Deep Learning Identifies an Inflammatory Clock which Predicts Multimorbidity, Immunosenescence, Frailty and Cardiovascular Aging in Humans

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ABSTRACT

While many diseases of aging have been linked to the immunological system, immune metrics with which to identify the most at-risk individuals are lacking. We studied the blood immunome of 1001 individuals aged 8-96 and developed a deep learning method based on patterns of systemic age-related inflammation. The resulting inflammatory clock of aging (iAge) tracked with multiple morbidities, immunosenescence, frailty and cardiovascular aging. We demonstrate that iAge is associated with exceptional longevity in a separate cohort of centenarians. The strongest contributor to this metric was the chemokine CXCL9, which was involved in cardiac aging, adverse cardiac remodeling, and decreased vascular function. Furthermore, aging endothelial cells in human and mice show loss of function, indicators of early cellular senescence and hallmark phenotypes of arterial stiffness, all of which are reversed by silencing CXCL9. In conclusion, we identify a key role of CXCL9 in age-related systemic chronic inflammation and derive a novel metric for age-related multimorbidity that can also be used for early detection of age-related clinical phenotypes.

INTRODUCTION

The important role of the immune system in the maintenance of human health and protection against infections has been recognized for over a hundred years. However, it was only in the past few decades that it has become apparent that inflammatory components of the immune system are often chronically elevated in aged individuals and associated with an increased incidence of cancer, cardiovascular disease, neurodegenerative disorders, and others[1-3]. From these observations, it has been postulated that inflammation plays a critical role in regulating physiological aging[4, 5]. Furthermore, the well-established nine hallmarks of aging[6]; [1]

genomic instability, [2] shortening telomere length, [3] epigenetic modifications, [4] loss of proteostasis, [5] deregulated nutrient sensing, [6] mitochondrial dysfunction, [7] cellular senescence, [8] stem cell exhaustion, and [9] altered intracellular communication, have all been shown to be linked to sustained systemic inflammation[7-16].

Contrary to the acute response, which is typically triggered by infection, chronic and systemic inflammation is thought to be triggered by physical, chemical or metabolic stimuli ("sterile" agents) such as those released by damaged cells and environmental insults, generally termed "damage-associated molecular patterns" (DAMPs). This type of inflammation is associated with aging and characterized by being low-grade and persistent, ultimately leading to collateral damage to tissues and organs[1, 17]. Despite the importance of this type of inflammatory reaction, there are currently no standard biomarkers to characterize a chronic inflammatory state, and studies have generally yielded conflicting results[18, 19].

Recent work from our group identified a novel cellular composite metric for immune aging (IMM-AGE) which was strongly associated with all-cause mortality[16]. Here, we have extended our studies to focus on soluble immune biomarkers and define the relation between systemic chronic inflammation and disease. We set out to establish a broad survey of immunity in over 1000 individuals (the Stanford 1000 Immunomes Project or Stanford 1KIP), wherein biological samples from 1001 subjects were obtained in the years 2007-2016 and comprehensively measured in a single facility, the Stanford Human Immune Monitoring Center (HIMC). At this center, peripheral blood specimens were processed and analyzed using multiple technological platforms for gene expression, serum cytokine levels, cell subset composition, cellular responses to multiple stimuli, and the seropositivity to cytomegalovirus infection. For

902 subjects, a comprehensive health assessment using a 53-feature clinical questionnaire was also obtained.

Given the well-established importance of chronic inflammation for many human diseases and the lack of standard measures[20], we used deep learning methods on blood immune biomarkers to construct a metric for age-related chronic inflammation (iAge). iAge is able to predict important aging phenotypes and provides novel insights into the mechanisms leading to vascular aging. In addition, this metric was associated with exceptional longevity in an independent cohort of centenarians and with all-cause mortality in the Framingham Heart Study. Last, we demonstrate that the most robust contributor to iAge, the interferon-related chemokine CXCL9, was associated with an upregulation of multiple inflammatory pathway genes, downregulation of proliferation pathways, and endothelial cellular senescence. Moreover, CXCL9 suppressed vascular function in aortic tissue from mice and correlated with subclinical cardiac remodeling and arterial stiffness in a validation study of healthy older adults.

Therefore, we have identified a novel soluble protein score for systemic chronic age-related sterile inflammation which tracks with multiple disease phenotypes in multiple cohorts and thus, could be used as a metric to healthy versus unhealthy aging. Our results also demonstrate a new link between inflammatory molecules of the immune system and vascular biology.

RESULTS

Study design of the Stanford 1000 Immunomes Project

During the years 2007 to 2016, blood samples were drawn from ambulatory subjects (N = 1001) (339 males and 662 females) from age 8 to 96 (**Supplementary Figure 1** and

Supplementary Figure 2) who had been recruited at Stanford University (the Stanford 1000 Immunomes Project, or Stanford 1KIP) for a longitudinal study of aging and vaccination[5, 21-29], and for an independent study of chronic fatigue syndrome[30]. Only healthy controls were included (see Methods). For all samples of the Stanford 1KIP, deep immune phenotyping was conducted at the Stanford Human Immune Monitoring Center (HIMC), where peripheral blood specimens were processed and analyzed using rigorously standardized procedures[31]. Serum samples were obtained and used for protein content determination (including a total of 50 cytokines, chemokines and growth factors) (N = 1001) and to assess cytomegalovirus status (N = 748), a major determinant of immune system variation[22, 25]. Peripheral blood mononuclear cells (PBMCs) or whole blood samples were used for the determination of gene expression (N = 397), cellular phenotypes and frequencies (N = 935), and for investigation of *in vitro* cellular responses to a variety of cytokine stimulations (N = 818). Extended clinical report forms were collected from 902/1001 subjects, of which 299 were males and 603 were females (**Supplementary Figure 1**). A total of 37 additional older adults (19 centenarians and 18 controls) from Bologna, Italy, were included and screened for serum proteins to derive iAge on these extremely long-lived humans.

