Robust shifts in S100a9 expression with aging: A novel mechanism for chronic inflammation

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The S100a8 and S100a9 genes encode a pro-inflammatory protein (calgranulin) that has been implicated in multiple diseases. However, involvement of S100a8/a9 in the basic mechanisms of intrinsic aging has not been established. In this study, we show that shifts in the abundance of S100a8 and S100a9 mRNA are a robust feature of aging in mammalian tissues, involving a range of cell types including the central nervous system. To identify transcription factors that control S100a9 expression, we performed a large-scale transcriptome analysis of 62 mouse and human cell types. We identified cell type-specific trends, as well as robust associations linking S100a9 coexpression to elevated frequency of ETS family motifs, and in particular, to motifs recognized by the transcription factor SPI/PU.1. Sparse occurrence of SATB1 motifs was also a strong predictor of S100a9 coexpression. These findings offer support for a novel mechanism by which a SPI1/PU.1-S100a9 axis sustains chronic inflammation during aging.

Aging is commonly associated with a state of chronic inflammation that contributes to DNA damage, atherosclerosis, stem cell senescence and cognitive decline. Although inflammation does not occur uniformly or within all mammalian tissues due to increased age, in some tissues aging leads to activation of immune-response pathways and the formation of lymphoid aggregates, particularly within perivascular regions. Mechanisms that underpin these processes are not well understood, however, and further work is needed to identify “hubs” within inflammatory and cytokine networks that drive these events. In recent years, two low molecular weight proteins, S100a8 (calgranulin A) and S100a9 (calgranulin B), have emerged as central inflammatory regulators capable of driving and responding to inflammation signals. On the one hand, S100a8/a9 mRNA and protein levels are markedly increased in response to cytokine stimuli. At the same time, S100a8/a9 reinforce inflammatory cascades by serving as leukocyte chemoattractants, inducing the expression of pro-inflammatory cytokines, triggering activation of NF-kB, and by serving as ligands that interact with and stimulate receptor for advanced glycation end products (RAGE). At the same time, S100a8/a9 reinforce inflammatory cascades serving as leukocyte chemoattractants, inducing the expression of pro-inflammatory cytokines, triggering activation of NF-kB, and by serving as ligands that interact with and stimulate receptor for advanced glycation end products (RAGE). The transcription factors STAT3 and NF-kB were independently identified as activators of S100a9 expression in both human and mouse cell types. Other TFs have also been identified as S100a9 regulators, although it remains
unclear whether their regulatory role is limited to humans alone, mice alone, or to a single cell type. Such factors include putative S100a9 activators C/EBPα and C/EBPβ, HIF-1α, GLI1, and SPI1/PU.1, in addition to putative S100a9 repressors BRCA1, AP-1, SATB1, and Arrt/HIF-1b. In the context of aging or disease, such DNA-binding factors may drive or suppress S100a9 expression, thereby modulating the intensity of age-dependent inflammation. This is one avenue towards development of therapeutic approaches, and indeed, pharmacological inhibition of S100a9 activators (e.g., NF-kB) has been linked to slowed tumor growth and delayed accumulation of senescent cells with aging.

We here show that aging leads to shifts in the abundance of S100a8/a9 in humans and mice, which involve multiple tissues and robust trends across mouse genotypes. These findings demonstrate that shifts in S100a8/a9 abundance are a feature of normal aging, suggesting a role for S100a8/a9 in age-associated inflammation that extends beyond their involvement in specific diseases (e.g., Alzheimer’s disease). We have further investigated mechanisms of S100a9 transcription using a large-scale integrative transcriptomics strategy, which has allowed us to systematically screen DNA-binding factors for association with S100a9 expression across many cell types and transformed cell lines (30 mouse cell types and 32 human cell types). Our approach provides objective statistical assessment of evidence for those TFs with known DNA binding affinities, and offers a means to distinguish cell type-specific patterns from more robust trends supported in multiple cell types. We thus illustrate a strategy that is generally useful for in silico study of mammalian gene regulation, and in the current application, our results provide systems-level insight into TFs and pathways that govern S100a9 transcription. Our findings, moreover, point towards a new mechanism for age-related chronic inflammation, in which over-production of S100a9, enforced by key transcription factors, triggers a feed-forward cycle that sustains a pro-inflammatory microenvironment with increasing age.

Results

Shifting in S100a8/a9 abundance are a robust feature of normal aging in mouse and human tissues. We used Affymetrix DNA oligonucleotide microarrays to evaluate gene expression in tail skin from young (5 months) and old (30 months) CB6F1 mice (n = 5 per age group and sex). Two of the top ten age-increased genes encoded S100 proteins, including S100a8 and S100a9 (data not shown). We confirmed this pattern using RT-PCR and showed that S100a8/a9 expression increased late in the lifespan between 17 and 30 months of age, with no significant change between 5 and 17 months of age (Figures S1A and S1D). Consistent with this, immunostaining did not detect S100a9 in young mice, but did detect S100a9 widely distributed in the epidermis, dermis and subcutaneous layers in old mouse skin (Figure S2). We expected to observe a similar pattern in human skin. However, analysis of a large microarray dataset indicated that S100a8/A9 expression decreased with age 1–2% per year (Figures S1B and S1C). We thus used RT-PCR to analyze an independent set of skin biopsies obtained from young (20–40 years old; n = 11 males and n = 6 females) and elderly human subjects (> 80 years old; n = 5 males and n = 10 females). This confirmed that S100A8/A9 expression decreased with age in human sun-protected skin (Figure S1E), in contrast to the trend observed in tail skin from CB6F1 mice (Figures S1A and S1D).

