC-seq, RNA-seq and flow cytometry data were generated. B Summary of samples, i.e., individual animals: 74 flow cytometry samples (39 B6, 35 NZO), 103 RNA-seq samples (49 B6, 54 NZO), and 90 ATAC-seq samples (38 B6, 52 NZO). C PCA results for flow cytometry (n = 33 cell subsets), ATAC-seq (n = 96,623

peaks) and RNA-seq (n = 18,294 genes). Tissue/cell type information was overlaid onto PC1 and PC2 dimensions and percentages indicates the explained variance with the given principal component. D The variances of the datasets were attributed to meta data (sex, age, strain and tissue/cell type) using principal variance component analyses (PVCA).

Figure 2

A Flow gating strategy for measuring the cell compositions of T-cell subsets (effector, effector memory (EM), naive and central memory (CM)). B Cell composition pie charts for B, CD4+ T and CD8+ T cells. Remaining cells were as designated “other myeloid” cells as they comprise mostly monocytes, DCs. C Summary for the association of age with a given cell type using linear models. Each cell type was colored according to the slope of change per unit of age (in months) and the direction of the slope - red for positive (increase with age) and blue for negative (decrease with age) slopes, with darker colors indicating steeper slope (i.e., more significant changes). Significant associations (FDR 5%) are marked with a dot. D, E CD8+ T EM cell percentages increase with age in PBL (D) and spleen (E) (more significantly in B6 vs. NZO); whereas CD4+ naive T cells decline with age in both tissues. F Age (in years) and cell composition association analyses in human PBMCs(9). Significant associations (FDR 5%) are marked with a dot. G Percent of naive CD8+ T cells versus age (in years) for men and women PBMCs. Note that declines are much more significant in men.

Figure 3

Figure 3

A Differentially expressed (top) and accessible (bottom) genes between old (18 mo) and young (3 mo) mice in PBL and spleen of B6 and NZO strains at FDR = 0.05 and $|\log FC| > 1$. Red indicates upregulated/opening with age, whereas blue indicates downregulation/closing. B Functional annotations of differential genes/peaks using GSEA; p values were calculated using 1000 permutations and corrected using the Benjamini-Hochberg procedure. Only the pathways that have FDR < 0.05 are shown with

bubbles. The “Inflammatory processes” (Inflammation I) module was upregulated/opening across tissue and strain types. CD4+ and CD8+ T cell module was downregulated especially in PBL, which might be due to decrease in T-cell proportions (Figure 2B). C Gene expression levels of differentially expressed genes (DEGs) among the “Inflammation I” module genes. Libraries were normalized using cpm function from edgeR package and the gene expression values were z-transformed. Notably, activation of Il1b and Fosl2 were among the most conserved signature of aging across all comparisons. D Il1b and E Fosl2 expression levels at different time points (3, 12, 18 months). Expression values were log(cpm) normalized.

Figure 4

Figure 4

A Differentially expressed (top) and accessible (bottom) genes between old (18 mo) and young (3 mo) mice in CD8+ T cells sorted from spleens of B6 and NZO strains at FDR = 0.05 and $|\log FC| > 1$. Red indicates upregulated/opening with age, whereas blue indicates downregulation/closing. B Functional enrichment of differential regions using Wikipathways; p values were calculated using hypergeometric p-value and were corrected using the Benjamini-Hochberg procedure. Only the pathways with significant

enrichment (FDR < 0.25) are represented. The IL2 signaling pathway module was upregulated across cell types and strains. C Gene expression level of genes in the IL2 signaling pathway across samples. Gene expression values were normalized (cpm) and were z-transformed. Asterisks denote statistically significantly upregulated genes with age that are common across strains and cell types. Note that Jun and Fos are among these genes. D Chromatin accessibility around Jun and Fos genes. Note that the accessibility increases with age across cell types and strains.

Figure 5

Figure 5

A Transcription factor (TF) footprinting enrichment analyses in mice. Left: 34 TF footprints were enriched among opening peaks. 27 were shared across tissue and cell types. Right: 20 TF footprints were enriched among closing peaks; these were cell-specific. (Adjusted $q < 0.05$ using BiFET). B Overlap between footprints enriched in opening peaks in human and mice. 6 proteins were common including five AP-1 members (FOS, FOSL2, JDP2, JUNB, JUND), and a co-factor of the complex NFE2L2. C Age-related changes (positive log FC indicates increase with age) in the chromatin accessibility levels of 6 shared TFs. Note the conserved accessibility increase across human and mice, strains, and tissues. AP-1 complex members are marked with an asterisk. D PCA plot of AP-1 complex genes in mice (34 genes shared in Table S6) separates old mice (18 mo, dark red) from young mice across all cell types (3 mo, yellow). E Percent of footprints detected for the shared 6 TFs in each age group and cell types. Counts are normalized with respect to the total number of footprints detected. TF footprint counts and percentages are listed in Table S7. F Functional enrichment of gene targets for selected TFs using immune modules in B6 PBL (complete results are in Table S8). Values show the percent of overlap between gene targets and module genes; significant enrichments are indicated with an asterisk (FDR < 0.05). Note the increase in NFkB pathway in older samples. G Enrichment of selected TF gene targets for the NFkB pathway genes across cell types and strains. (Enrichment FDR $< 0.05^*$, 0.01^{**} , 0.001^{***}) H Functional enrichment of gene targets for selected TFs using immune modules in human PBMCs. Values show the percent of overlap between gene targets and module genes; significant enrichments are indicated with an asterisk (FDR < 0.05).

Figure 6

Figure 6

A UMAP for the Tabula Muris Senis spleen data using 3- and 18-month old B6 mice (n=2, per age point) with original annotations. Cell types that have less than 100 cells were removed from the down-stream analyses, resulting in 4 major cell types; B cells, Macrophages, Natural Killer (NK) cells, and T cells (n=10381 cells). B The expression levels of the AP-1 complex subunits (Jun, Fos, Fosl2) in log-normalized scale in 3m and 18m cells. Note the increase in expression for all cell types. C Percentage of cells

expressing the AP-1 complex subunits across young and old cells. Note that these molecules are expressed more with age across all cell subsets. D c-JUN binding levels upon different stimuli (anti-CD3+ anti-CD28, LPS and poly(I:C)) for 3- and 18- months B6 mice. c-JUN binding levels were normalized to the non-stimulated samples (complete data in Table S10). E Western blot of p-c-JUN and c-JUN proteins for nuclear and cytosolic extracts of male B6 mice. Note that c-JUN levels increase with age – especially upon poly(I:C) stimulation. F Number of footprints detected around the Il6 gene (100Kb upstream and 100Kb downstream of the TSS from each side) in B6 PBL and spleen. Note the increased AP-1 activity around this molecule with age. G IL6 cytokine levels secreted by splenocytes of 3- and 18-month-old B6 mice (Table S11). Note the increased production of the cytokine with age upon poly(I:C) stimulation.

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