Deep Learning Analysis of Circulating Immune Biomarkers to Create an Inflammatory Clock of Aging

Given the increasingly recognized effect of systemic chronic inflammation in the development of a wide variety of diseases associated with aging, especially in cardiovascular disease[5, 32], we set out to construct a metric for age-related chronic inflammation that could summarize an individual's inflammatory burden. This type of inflammation is thought to be a

maladaptive response to tissue damage, metabolic dysfunction, and environmental insults[1, 17]. In contrast to the acute inflammatory response, for which a number of secreted molecules (such as C-reactive protein, IL-1β, IL-6, and TNF-α) have been validated, for age-related chronic inflammation there is no standard cytokine signature[18-20]. Thus, we undertook an unbiased approach to compactly represent the non-linear structure of the cytokine network. To do so, we developed a deep learning method called guided auto-encoder (GAE). The final model used consists of a 2 layer fully connected neural network with 5 nodes in each layer. The GAE (see Methods) is a type of deep neural network which utilizes non-linear equations and effectively eliminates the noise and redundancy in the data, yet retaining the most relevant biological information from the circulating immune protein data.

To test the robustness and quality of the GAE method, we compared the accuracy of the GAE against other widely used dimensionality reduction methods that use linear equations, such as the Elastic Net, Gradient Boosting Decision Tree (GBDT), and principal component analysis (PCA), as well as those involving non-linear equations, such as plain auto-encoders and neural networks (**Supplementary Figure 3A-C**). We employed 5-fold cross validation and measured the predictive performances of each method on the test set. Overall, the GAE method outperformed other methods in predicting chronological age ($P < 0.05$) with the exception of the classic neural network ($P = 0.54$) (**Supplementary Figure 3B**). The average reconstruction errors on the test set for prediction of age and circulating immune protein data were 15.2 years and 0.26 (normalized), respectively. These results indicate that the phenomenon of low-grade chronic inflammation in aging humans is best modeled using non-linear methods, which take into account the network structure and redundancy in immunological protein biomarkers. This novel metric for chronic inflammation accurately predicts chronological age in the population

(**Figure 1A**) using the total inflammatory burden as measured by the level of circulating immune proteins (**Supplementary Figure 4**).

The Inflammatory Clock of Aging (iAge) Predicts Multimorbidity and Frailty

To gain further insights into the effect of age-related chronic inflammation on age-related pathology, we computed a regression analysis between the total number of age-related diseases (multimorbidity) and iAge. Multimorbidity has become the gold standard in aging research since it represents the accumulation of physiological damage in an individual[33]. The items analyzed included different diseases of different physiological systems: cancer, cardiovascular, respiratory, gastrointestinal, urologic, neurologic, endocrine-metabolic, musculoskeletal, genital-reproductive and psychiatric dysfunctions. All these disease features were binary. For these analyses, we controlled for age, BMI, sex, CMV (cytomegalovirus) and high cholesterol (also binary category), because of the reported effect of each of these variables in the etiology of age-related pathologies. We observed a significant correlation between iAge and multimorbidity in the older adults in this study (>60 years old) ($N = 285$, $P < 0.001$) (**Figure 1B**). This highlights the key role of iAge in the accumulation of physiological damage during aging.

Next, we envisioned an unbiased approach to select predictors of multimorbidity based on the available data for all 902 Stanford subjects while controlling for the age effect. To do so, we used a shrinkage method for variable selection by cross-validation, called the Elastic Net, which has been increasingly used in immunology, aging, and other medical fields over the past years[34]. We applied differential penalties for each potential predictor such that the machine learning procedure would 'force' age to be selected while imposing a stringent penalty to all other features so that the variables selected do not correlate with age (**Supplementary Figure**

5A). The Mean Absolute Error (MAE) for the prediction of multimorbidity was 0.41 (**Supplementary Figure 5B**). The top features with the largest coefficients include iAge, high cholesterol and BMI (**Supplementary Figure 5C**). In addition, immune parameters such as total CD8 (+) T cells, plasmablasts, transitional B cells such as IgD+CD27- and IgD-CD27- B cells (negative predictors), effector CD8 (+) T cells, total lymphocytes, monocytes, and central memory T cells (positive predictors) were predictive of multimorbidity (**Supplementary Figure 5D**). Collectively, these results show that the inflammatory clock is a metric for overall health linked to multiple diseases associated with aging.

To longitudinally assess the importance of iAge in age-related functional deterioration, we calculated iAge in a subgroup of 29 older adults in year 2010 and a frailty score including time-up-and-go[35] was measured in 2017 for the same subjects. Using a linear regression model where frailty score in 2017 was regressed onto iAge calculated in 2010 and controlled for chronological age, gender, BMI and CMV status, we found that iAge from 2010 was predictive of frailty score in 2017 ($R^2 = 0.81$, $P < 0.001$). (**Figure 1C**). Strikingly, the contribution of iAge to the frailty score was significantly stronger than that of calendar age (**Figure 1D**).

Lower Inflammatory Clock index in Centenarians

Next, we explored the relationship between inflammatory age and exceptional longevity. We computed an inflammatory index in an additional cohort of 37 subjects, 18 of which were 50-79 years old and 19 centenarians, except for one individual who was 99 years old at the time of blood extraction. To do so, we first ranked both cohorts in terms of their chronological age (cAge) and their iAge. For each subject, we then computed the difference of their rank [cAge] and rank [iAge] and used this difference (iAge index) to stratify subjects into high and low, if they were above or below the population rank mean, respectively. Last, we calculated

enrichment for exceptional longevity in the low iAge index group (subjects with most protective phenotypes) by hypergeometric test. 68% (13/19) centenarians were in the low rank group (P = 0.028) whereas only 31% (6/19) were in the high rank group. In contrary, there were 77% (14/18) of controls in the high rank versus 23% in the low rank group (**Figure 1E**), which indicates that regardless of their chronological age, centenarians have a protective iAge index phenotype. This indicates that iAge is associated with exceptional longevity.