We further investigated the effects of aging on S100 expression in other mouse and human tissues. The AGEMAP study was a large-scale effort designed to identify age-associated gene expression patterns across 16 tissues from C57BL/6 mice (1, 6, 16 and 24 months of age). However, inspection of the AGEMAP S100a9 expression pattern revealed no trend towards elevated expression across 16 tissues (Figure S3). Since AGEMAP was limited to a single inbred genotype (C57BL/6) and used cDNA arrays that lacked probes to detect several S100 genes (e.g., S100a8), we assembled datasets that used whole-genome Affymetrix arrays to evaluate gene expression in young and old tissues (Figure 1). These data revealed that, in both mice and humans, all S100 gene family members were significantly altered by aging in at least one tissue (P < 0.05), with the strongest and most consistent effects observed for S100a8 and S100a9 (Figure 1). These results were further supported by our studies of multiple organs from CB6F1 mice, which showed an increase in S100a9 expression with age (heart, liver, lung and kidney) (Figure S4). Additionally, staining of tissues using an anti-S100a9 antibody revealed a trend towards

Figure 1 | Shifts in the expression of S100a8 and S100a9 are a robust feature of aging in human and mouse tissues (meta-analysis of microarray data). The effects of aging on the expression of S100-encoding mRNAs were evaluated in human (left) and mouse (right) tissues. All human data was generated using the Affymetrix Human Genome U133 Plus 2.0 array platform (29 experiments), and all mouse data was generated using the Affymetrix Mouse Genome 430 2.0 array platform (34 experiments). Data were obtained from the Gene Expression Omnibus or ArrayExpress databases. Colors denote the estimated fold-change over 40 years in humans (left; old/young) or over 2 years in mice (right; old/young). Triangles denote significant effects of aging on the expression of the listed gene (row) with respect to the indicated tissue (column) (P < 0.05 or FDR < 0.05).
increased S100a9 protein in liver (males and females, Figures S5 and S6), kidney (males only; Figure S7) and lung (males and females, Figure S8).

An in silico procedure for identifying transcription factors and motifs that regulate S100a9 expression in 30 mouse and 32 human cell types. Our data identified shifts in the abundance of S100a9 mRNA and protein as a robust feature of intrinsic mammalian aging. Given that expression of S100a9 was often elevated with age (Figure 1), we hypothesized that such an increase could facilitate age-associated inflammation and the formation of lymphocyte aggregates in older tissues, as has been described in prior work. To identify mechanisms that may mediate increased S100a9 expression with aging, therefore, we developed an in silico procedure to identify transcription factor binding sites that predict S100a9 coexpression in mouse and human cell types (Figure 2). For this purpose, we assembled manually curated datasets for each of 30 mouse cell types and 32 human cell types. Our strategy was to identify S100a9 coexpressed genes for each mouse and human cell type (Figures 2A and 2B), yielding the cell type-specific sub-network surrounding S100a9 within the genome-wide coexpression network (Figure 2C). We then used generalized additive logistic models (GAM) to identify motifs for which motif frequency in the 2 KB upstream region predicted S100a9 coexpression within each cell type (Figure 2D). For these analyses, we screened a dictionary consisting of 1209 motifs, where each motif corresponded to a mouse or human DNA-binding protein (see Methods). In addition to cell type-specific analyses, we analyzed a “composite” S100a9 coexpression network, which was generated by integrating trends across all cell types in mouse or human, respectively (see Methods).

Genes coexpressed with S100a9 in mouse cells are located in regions enriched with binding sites for ETS transcription factors (e.g., SPI1/PU.1) but with sparse occurrence of SATB1 sites (AATTTTT). Genes coexpressed with S100a9 in the composite network were enriched with DNA motifs bound by members of the ETS transcription factor family (Figures 3 and S9). Of the ten most significant motifs in the composite network, nine were recognized by ETS family members (e.g., SPI1, ETS1, ETS2), and four of these were associated with the ETS member SPI1/PU.1 (Figure 3). The top-ranked motif in the composite network, SPI1/AGGAAGT/MA0080, was significantly enriched among S100a9 coexpressed genes in 14 of 30 cell types (P < 0.05), although we identified another SPI1/PU.1 motif that was significantly enriched among S100a9 coexpressed genes in 25 of 30 cell types (SPI1/GGAAG/MA0080; Figure 3). Additionally, we identified five cell types for which the most significantly enriched motif was associated with SPI1/PU.1 (hematopoietic stem cells, B-cells, thymocytes, CD8+ T-cells and CD4+ T-cells; Figure 3). These analyses were based upon 2 KB upstream regions, but further analyses of other regions also supported an association between ETS family motifs and S100a9 coexpression (e.g., conserved regions 2 KB upstream, intronic regions and all non-coding intergenic regions; Figures S9A – S9C). Additionally, we performed an alternative analysis to determine which k-mers (of length 2, 3, 4 or 5) best distinguished enriched (P < 0.05 with Z > 0) from non-enriched (P > 0.05) motifs (2 KB upstream region). In the composite network, enriched motifs differed from non-enriched motifs in that they matched the 5-mer AGGAA|TTCTCT (Figure S9D). This 5-mer matches the GGA(A/T) core motif recognized by ETS family members and resembles the consensus sequence for several SPI1/PU.1 binding sites (Figures 3 and S9).