To further validate the clinical implication of the iAge score, we leveraged the data from the Framingham Heart Study[36], a longitudinal cohort tracking thousands of individuals for decades. Since there were no sufficient proteomics data to directly estimate iAge in the cohort, we derived a gene expression signature of iAge using available data from 397 subjects in our study and performed an enrichment analysis of the derived gene signature on each sample in the Framingham Heart Study (see Methods). We observed that the iAge gene-signature was significantly associated with all-cause mortality following adjustment to multiple covariates associated with mortality, including age, gender, smoking, cholesterol levels, blood pressure, diabetes, and existence of a cardiovascular disease (P = 0.02, cox proportional hazard model, N = 2,290 individuals).

The Inflammatory Clock of Aging (iAge) is Correlated with Immunosenescence

Canonical acute inflammation proteins such as C-reactive protein and Interleukin-6 have been associated with immunosenescence in previous studies[37, 38], but the relationship with systemic chronic inflammation (SCI) has not yet been established. To investigate this link, we first used the frequency of naïve CD8 (+) T cells, a well-known marker for immunosenescence, and estimated the contribution of iAge after controlling for Age, CMV, and sex by a multiple

regression model. Not surprisingly, age was the strongest contributor to changes in naïve CD8 (+) T cells followed by iAge, CMV (negative contributors) and sex (frequency of total CD8 (+) T cells in females was 24% vs. 30% in males) (**Figure 2A**).

To examine the effect of chronic inflammation in the immune response, we used a multiplex assay of phosphorylated STAT molecules in PBMCs following different stimulations *in vitro*[39] to evaluate activated components of the JAK-STAT signaling pathway, which is the foremost signaling mechanism for a wide array of cytokines and growth factors[40]. PBMCs were stimulated *ex vivo* with the cytokines IFN-α, IL-6, IL-10, and IL-2 and subsequently stained with antibodies specific for the phosphorylated forms of STAT proteins. The fold-increase of phospho-STAT1, phospho-STAT3 and phospho-STAT5 were measured in the B cells, total CD4 (+) T cells (including CD45RA(+) and CD45RA(-) subsets), total CD8 (+) T cells (including CD45RA(+) and CD45RA(-) subsets), and monocytes of 818 individuals, totaling 96 conditions. We conducted multiple regression analysis controlling age, CMV and sex (see methods). Strikingly, there was a general decrease of the B cell and T cell responses to stimuli and an overall potentiation of monocyte response associated with increasing iAge (combined $P < 10^{-5}$) (**Figure 2B**). These results demonstrate that iAge correlates with an established biomarker of immune senescence (naïve $CD8^+$ T cell frequency) and with PBMC signaling characteristics *in vitro.*

CXCL9 is an Important Component of the Inflammatory Clock

In order to isolate the factors contributing the most to iAge, we computed the most variable jacobians (the first-order partial derivative of iAge). We found both positive and negative contributors to iAge (**Figure 3A**), where the top 15 most variable jacobians were

CXCL9, EOTAXIN, Mip-1α, LEPTIN, IL-1β, IL-5, IFN-α and IL-4 (positive contributors), and TRAIL, IFN-γ, CXCL1, IL-2, TGF-α, PAI-1 and LIF (negative contributors). Interestingly, canonical markers of acute infection such as IL-6 and TNF-α were not major contributors to iAge, indicating that, except for IL-1β, infection-driven inflammatory markers of the acute inflammatory response do not contribute to age-related chronic inflammation. Given that the most positive contributor to iAge was CXCL9, we set out to explore the importance of CXCL9 and its role in aging. First, we compared CXCL9 levels between different age groups with the one-way ANOVA test, which shows a statistically significant difference ($P < 10^{-15}$) (**Figure 3B**). To better define the age at which CXCL9 commence to rise in the population, we applied the pairwise Tuckey Honest Significant Differences Test and found that only after the age of 60 do we observe a pronounced significant change in the levels of this protein **(Figure 3B)**. Finally, in a univariate regression analysis, we found that age positively correlates with CXCL9 ($R2 = 0.1$, $P < 10^{-16}$) (**Supplementary Figure 6**). Taken together, these results suggest that CXCL9 is an important factor in age-related chronic inflammation.

CXCL9 Correlates with Cardiovascular Aging in Otherwise Healthy Adults

In a validation cohort, we strived to verify the results that CXCL9 is an important contributor to aging phenotypes. More importantly, previous research has implicated CXCL9 in cardiovascular aging[41-44], so we explored its role in cardiovascular aging using the cohort. We conducted a follow-up study in a group of 97 extremely healthy adults (age 25-90 years old) matched for cardiovascular risk factors (including conserved levels of high-sensitivity C-reactive protein) (**Supplementary Table 1**) who were selected from a total of 151 recruited subjects using strict selection criteria (see Methods). In this healthy cohort, inflammation markers were

measured using a 48-plex cytokine panel. Only 6 out of 48 circulating immune proteins were significantly correlated with age ($P < 0.05$) and among these, CXCL9 was again the largest contributor to age-related inflammation (**Supplementary Figure 7**), supporting the findings observed in the 1KIP cohort. In addition, IL-11Rα, CXCL10, and HGF increased with age, while CXCL1 and LIF decreased (**Supplementary Figure 7).** All these changes were in the same direction to those observed in the 1KIP cohort.

Individuals in the validation cohort were subjected to cardiovascular assessment, including pulse wave velocity (PWV) testing, a measure of vascular stiffness, and relative wall thickness (RWT), a surrogate measure of cardiac remodeling (see Methods). We then performed multiple regression hierarchical analysis using the six selected inflammatory markers associated with aging in this cohort and the cardiovascular measurements (PWV and RWT) controlling for age, sex, BMI, heart rate, systolic blood pressure, fasting glucose, and total cholesterol to HDL ratio. At a $P < 0.01$, we found a modest positive correlation ($R = 0.22$) between CXCL9 and the cardiovascular aging PWV and RWT ($R = 0.3$) (**Figure 3C-F**). We also found a negative correlation between LIF and PWV ($R = -0.27$), and RWT ($R = -0.22$).

PWV as a measure of arterial stiffness and organ damage is a better predictor of future cardiovascular events and all-cause mortality than conventional measures of CVD risk factors such as age, sex, blood pressure, lipids, BMI, and smoking status[45, 46]. Recent studies show that as PWV increases, the microvasculature of target organs such as brain and kidney are damaged[47]. Here we show that the biggest contributor to iAge, the chemokine CXCL9 positively correlates with PWV. Therefore, taken together, these results show that subclinical cardiac tissue remodeling and increased arterial stiffness can be found in otherwise healthy individuals with elevated levels of CXCL9 levels and low levels of LIF.

CXCL9 Increases with Age in Human Blood Endothelial Progenitor Cells

Long-standing evidence has suggested a role for the endothelium in the etiology of hypertension and arterial stiffness[48, 49]. More recent work has also shown that advanced signs of cardiovascular aging such as tissue remodeling, and cardiac hypertrophy are often preceded and may be initiated by the malfunctioning of aged endothelia[50-52]. We explored potential contribution of CXCL9 towards cardiovascular aging through endothelial cells. First, we assessed the levels of CXCL9 in young and old individuals by isolating their blood endothelial progenitor cells (BECs) (**Supplementary Figure *8*A**). Quantitative PCR analysis of the BECs from young and old individuals showed a significant increase in CXCL9 levels in old patients when compared to young patients (**Figure 4B**). Importantly, a comprehensive characterization of these BECs from both these cohorts showed impairment of endothelial function in old individuals when compared to young. To measure endothelial function, we examined the ECs ability to form networks of tubular structures[53, 54], produce nitric oxide (NO)[55], and incorporate acetylated low-density lipoprotein (Ac-LDL)[56]; together, the assays robustly assess the health of the endothelial cells. Comparing ECs from old and young individuals, we found that BECs from old patients showed reduced capacity to form networks of tubular structures (**Supplementary Figure 8B** and **Figure 4B**), reduced capacity to produce NO (**Figure 4C**), and a reduced capacity to incorporate Ac-LDL (**Figure 4D**).

Similar experiment was conducted in mice. Aortas from young (3-4 month) and old mice (2 yr.) were excised, digested and cultured in EC medium (**Supplementary Figure 8C**). Once confluent, ECs from both young and old mice were assessed for CXCL9 expression and function. As expected, ECs isolated from old mice showed higher levels of CXCL9

(**Supplementary Figure 8D**), while at the same time showed impaired EC function as evident by decreased tube formation (**Supplementary Figure 8E-F**). These results demonstrate a concomitant increase in CXCL9 in the endothelia and EC dysfunction associated with aging both in humans and mice.

Inhibition of CXCL9 Rescues Endothelial Cell Dysfunction

Next, we investigated how the increased in CXCL9 in older ECs is related to endothelial dysfunction. In these experiment, we used a well-established model for endothelial aging[57, 58] by generating human induced pluripotent stem cells (hiPSCs) from fibroblasts obtained from 5 independent donors[59], and subsequently differentiated them into endothelial cells (hiPSC-ECs)[60]. The CXCL9 receptor, $G\alpha_i$ protein-coupled protein CXCR3, was expressed in endothelial cells but not in cardiomyocytes (**Supplementary Figure 10**). We used lentiviral infection of CXCL9 sequence-specific short hairpin RNA to knockdown expression of CXCL9 in hiPSCs (CXCL9-KD). As a control, we also infected hiPSCs with nonsense-sequence shRNA (Scramble), and subsequently, both cultures were differentiated to ECs. CXCL9 expression, as analyzed by qPCR, was reduced by ~75% in CXCL9-KD hiPSC-ECs compared to Scramble hiPSC-ECs (not shown). CXCL9-KD and Scramble hiPSC-ECs were serially cultured to passage 8 in a time-course experiment to mimic cellular aging.

We then investigated the functional impact of increased inflammation and decreased proliferation in endothelial aged cells in a model for angiogenesis by measuring endothelial cell capacity to form networks of tubular structures[54], the production of NO, and uptake of Ac-LDL. iPSC-ECs at passage 8 showed significantly impaired tube formation when compared to early passages of iPSC-ECs including passage 0 and 2. As early as passage 4, endothelial cells lose

their capacity to form tubes, which can be partially restored when CXCL9 is knocked-down (**Figure 4E** and **Supplementary Figure 11**). Next, we assessed the capacity of these early or late passaged iPSC-ECs to produce NO or uptake acetylated LDL. As seen in **Figure 4F** and **G**, it can be clearly observed that late passaged iPSC-ECs fail to produce NO in response to acetylcholine or uptake Ac-LDL, respectively, when compared to early passages of iPSC-ECs. Importantly, the knockdown of CXCL9 (CXCL9-KD) in iPSC-ECs rescued the EC dysfunction in late passages of iPSC-ECs (P6 and P8), suggesting an important role of CXCL9 and EC phenotype. It is also noteworthy that when comparing tube formation, NO production, and uptake of Ac-LDL in Scramble at passage 0 vs. CXCL9 at passage 8, there are statistical significance in all three metrics ($P<0.01$). This suggests that while the knock down of CXCL9 rescues endothelial dysfunctions by passage 8, it cannot restore ECs' function completely to the level of healthy ECs at passage 0. Altogether, these results are consistent with previous findings that show age-dependent endothelial dysfunction, fewer T cells, and impaired vasodilation with advanced age in animal models and that angiogenesis requires the migration and proliferation of endothelial cells[61]. Taken together these results demonstrate that CXCL9 has a profound effect and thus a new role for this chemokine in angiogenesis and endothelial cell function during cardiovascular aging.