Cross-validation was used to determine how well single-motif regression models could predict S100a9 coexpression. This showed that the “best model” (Akaike information criterion, AIC) was able to predict S100a9 coexpression for the composite network and 20 of 30 mouse cell types (i.e., observed and null AUC distributions at least 1 SD apart) (data not shown). Performance improved, however, when we analyzed two-motif models (Figure 4). For bivariate motif models, there was strong separation between observed and null AUC distributions for 26 of 30 cell types (Figure 4). The best-performing bivariate models for each cell type often related increased abundance of ETS-associated motifs to increased probability of S100a9 coexpression (Figure 4). Surprisingly, however, for 6 of 30 cell types, decreased occurrence of SATB1 binding sites was predictive of S100a9 coexpression (VSSATB1_Q5/AATTTT; splenocytes, keratinocytes, lymphatic endothelial cells, pancreatic islets, 3T3-L1 cells and RAW266 cells) (Figure 4). This association was robustly observed across 29 of 30 cell types (upstream 2 KB region; P < 0.05 with Z < 0), indicating that genes coexpressed with S100a9 in mouse cells are associated with genomic regions that are deficient with respect to SATB1 binding sites.

The region 150–250 BP upstream of the mouse S100a9 gene contains the highest concentration of transcription factor binding sites that predict S100a9 coexpression. The complete statistical profile of all transcription factor binding sites we evaluated provides a tool for detecting loci with cell type-specific regulatory potential. We thus scanned the 2 KB region upstream of the mouse S100a9 gene to determine which region contained the greatest concentration of motifs enriched among S100a9 coexpressed genes (Figure 5). For the composite network, and all 30 cell types, sliding window analyses identified a high-scoring locus 150–250 BP upstream (mm9, chr3, 90499762–90499862) (Figure 5). We identified 19 motifs within this region for which increased occurrence was associated with S100a9 coexpression in the composite network (Figure S10; P < 0.047 with Z > 0). Further inspection uncovered a 13 base element (5-AC'TCCCTTCTTCCAT; mm9, chr3, 90499826–90499838) with binding sites for several significant motifs, including multiple matches to motifs recognized by SPI1/PU.1 and STAT transcription factors (Figure S10).

GC-rich motifs and bindings sites for ETS transcription factors (e.g., SPI1/PU.1) predict S100A9 coexpression in diverse human cell types. We repeated our screen of 1209 transcription factor binding sites (Figure 2), except we attempted to identify motifs predictive of S100A9 coexpression in 32 human cell types (2 KB upstream regions). A motif-by-motif comparison of Z statistics from 13 corresponding cell types in mouse and human revealed good correspondence in 12 cases (0.21 ≤ r ≤ 0.64; P < 0.001), and overall, correspondence between results from mouse and human composite networks was convincing (r = 0.43; P = 10^-49) (Figure S11). In line with this, several motifs supported in our analysis of mouse cells were bolstered by trends detected in human cells (Figures 6 and S12). For instance, two motifs associated with ETS transcription factors were consistently enriched among S100A9 coexpressed genes (VSETS2_Q6/CTTCCCTG and SPI1/GGAAG/MA0080), with significant trends detected for 28 of 32 human cell types including the human composite network (2 KB upstream regions; P < 0.05 and Z > 0) (Figure 6). This pattern was also supported by evaluation of conserved 2 KB upstream sequences, intronic sequences, and complete non-coding intergenic sequences (Figures S12A–S12C). Interestingly, a strong trend in human cells was enrichment of GC-rich motifs in genomic regions associated with S100A9 coexpressed genes (e.g., V$iMOVOB_01/GGGGG$ and V$SSP1_Q2_01/CCCCCGGGG$) (Figure 6). Consistent with this, we searched for features that distinguished significantly enriched motifs (P ≤ 0.05 with Z > 0) from all other motifs, and found that enriched motifs more closely matched the 4-mer CCCCC (Figure S12D). This trend was further supported by cross-validation analyses in which we identified bivariate models that best predicted S100A9 coexpression (AIC criterion; see Figure 7). For 7 of 32 cell types, increased abundance of an ETS transcription factor motif was...
Figure 2 | In silico strategy for identifying cis-regulatory mechanisms controlling S100a9 expression. The figure illustrates a general procedure for identifying a cluster of S100a9-coexpressed genes (parts A – C), which can then be evaluated to identify TF binding sites that occur at disproportionately high frequency within associated genomic sequences (part D). The procedure is here illustrated for a single cell type (mouse chondrocytes), but we have applied the methodology across a broader panel of 30 mouse and 32 human cell types. In the first step (A), a foreground set of S100a9-coexpressed transcripts is identified. This is done by calculating the Spearman rank correlation ($\rho$) between each transcript and S100a9, and then ranking all transcripts by the magnitude of $\rho$. The dashed red line shown in (A) represents the segment with minimal distance between the origin (lower left corner) and the curve shown in the figure. This red line serves to define the foreground set of S100a9-coexpressed genes (dark grey region). In part (B), this S100a9-coexpression cluster is illustrated with respect to the 53 microarray samples used to calculate Spearman rank correlations shown in (A), where each microarray sample was generated by hybridization with cDNA derived from mouse chondrocytes. The foreground set of S100a9-coexpressed genes can thus be viewed as the local sub-network that surrounds S100a9, as illustrated in (C). In the final step (D), a generalized additive logistic model (GAM) is used to identify significant associations between S100a9 coexpression and the number of TF binding sites present within the 2 KB region upstream of the transcription start site (or other genomic regions). In GAM models, the probability of S100a9 coexpression is modeled (vertical axis) as a function of two variables $x_1$ and $x_2$, where $x_1$ is the length of unmasked sequence scanned for a given gene and $x_2$ is the number of TF binding sites identified in the upstream region. GAM models were fit for each of 1209 TF binding sites, and a significant association between S100a9 coexpression and binding site occurrence was evaluated based upon significance of the coefficient $\beta_2$. 
predictive of S100A9 coexpression in the best-performing bivariate model (e.g., SPI1/PU.1, ETS1, ETS2 and ELK1). However, for approximately half of the 32 cell types, bivariate models related increased frequency of a GC-rich motif to increased probability of S100A9 coexpression (Figure 7).

A region 930 - 1030 BP upstream of the human S100A9 gene contains the highest concentration of TF binding sites that predict S100A9 coexpression. Using results from our in silico screen of transcription factor binding sites in 32 human cell types, we scanned the 2KB region upstream of S100A9 to identify loci containing a high density of motifs enriched among S100A9 coexpressed genes. For most cell types, the first 500 BP upstream contained abundant matches to significantly enriched motifs (Figure S13). Surprisingly, however, the region 930 – 1030 BP upstream of S100A9 contained the greatest concentration of binding sites for high-scoring motifs (hg19, chr1, 153329299 – 153329399; Figure S13), with matches to 43 motifs significantly associated with S100A9 coexpression in the composite network (P < 0.05 with Z > 0; Figure S14). More than half of these motifs (24 of 43) matched a 19 base pair GC-rich sequence within this region (5-GCCGTGGGGGCGGGCAGGA-3; hg19, chr1, 153329306–153329324).

Amplification of a SPI/PU.1-S100a9/A9 inflammatory axis in aged tissues and a model for S100a9/A9-mediated chronic inflammation with aging. Our findings, based upon an integration of transcriptome data from a diversity of mammalian cell types, implicate the ETS transcription factor SPI1/PU.1 as a driver of S100a9/A9 expression. We investigated whether accumulation of S100a9 with aging in CB6F1 mice was associated with elevation of SPI1/PU.1 (Figure 8). In skin, there was no significant elevation of SPI1/PU.1 mRNA with aging (Figure 8A), although histochemical analysis of old skin identified cells staining positive for both SPI1/PU.1 and S100a9 (Figure S15). For other tissues (liver, kidney and lung), RT-PCR

Figure 3 | Top-ranked transcription factor motifs that predict S100a9 coexpression (30 mouse cell types). Top-ranked motifs are listed in the left margin and were selected based upon three criteria. First, we identified those motifs for which an increased number of occurrences significantly increased the probability of S100a9 coexpression in the composite network (i.e., lowest p-values in composite with Z > 0; left margin labels with black font). Second, we identified those motifs for which an increased number of occurrences significantly increased the probability of S100a9 coexpression across the largest total number of cell types (i.e., largest number of up-triangles per row; left margin labels with red font). Third, for the 10 cell types that most consistently expressed S100a9 above background in microarray samples (i.e., neutrophils, ..., monocytes), we identified the single motif most significantly associated with S100a9 coexpression (i.e., lowest p-value for each cell type with Z > 0; left margin labels with blue font). Positive Z statistics (red heatmap colors) indicate that increased motif occurrence within 2 KB upstream regions was associated with increased probability of S100a9 coexpression. Negative Z statistics (green heatmap colors) indicate that decreased motif occurrence 2 KB upstream was associated with increased probability of S100a9 coexpression. For each cell type (columns), the percentage shown in parentheses is the fraction of microarray samples for which S100a9 was expressed above background.
analyses revealed significant elevation of SPI1/PU.1 mRNA with aging in males (Figures 8B–8D), in correspondence with tissue-specific trends observed for S100a9 mRNA (Figure S4).

Further histochemical analysis of lung tissue demonstrated increased SPI1/PU.1 protein in older mice and concordance between SPI1/PU.1 and S100a9 staining patterns, with increased SPI1/PU.1 in nuclei and increased S100a9 in cytoplasmic regions (Figure 8E). We hypothesized that the SPI1/PU.1-S100a9 cells were predominantly leukocyte foci; however, although we could detect macrophages (F4/80), neutrophils and T-cells (CD3) in old lung, stains for these cell types showed only minimal overlap with the S100a9 distribution (Figures S16, S17 and S18). This suggested additional sources for S100a9 in old lung, potentially including senescent epithelial cells or other inflammatory cell types (e.g., B-cells or NK-cells). Our findings, taken together, lend support to a model for the development of chronic inflammation and leukocyte cluster formation during aging, sustained by continued over-production of S100a9, which is driven by the action of SPI1/PU.1 and other TFs in resident and/or infiltrating cell types (Figure 8F).