CXCL9 Governs Inflammation and Proliferation Pathways in Aged Endothelium

In the time-course experiment where CXCL9-KD and Scramble hiPSC-ECs were serially cultured to passage 8, RNA was also extracted at every other passage for bulk RNA-seq transcriptome analysis (see Methods). We observed a time-dependent increase in CXCL9 transcript levels up to ~4-fold at passage 8 compared to cells obtained from cultures at day 0, and

a substantial reduction of CXCL9 expression in CXCL9-KD hiPSC-ECs (**Figure 5A**). Fast Gene Set Enrichment Analysis (FGSEA) pathway enrichment analysis in aged cells revealed an upregulation of genes in hallmark inflammatory pathways and downregulation of genes in hallmark cell proliferation pathways (**Figure 5B**). This profile is indicative of an early cellular senescence phenotype[62-66]. CXCL9-KD showed a complete reversal of this early cellular senescence phenotype with upregulation of proliferative pathways and downregulation of inflammatory pathways when compared to Scramble hiPSC-ECs (**Figure 5C-E**). Examples of these inflammatory and proliferation hallmark pathways include the IFN-γ response and E2F Targets, respectively (**Figure 5D** (Scramble)**, Figure 5E** (CXCL9-KD)**).** Such functional impact of increased inflammation and decreased proliferation in endothelial aged cells could contribute to the impaired tube formation and endothelial dysfunction observed in the experiments described previously.

CXCL9 Impairs Vascular Function and Contributes to Arterial Stiffness

To further explore CXCL9's role in cardiovascular aging in our *in vitro* endothelial cell aging model, we focused on the molecules that are related to a surrogate of cardiovascular risk, arterial stiffness. Endothelial cell dysfunction has been shown to strongly affect arterial stiffness via cellular adhesion molecules (CAMs), matrix metalloproteinases (MMPs), and collagen molecules (COLs)[67]. Collagen deposits during inflammation-driven fibrosis lead to a reduced structural compliance and flexibility[67, 68] and extracellular matrix components of the vasculature are regulated by MMPs and their upregulation is a sign of disease[69]. Increased vascular stiffness also occurs directly via MMPs, which contribute to endothelial dysfunction by depressing endothelial flow-mediated dilation, worsening the response to vascular injury, affecting

angiogenesis, and promoting atherosclerotic plaque formation[70]. Finally, cellular adhesion molecules are integral to the migration of leukocytes to the point of inflammation[71]. Given the role of CAMs, MMPs, and COLs in endothelial dysfunction, we compared their gene expression of Scramble hiPSC-ECs at passage 0 vs. passage 8. We found a substantial up-regulation of CAM, MMP, and COL genes related to arterial stiffness at passage 8 (**Figure 6A**). Except for most COL genes, this vascular stiffness gene profile is reversed in CXC9-KD cells, which suggests that silencing of this single gene can restore endothelial cell phenotype (**Figure 6B**).

Since the genes related to arterial stiffness are upregulated in Scramble passage 8 but their expressions are largely attenuated in CXCL9-KD, we hypothesized that there might be a causal effect between arterial stiffness and increase expression of CXCL9. To test this, we incubated mouse thoracic aortic sections with increasing concentrations of recombinant mouse CXCL9 and assessed cellular contractibility by incubating vessels with the prostaglandin agonist U46619 and measuring relaxation curves by isometric myography[72]. As shown in **Figure 6C**, a dose-dependent effect of CXCL9 is observed on vasorelaxation in treated aortas versus controls, which validates our findings of the effect of CXCL9 on the arterial stiffness gene expression phenotypes. The same experiment was conducted in young vs. old mice using only one dose of CXCL9 (1ng/ml). As seen in **Figure 6D**, aortic rings excised from old mice showed impaired vascular relaxation when compared to young mice in response to acetylcholine. However, aortic rings from both young and old mice when incubated with CXCL9 exhibited impaired vascular relaxation. These results demonstrate a central role for CXCL9 in vascular dysfunction, and thus likely contributes to arterial stiffness and premature aging *in vivo*.

Age-related Elevation in CXCL9 Leads to Endothelial Cell Senescence

The lack of angiogenesis, impaired production of NO, and dysfunctional uptake of Ac-LDL indirectly suggested a cellular senescence phenotype that could be rescued by knocking down CXCL9 as iPSC-EC is passaged. To directly explore the role of CXCL9 in cellular senescence, we assessed the proliferation rate and cellular senescence markers in scramble and CXCL9-KD iPSC-ECs at different passages. First, we assessed the kinetic profile of iPSC-ECs from scramble and CXCL9-KD cells every 24 hrs. for up to 4 days. Briefly, equal number of scramble and CXCL9-KD iPSC-ECs from passage 0 and passage 8 were seeded in a 96-well plate and cells were quantified using Cytation 5 cell imaging multi-mode reader where individual cells were counted every 24 hrs. by imaging 4′,6-diamidino-2-phenylindole (DAPI)-positive cells. As seen in **Figure 7A**, the kinetic profile of iPSC-EC proliferation over four days showed a significant increase in the proliferation rate in P0 iPSC-ECs when compared to P8 iPSC-ECs. Importantly, when CXCL9 was inhibited in P8 iPSC-ECs (CXCL9-KD), the proliferation rate showed a significant increase when compared to Scramble-treated cells.

Next, we assessed the senescence-associated β-galactosidase (SA-β-gal) activity in Scramble or CXCL9-KD iPSC-ECs at different passages to determine the cellular senescence in these cells. Cell lysates were collected, and SA-β-gal activity measured using a fluorometric substrate. Fluorescence was measured at 360 nm (excitation) / 465 nm (emission). As expected, Scramble iPSC-ECs showed an increase in SA-β-gal activity with every passage from P0 to P8, suggesting an increase in cellular senescence. However, CXCL9-KD iPSC-ECs showed a significant decrease in their SA-β-gal activity at later passages when compared to scramble, suggesting a direct link between CXCL9 expression and cellular senescence (**Figure 7B**).

Finally, we examined the capacity of Scramble and CXCL9-KD iPSC-ECs to form capillaries *in vivo* when injected subcutaneously in immunodeficient mice[73]. Early and late

passaged iPSC-ECs from both scramble and CXCL9-KD groups were placed in matrigel and injected subcutaneously into the lower abdominal region of SCID mice. Following 2 weeks, these matrigel plugs were excised, fixed and stained for human CD31. As seen in **Figure 7C**, immunohistochemical images showed formation of capillaries in Scramble and CXCL9-KD iPSC-ECs at passage 0, however passage 8 (late passaged) Scramble iPSC-ECs failed to show sprouting *in vivo* (**Figure 7C-D**). In contrast, passage 8 CXCL9-KD iPSC-ECs showed significantly improved *in vivo* angiogenesis, suggesting an important role of CXCL9 in EC senescence.

DISCUSSION

In this study, we conducted extensive immune monitoring in a large cohort of 1001 subjects to identify immune biomarkers of aging and developed reference values for age-related chronic inflammation. We used artificial intelligence to create a compact representation of these biomarkers and derived an "inflammatory clock" of aging, which takes into account the non-linear relationship and redundancy of the cytokine network. This novel metric tracked with multiple aging phenotypes in the general population and thus, it has strong potential for translational medicine, as it could be used as a diagnostic tool for identifying those at risk for both non-communicable and infectious diseases.

Our non-linear GAE method was optimal for the identification of iAge and its contributors. As other deep learning methods GAE is capable of capturing complex relationships between analytes. Similar methods striving to extract signatures of aging have been described in different systems ranging from genome-wide association studies (GWAS) to proteomics. We summarize a few notable aging clocks in **Supplementary Table 3**. In brief, an epigenetic clock

using markers measuring DNA methylations on CpG sites have been used to calculate an epigenetic age that were able to predict all-cause mortality[74, 75]. It has also been associated with age-related diseases such as frailty, Alzheimer's Disease, Parkinson's Disease, cancer. Other clocks such as transcriptomic and microRNA clocks have also shown to successfully capture aspects of the aging process that are different from epigenetic clocks. Instead of associated with all-cause mortality or disease, transcriptomic clocks are associated with IL-6, albumin, lipids, and glucose levels [76]. There have also been attempts to derive proteomics clocks and metabolomic clocks[77-82] of clinical relevance; but iAge allows for new discoveries in the immune system. iAge derived from immunological cytokines gives us an insight into the salient cytokines that are related to aging and disease. An important difference compared with other clocks is that iAge is clearly actionable as shown by our experiments in CXCL9 where we can reverse aging phenotypes. More practical approaches range from altering person's exposomes (lifestyle) and or the use of interventions to target CXCL9 and other important biomarkers described here.

Recent advancements in deep learning beyond traditional machine learning methods has provided enormous opportunities to model biological age. Some of the most popular deep learning architectures used to estimate biological age have been recurrent neural networks (RNN), convolutional neural networks (CNN), generative adversarial networks (GAN), and deep artificial neural networks (ANN). RNNs have been used on face attributes and physical activities to estimate biological age.[83] Although the modality is not in the realm of biological markers, RNNs have potential to garner results in biological data that requires positional relationships like epigenetic age. CNNs and GANs have both been used to abstract facial attributes to predict chronological age.[84, 85] GANs and CNNs are exceptional in abstracting images to distill useful information. Future applications of GANs and CNNs can be applied in other biological images

such as MRIs. However, for now, these models have been proof of concepts that they can accurately estimate chronological age. They might not necessarily predict the health or life span of individuals. The deep learning models that have been applied to modality used in this paper are the deep artificial neural networks. ANNs have been applied to blood biochemistry markers and cell counts to derive biological age.[86, 87] The results showed that such clocks were able to predict for all-cause mortality, potentially finding novel biomarkers to intervene and steer individuals toward a healthier life.

Some of the limitations of biological clocks in general is that these do not directly provide the mechanism by which they work. Whereas it is possible to infer causality between aging and molecular biomarkers especially in the context of longitudinal or time-series data, individual biomarkers selected from biological aging clock need to be experimentally tested to elucidate the underlying mechanism as we have done in this study. Our guided autoencoder (GAE) algorithm, a deep learning method that efficiently deals with the network structure and non-linear behavior of the inflammatory response can extract high-level complex abstractions as 'data representations' using non-linear functions and it is well-suited for the analysis of complex systems where most behaviors are non-linear, context-dependent and organized in a distributed hierarchical fashion[88]. In our case, this method outperformed other commonly used linear-modeling methods such as the elastic net and PCA, and also other non-linear approaches such as plain autoencoder[89] (**Supplementary Figure 3B**). The correlation between age and iAge was 0.78 ($P<10^{-16}$) (**Figure 1A**), which is lower than that of the recently reported 'proteomic age' metric (R = 0.92)[90]. However, in contrast with proteomic age, which did not report disease associations, we find that iAge tracks with multiple diseases and immunosenescence. In particular, we find a strong association between high chronic inflammation and poor acute *ex*