Discussion
The S100A8–S100A9 heterocomplex (calgranulin) was recently identified as a factor contributing to amyloid plaque accumulation and poor cognitive performance in an Alzheimer’s mouse model. This finding has called attention to the significance of S100A8-S100A9 in cognitive aging, but a broader role for S100A8-S100A9 in intrinsic aging has not been established. In this study, we have shown that shifts in the abundance of S100a8/S100a9 mRNA with aging occurs in multiple tissues (e.g., skin, lung, liver), while in humans, there is prominent elevation of S100A8/S100A9 expression in the central nervous system (Figure 1). These trends are likely to have consequences for development of
chronic inflammation and formation of lymphocyte foci during aging. It was therefore of interest to identify transcription factors that may control S100a9 expression, since targeting such factors could provide a basis for effective therapies to treat persistent inflammation, providing a long-term strategy to encourage healthy aging.

Laboratory investigations of S100a9 transcriptional regulatory mechanisms, however, have been limited to only a few cell types, and large-scale experimental study of such mechanisms across many cell types is not yet feasible. We therefore developed an integrative transcriptomics strategy to systematically identify candidate cis-regulatory mechanisms driving S100a9 expression in 62 mouse and human cell types (Figure 2). Our approach provides an unsupervised and scalable strategy for identifying transcription factors associated with S100a9, and more broadly, with the cell type-specific coexpression networks in which S100a9 is embedded (Figure 2C).

In previous work, STAT3, NF-κB, C/EBPz and C/EBPβ1, HIF-1α, GLI1 and SP1/Pu.1 have been identified as activators of S100a9 expression, while BRCA1, AP-1, SATB1 and Arnt/HIF-1β have been identified as S100a9 repressors. In addition, multiple lines of evidence support the chromatin-associated enzyme PARP-1 as an activator of S100a9 transcription. For each cell type, the 2 KB region upstream of the S100a9 transcription start site was scanned for matches to the TF binding sites within our dictionary of 1209 motifs. If a match was identified, the matching region was assigned a cell-type-specific score (proportional to the Z statistic calculated for that motif and cell type), which quantified the degree to which increased motif occurrence increased the probability of S100a9 coexpression. If more than one motif matched at a given position, the highest score was assigned. A sliding window analysis was then used to identify regions with greatest concentration of high-scoring base pairs (dark grey = best 400 BP window; yellow = best 200 BP window; orange = best 100 BP window; red = best 50 BP window). The right margin (blue symbols) lists the individual motif, with at least one match 2 KB upstream, for which increased motif occurrence was most significantly associated with S100a9 coexpression (i.e., lowest p-value with Z > 0).
ample support in our analyses, while both factors have been implicated in S100a9 regulation by prior experimental work as well35,38. SPI1/PU.1 is an ETS family transcription factor that, like other ETS factors, exhibits affinity for sequences featuring a GGA(A/T) core motif43. In our study, motifs containing this element were frequently overrepresented among S100a9 coexpressed genes, indicative of an association between ETS family transcription factors and S100a9 coexpression, and overall, the strongest trends were associated with motifs recognized by SPI1/PU.1 (see Figure S20 summary). The region upstream of the mouse and human S100a9 gene contains multiple SPI1/PU.1 motifs, although it is unknown whether SPI1/PU.1 directly interacts with these sites; however, one study reported 10-fold increased expression of S100a9 following retroviral replacement of SPI1/PU.1 in mouse myeloid cells35. Basal expression of SPI1/PU.1 is highest in lymphoid cells, but its expression can also be induced by cytokines such as IFNγ45. Within aging tissues, the role of SPI1/PU.1 in driving increased S100a9 expression would most likely occur in the context of local inflammatory reactions, such as those involving microglia in the central nervous system46, or perivascular formation of leukocyte clusters consisting of infiltrating monocytes, B-cells, T-cells, dendritic cells and macrophages47. Formation of such lymphoid structures in vascular tissues provides a platform for subsequent expansion of inflammatory cell populations, which is likely to at least partly account for elevation of S100a8 and S100a9 with aging (Figures 1 and S4). Within these contexts, production of S100a9 by resident and/or infiltrating myeloid cells, enforced by SPI1/PU.1, may serve as a feed-forward process that both activates and is responsive to inflammatory signals, ultimately providing a mechanism by which inflammatory reactions become self-sustaining and chronic (Figure 8F). Nevertheless, we expect that the contribution of this mechanism to inflammatory processes will vary among tissues, or potentially, may differ between the sexes4. For instance, in kidney and lung, our data show that expression of the gene encoding SPI1/PU.1 is more strongly increased in males as compared to females (Figures 8C and 8D).