534 *vivo* immune responses, which is consistent with previous reports showing that high levels of
535 baseline inflammatory markers correlate with weaker responses to hepatitis B and herpes zoster
536 vaccine formulations[15, 91]. Similarly, chronic inflammation was, at least in part, responsible for a
537 reduced JAK-STAT response to cytokine stimulations in various leukocyte populations in our
538 previous studies of aging[28]. Despite the proven utility of the cytokine stimulation assays used in
539 our study with respect to an individual's overall immune competence versus immune
540 senescence[5, 24, 25], a limitation of the assay relates to the stimuli used here which may not
541 completely mirror the physiological stimuli that act on specific immune cell subsets *in vivo.* For
542 example, while the stimuli we used strongly activate the memory compartment of bulk CD8 (+)
543 and CD4 (+) T cells, these act relatively weakly on naïve T cells. Additional cell subsets that are
544 poorly activated by the cytokines used in our study are Th1 CD4 (+) T cells, which can be
545 activated by IL-12 and IL-18, or Th17 CD4 (+) T cells, which respond to other cytokine
546 stimulations such as IL-1β or IL-18 in concert with IL-23 to produce Th17 associated cytokines.
547 Recent findings from our group[28],[16] placed the immune system in the center of aging
548 phenotypes. Similar to our previous findings on the cytokine response score for diastolic
549 dysfunction and atherosclerotic burden[28], our inflammatory clock metric specifically hones in on
550 the crucial role that the immune system and systemic chronic inflammation play in the
551 accumulation of diseases of aging, with a focus on cardiovascular aging. Unlike other metrics of
552 'biological' age which do not offer a clinically relevant metric[92] we demonstrate that iAge
553 predicts multimorbidity, and therefore can be used as a biological surrogate of age-related health
554 vs. disease. iAge is directly associated with multiple disease phenotypes including cardiovascular
555 aging, frailty, immune decline and exceptional longevity. In our recent work[16], we combined
556 cellular phenotypes to describe subject- and population-level immune aging phenotypes (IMM-

AGE) which correlated with iAge. This suggests that future research should leverage both immune-age scores to propose a unified metric that reflects multiple aspects of immune aging, thus potentially providing a better clinical predictive value.

A major contributor to the inflammatory clock, CXCL9, was validated as an indicator of cardiovascular pathology independent of age. CXCL9 is a T-cell chemoattractant induced by IFN-γ and mostly produced by neutrophils, macrophages, and endothelial cells. Despite prior data showing that CXCL9 and other CXCR3 ligands are significantly elevated in hypertension and in patients with left ventricular dysfunction[41], we find that CXCL9 is mainly produced by aged endothelium and predicts subclinical levels of cardiovascular aging in nominally healthy individuals. CXCL9 has previously been associated with aging in different aspects. Specific association studies of human serum cytokine levels have found CXCL9 to increase with age[93-96]. Other studies exploring certain areas of the human body such as aqueous humor has also found an increase in CXCL9 in older populations[97]. Human serum gene expression level and gene expression level in rat pancreatic islets have also been shown to increase with age[90,98]. Studies exploring serum cytokine levels in diseases comparing old and young populations have also been conducted. CXCL9 display an age-dependent profile in Chagas disease[99] and Atopic dermatitis[100]. It has also been shown to be associated with fall in older population[101]. Similarly, in mouse model, CXCL9 response to bacterial stimulation at old age is attenuated[102]. At least two sources of CXCL9-mediated inflammation can ensue with aging based on our findings; one that is age-intrinsic and observed in aging endothelial cells, and one independent of age (likely as a response to cumulative exposure to environmental insults) and found in the validation cohort of 97 apparently healthy adults. Interestingly, we did not find any significant correlation between known disease risk factors reported in the study (BMI, smoking, dyslipidemia) and the levels of

CXCL9 gene or protein expression. We thus hypothesize that one root cause of CXCL9 overproduction is cellular aging *per-se*, which can trigger metabolic dysfunction - as shown in many previous studies of aging - with production of damage-associated molecular patterns (DAMPs). Examples of these include adenosine, adenine and N4-acetylcytidine as demonstrated in our previous longitudinal studies of aging[5]. These DAMPs can then act through the inflammasome machinery, such as NLRC4, to regulate multiple inflammatory signals including IL-1β and CXCL9[103].

Our data also place the endothelium as a central player in cardiovascular aging in agreement with a previous study that showed that cardiac hypertrophy is mostly driven with endothelial dysfunction[104]. They also suggest that endothelial cells may be one source of inflammation, but it is also possible that cardiomyocytes play a role since in models of acute myocardial infarction there is activation of the inflammasome NLRP3 in these cells[105]. Last, the hypertrophic response may also be affected by the adaptive immune response as was suggested both in non-ischemic heart failure (HF) patients and in mice with HF induced by transverse aortic constriction (TAC), where T cells exhibited enhanced adhesion to activated vascular endothelium. Moreover, T cell-deficient mice (TCRα(-/-)) subjected to the TAC method had preserved left ventricular (LV) systolic and diastolic function, reduced LV fibrosis, hypertrophy and inflammation, and improved survival compared with wild-type mice which demonstrates that the adaptive immune system is also involved in the progression to severe cardiovascular aging phenotypes[106].

Since endothelial cells but not cardiomyocytes expressed the CXCL9 receptor, CXCR3 (**Supplementary Figure 10**), we hypothesize that this chemokine acts both in a paracrine fashion (when it is produced by macrophages to attract T cells to the site of injury) and in an

603 autocrine fashion (when it is produced by the endothelium) creating a positive feedback loop. In
604 this model, increasing doses of CXCL9 and expression of its receptor in these cells leads to
605 cumulative deterioration of endothelial function in aging. Moreover, the silencing of CXCL9 in
606 endothelial cells resulted in a reversal of the high inflammation/low proliferation early
607 senescence phenotype, which suggests by tackling CXCL9 it may be possible to delay the onset
608 of endothelial cell senescence. It is also interesting to note that IFN-γ, a direct agonist to
609 CXCL9, did not increase in expression in our cellular aging RNA-seq experiment, suggesting
610 that there are triggers of CXCL9 (other than IFN-γ) which play a role in cellular senescence in
611 the endothelium, that are currently unknown. However, in our 1KIP study, IFN-γ was in fact the
612 second most important *negative* contributor to iAge, which could be explained by the cell
613 priming effect of cytokines, where the effect of a first cytokine alters the response to a different
614 one[107-109]. In a more recent and refined version of this model (the high baseline-low output model
615 for chronic inflammation and the acute response) we show that sustained levels of inflammatory
616 mediators lead to a non-functional constitutive phosphorylation of signaling pathways with
617 saturation of phosphorylation sites in signaling proteins (such as the JAK-STAT system), which
618 results in a lowered delta phosphorylation in response to acute stimuli and subsequent
619 dampening of the immune response to infections or vaccination[28].