SATB1 (special AT-rich sequence binding protein 1) is a chromatin organizing factor that controls gene expression by packaging chromatin into dense loops and by facilitating the aggregation of chromatin remodeling enzymes in specific domains48. Absence of SATB1 binding sites (AATTTT) was a strong predictor of S100a9 coexpression for nearly every cell type we considered (see Figure S21 summary). This was surprising, since initially, we expected that S100a9 coexpression would be closely associated with increased abundance of certain motifs, rather than their absence. However, absence of SATB1 binding sites in regions surrounding S100a9 coexpressed genes may allow for an expanded chromatin structure that

Figure 6 | Top-ranked transcription factor motifs that predict S100A9 coexpression (32 human cell types). Motifs listed in the left margin were selected based upon the three criteria described in the Figure 3 legend. Positive Z statistics (red heatmap colors) indicate that increased motif occurrence within 2 KB upstream regions was associated with increased probability of S100a9 coexpression. Negative Z statistics (green heatmap colors) indicate that decreased motif occurrence 2 KB upstream was associated with increased probability of S100a9 coexpression. For each cell type (columns), the percentage shown in parentheses is the fraction of microarray samples for which S100a9 was expressed above background.

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increases accessibility of proximal cis regulatory elements\(^3\). In mouse keratinocytes, SATB1 binding occurs near the epidermal differentiation complex containing the \(S100a9\) gene, leading to a compressed chromatin conformation\(^3\). This action is proposed to repress gene expression, since SATB1-null mice exhibit greater than 5-fold elevation of \(S100a8\) and \(S100a9\)\(^3\). In our study, SATB1 motifs were under-represented in regions proximal to \(S100a9\) coexpressed genes. One possible explanation for this result is that an expanded chromatin structure, facilitated by a deficiency of SATB1 binding sites, is instrumental for cis regulation of \(S100a9\) and other co-regulated genes. Such indirect regulation of \(S100a9\) would represent an elegant instance in which a pro-inflammatory transcript is controlled by the balance of two sequence-mediated processes – the higher order control of chromatin packaging by SATB1 – and secondly, by localized interactions between cis regulatory elements and their activating transcription factors (e.g., SPI1/PU.1).

Transcriptional regulation can depend upon the cooperative interactions among DNA-binding proteins recruited to a given locus\(^4\), and such interactions are especially common among the ETS family transcription factors\(^5\). Since an abundance of motifs was significantly associated with \(S100a9\) coexpression in most cell types, we considered whether such motifs were co-localized in compact sequence regions upstream of the mouse or human \(S100a9\) gene. In mouse, the region 150–250 BP upstream of \(S100a9\) contained a high density of motifs significantly enriched among \(S100a9\) coexpressed genes (Figure 5). Within this region, we have highlighted a 13 BP element with overlapping binding sites for SPI1/PU.1 and STAT3 (Figure S10). The forward strand (5-ATGGAAGGGAAGT-3) features multiple binding sites for SPI1/PU.1 (i.e., AGGAAGT and GGAAG), while the reverse strand (5-ACTTCCCTTCCAT-3) features multiple STAT3 binding sites (i.e., TTCC) (Figure S10). It is interesting to speculate that cooperative interactions between STAT3 and SPI1/PU.1 may occur at this locus, particularly since these factors belong to the same transcriptional regulatory network, in which transcription of the gene encoding SPI1/PU.1 is induced by STAT3\(^4,5\). With regard to the human \(S100A9\) gene, regulatory elements have previously been identified less than 600 BP upstream from the transcription start site\(^3,2\). Surprisingly, however, we identified a region 930–1030 BP upstream as most strongly enriched with matches to those motifs overrepresented among \(S100A9\) coexpressed genes (Figure S13). A key feature of this region was a 19 BP GC-rich element including motifs recognized by OVOL2/MOVOB, WT1 and SP1 (Figure S14). This element was also located 12 BP from a SPI1/PU.1 binding site, raising the possibility of interactions between SPI1/PU.1 and a partner factor with affinity for GC-rich motifs\(^5\). The importance of the \(S100a8-S100a9\) heterocomplex as a mediator of acute inflammatory reactions is well established\(^6-12\), but the role of \(S100a8–S100a9\) in low grade chronic inflammation during...
aging has not been investigated. Many genes have been reported to increase or decrease expression with aging. However, when data are compared among multiple laboratories, few genes exhibit robust trends similar to those we have described for S100a8 and S100a9. This paper, therefore, supports a role for S100a8–S100a9 in the process of mammalian aging, and emphasizes the need for further studies of S100a8–S100a9 as a mediator of age-associated inflammation. Within this context, we have developed a strategy for the large scale and cell type-specific identification of cis regulatory mechanisms. At present, many transcription factor target gene annotations are generic, with a certain factor X recognized as regulating a target gene Y, but without any reference to the cell type in which this interaction takes place. Such TF-target relationships, however, may differ among the heterogeneous collections of cell types that interact in most mammalian disease processes. This approach developed here illustrates one strategy for generating cell type-specific hypotheses of mammalian gene regulation. We anticipate that this strategy will become more powerful in future work, as we build a more comprehensive knowledge of binding affinities for mammalian transcription factors, leading to increasingly refined motif dictionaries that can be used to interrogate coexpression networks. This can facilitate the construction of multicellular gene regulatory networks that, by linking inter-cellular interactions with the intracellular mechanisms governing transcription, may better capture the dynamic processes that drive aging and disease in mammals.

Methods

Ethics statement. This study was conducted in compliance with good clinical practice and according to the Declaration of Helsinki principles. Informed written consent was obtained from all human subjects, under protocols approved by the University of Michigan institutional review board (HUM00037994). All animal protocols were approved by the University of Michigan committee on the use and care of animals (018 ARF 5614).

Meta-analysis of age-associated gene expression patterns. The effects of aging on gene expression in mice were evaluated using the Affymetrix Mouse Genome 430 2.0 array, while the effects of aging in humans were evaluated using the Affymetrix Human Genome U133 Plus 2.0 array. Expression analyses of C57BL/6J liver, C57BL/6 aorta, and human sun-protected skin biopsies were carried out as described in an earlier publication, and raw data are available from Gene Expression Omnibus (GSE35322 and GSE13355).

Figure 8 | Aging increases mRNA and protein levels of SPI1/PU.1 with an overlapping distribution of SPI1/PU.1 and S100a9 in older tissues (CB6F1 mice). RT-PCR was used to evaluate the expression of SPI1/PU.1 mRNA in (A) tail skin, (B) liver (C) kidney and (D) lung of CB6F1 mice scarified at 5 or 30 months of age. Expression was normalized to 18S ribosomal RNA (Rn18s). An asterisk symbol denotes a significant difference between young and old mice of the same sex (P < 0.05; two-tailed t-test). In part (E), immunostaining for SPI1/PU.1 showed increased abundance of SPI1/PU.1 in older tissues (top panels), with increased nuclear SPI1/PU.1 and elevation of S100a9 in cytoplasmic regions (bottom panels: red = SPI1/PU.1; green = S100a9; blue = DAPI). (F) Proposed model by which over-production of S100a9 engenders a pro-inflammatory microenvironment, with sustained activation of the RAGE and NF-κB, leading to recruitment and migration of leukocytes into local tissues. This in turn leads to further infiltration by inflammatory cell types that actively transcribe S100a9, driving a self-reinforcing cycle that sustains inflammation and lymphoid aggregation with aging.
Motif dictionary. We have described an in silico procedure for screening sets of S100a9 coexpressed genes in order to identify motifs over- or under-represented among genomic sequences associated with such genes (e.g., 2 KB upstream regions; see Figure 2). An important first step for implementing this procedure was to generate a high-quality dictionary of motifs for mouse and human TFs and other DNA-binding proteins or complexes. For this purpose, an initial set of 2105 probability matrices representing binding preferences of known DNA-binding proteins or complexes was obtained from the Jaspar4, UnipROBE5 and TRANSFAC6 databases, including 145 matrices from the Jaspar CORE vertebrate collection, 295 matrices from UnipROBE, and 1665 matrices from TRANSFAC. Of the 1665 TRANSFAC matrices, we excluded 846 that were not associated with vertebrate species or were already included among those obtained from UnipROBE. This yielded a total of 1259 matrices from all three databases. Each matrix was trimmed to remove columns with low information content at the flanks. Starting at each flank, columns were removed until two consecutive columns with information content greater than 0.25 was encountered. For 9 matrices, this trimming procedure engendered a low information content matrix with fewer than five columns, and such matrices were excluded from further analysis. For the remaining 1250 matrices, a redundancy search was performed to identify very similar matrix models. We identified a total of 41 matrices that shared the same non-redundant consensus sequence with another matrix, where the difference in base-specific probability estimates differed by less than 0.20 on average. These 41 matrices were removed from consideration, leaving a final set of 1209 matrix models with limited redundancy. These 1209 matrix models were included in our screening procedures. Motif labels shown in our results (e.g., Figure 2A) are associated with the consensus sequences for each matrix, rather than the specific binding protein or protein complex. An identifier corresponding to the matrix ID used in the source database (JASPAR, UnipROBE or TRANSFAC), and the representative consensus sequence. JASPAR IDs start with “MA”, TRANSFAC IDs start with “V$”, and UniPROBE IDs (JASPAR, UniPROBE or TRANSFAC), and the representative consensus sequence. For each cell type and motif, a 0–1 additive logistic models (GAM) were used to test for a significant association between motif frequency and coexpression probability while controlling for variation in the coexpression and counts reflecting the number of motifs associated with S100a9 coexpression pattern. This increased the size of the foreground probe sets based upon the approach described in Figure 2A. The foreground set of S100a9/49 coexpressed genes was defined as the non-redundant set of genes associated with those probe sets coexpressed with S100a9/49 for a given cell type. Since our ranking procedure included only coexpression, foreground gene sets were ranked based upon the level of positive correlation with the S100a9/49 expression pattern (r > 0), excluding genes with a negative or inverse correlation with the S100a9/49 expression pattern (r < 0).

Statistical analyses required that we define a background gene set for each cell type, where this set contained genes for which there was little evidence of either positive or negative correlation with the S100a9/49 expression pattern. To identify this background set of genes, we defined two gene sets (A and B), where A contained genes positively correlated with the S100a9/49 expression pattern and B contained genes negatively correlated with the S100a9/49 expression pattern. The background gene set B was defined as all genes that were not positively or negatively correlated with S100a9/49 and were included as set A described in detail above. The procedure was repeated for all probe sets and genes for which there was little evidence of either positive or negative correlation with the S100a9/49 expression pattern. This procedure also ensured that foreground and background gene sets would be distinct for each cell type.

Composite sets of S100a9/49 coexpressed genes were identified following the same ranking procedure shown in Figure 2A. However, probe sets were ranked according to their (weighted) average correlation with S100a9/49 across all 30 mouse cell types or all 32 human cell types. For any one probe set, the Pearson correlation between foreground probe sets and scores for the probe set associated with S100a9/49 (1448756_at; human probe set id: 203535_at) was independently calculated for all cell types. A weighted average of correlation estimates was then calculated, with greater weight assigned to correlations estimated from cell types for which more data was available (e.g., macrophages, n = 393 samples for mice), with less weight assigned to correlations estimated based upon cell types for which less data was available (e.g., skin fibroblasts, n = 9 samples for mice). The procedure was repeated for all probe sets, which were then ranked according to the weighted average correlation (similar to Figure 2A). Because average correlation estimates calculated from many cell types tended to generate a steep curve (Figure 2A), one further modification was that probe sets were ranked according to r 1 rather than r 1, 2. This increased the size of the foreground probe sets and gene sets obtained for both composite and cell type-specific foreground gene sets.

Semi-parametric generalized additive logistic models. Semi-parametric generalized additive logistic models (GAM) were used to test for a significant association between S100a9/49 coexpression and counts reflecting the number of motifs associated with genes in foreground and background gene sets. For each cell type and motif, a 0–1 response variable was generated to denote whether a gene belonged to the foreground gene set (coded 1) or background gene set (coded 0). Two predictor variables were included within each model, including log-transformed length of sequence scanned for each motif (s 1) and the number of motif sites detected within the sequence (s 2) (Figure 2D). Length of sequence scanned varied among genes due to masking or inherent differences in the amount of intronic sequence or adjacent intronic sequence. Including s 1 within models was thus necessary to estimate the effect of motif frequency on coexpression probability while controlling for variation in the length of sequence scanned for each gene44. For each GAM model, s 1 was included as a non-parametric term with cubic spline smoothing, while s 2 was included as a semi-parametric term. Model fitting was performed using the backfitting procedure with the binomial family error distribution and logit link function. Given these procedures, we assumed that the logit of the probability of coexpression is linearly associated with s 2; however, we make no such assumptions involving s 1. While we did initially specify s 1 as n = 393, and therefore, we evaluated the effect of this choice using the n = 9 samples for mice. In our preliminary work, we noted that the largest residuals for fitted models usually occurred for genes with extremely short or long sequences. To improve overall fit, therefore, models were fit after excluding sequences of extreme length (1–5% excluded depending upon the genome region scanned). The association between motif frequency and S100a9/49 coexpression probability was evaluated based upon the coefficient estimate associated with s 2 for each cell type. For each cell type, a Z statistic was generated, with Z > 0 indicating that increased motif frequency was associated with increased probability of S100a9/49 coexpression, and Z < 0 indicating that decreased
motif frequency was associated with decreased S100a9/c-Jun coexpression. Given the large number of genes included within the procedure, Z statistics were assumed to follow a normal distribution under the null hypothesis of no association between motif frequency and S100a9/c-Jun coexpression1. P-values were thus calculated for each motif using Z statistics and the standard normal distribution. For each cell type, separate models were fit for all 1209 motifs included within our dictionary, leading us to expect that ~60 significant motifs would be identified by chance for a given cell type (z < 0.05). In our results, therefore, we have also reported significance calls based upon a more stringent FDR threshold, which was calculated directly from raw p-values by applying the Benjamini-Hochberg correction2.

We have highlighted motifs most significantly enriched among S100a9 coexpressed genes in the composite network, motifs most frequently enriched among S100a9 coexpressed genes in the ten cell types with highest S100a9 expression (Figures 3, S6, S9, and S12). In some cases, top-ranked motifs shared similar features; for instance, in Figure 6, several motifs have high GC content. We therefore performed secondary analyses to determine which motif features best distinguish enriched motifs (P < 0.05 with Z > 0) from other non-significant motifs (Figures S9D and S12D). For these analyses, we assigned a score to each motif that was proportional to how well it matched k-mers of length 2, 3, 4, or 5. This was done for each motif and each of the 690 possible non-redundant k-mers of length 2, 3, 4, or 5. Given the number of pixels assigned to background (0) or non-background (1) (Figures 8E, S2, S5, S6, S7, S8, S15, S16, S17 and S18). We further investigated the distribution (i.e., function “normalmixEM” from R package “mixtools”). After an appropriate threshold was determined for an image, the average red or green intensity within each of these regions was compared against the appropriate background region.

**Image quantification**

Diaminobenzidine and fluorescent staining intensities were quantified to facilitate interpretation and objective comparison among tissues and across image sets. Image quantification is the blue intensity on the RGB scale. The distribution of Xmax is the green channel color intensity, and the blue intensity on the RGB scale. The distribution of Xmax is the green channel color intensity.

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Author contributions

W.R.S., A.J. and J.E.G. conceived the framework, analyzed the data, and wrote the manuscript. X.X., A.L. and P.R. processed tissue samples and performed wet lab procedures. J.J.V. and G.F. participated in drafting the manuscript and provided critical input. All authors read, edited, and approved the final manuscript.

Additional information

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