620 In conclusion, by applying artificial intelligence methods to deep immune monitoring of
621 human blood we generate an inflammatory clock of aging, which can be used as a companion
622 diagnostic to inform physicians about patient's inflammatory burden and overall health status,
623 especially those with chronic diseases. Furthermore, our immune metric for human health can
624 identify within healthy older adults with no clinical or laboratory evidence of cardiovascular
625 disease, those at risk for early cardiovascular aging. Lastly, we demonstrate that CXCL9 is a

626 master regulator of vascular function and cellular senescence, which indicates that therapies
627 targeting CXCL9 could be used to prevent age-related deterioration of the vascular system, and
628 likely of other physiological systems as well.

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DISCLOSURES

David Furman and Mark M Davis are co-founders of Edifice Health Inc., a company that utilizes iAge.

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Figure 1

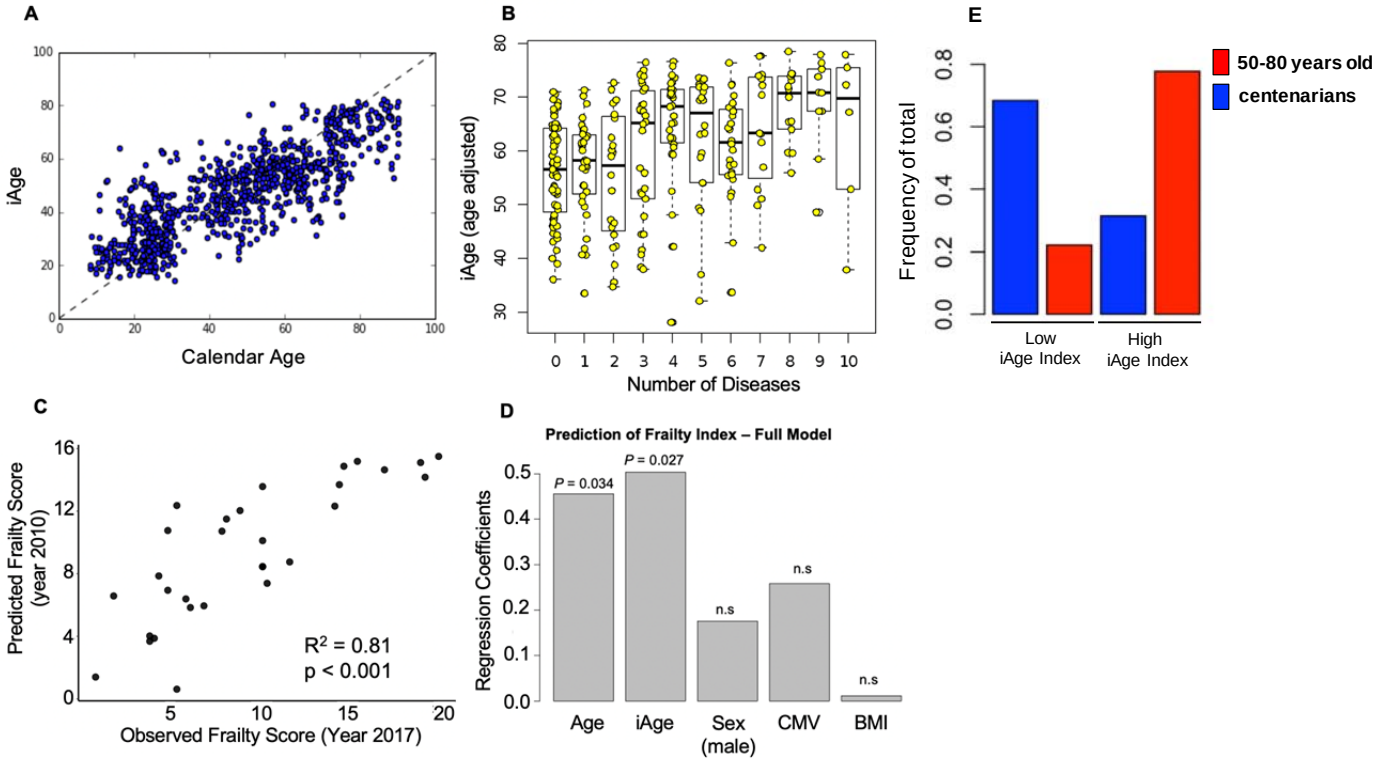

Figure 1. The inflammatory clock of aging tracks with multimorbidity, frailty and exceptional longevity. Using a guided auto-encoder method on 50 circulating immune proteins, we derived inflammatory clock of aging (iAge) which predicts chronological age (**A**). Ten age-related disease items were selected to characterize the clinical significance of iAge. The items analyzed included different diseases and physiological systems: cancer, cardiovascular, respiratory, gastrointestinal, urologic, neurologic, endocrine-metabolic, musculoskeletal, genital-reproductive and psychiatric. All these disease features were binary. After adjusting for co-variates, iAge is significantly correlated with multimorbidity in the older population analyzed (>60 years old, N = 285) (**B**). For a subset of older adults (N = 29), frailty was assessed in 2017 using a modified frailty score (see Methods). iAge measured in year 2010 predicts the frailty score 7 years in advance (**C**) and better than calendar age ($p < 0.05$ by likelihood ratio test for model comparison) (**D**). Comparison of the iAge index (rank cAge minus rank iAge) was computed between a group of older adults (N = 18, age range 50-79 years old) and centenarian subjects (N = 19, age range 99- 107). Centenarians were over-represented in subjects with low iAge index (protective phenotype) whereas the control older adults' group were over-represented in subjects with high iAge index(**E**).

Figure 2

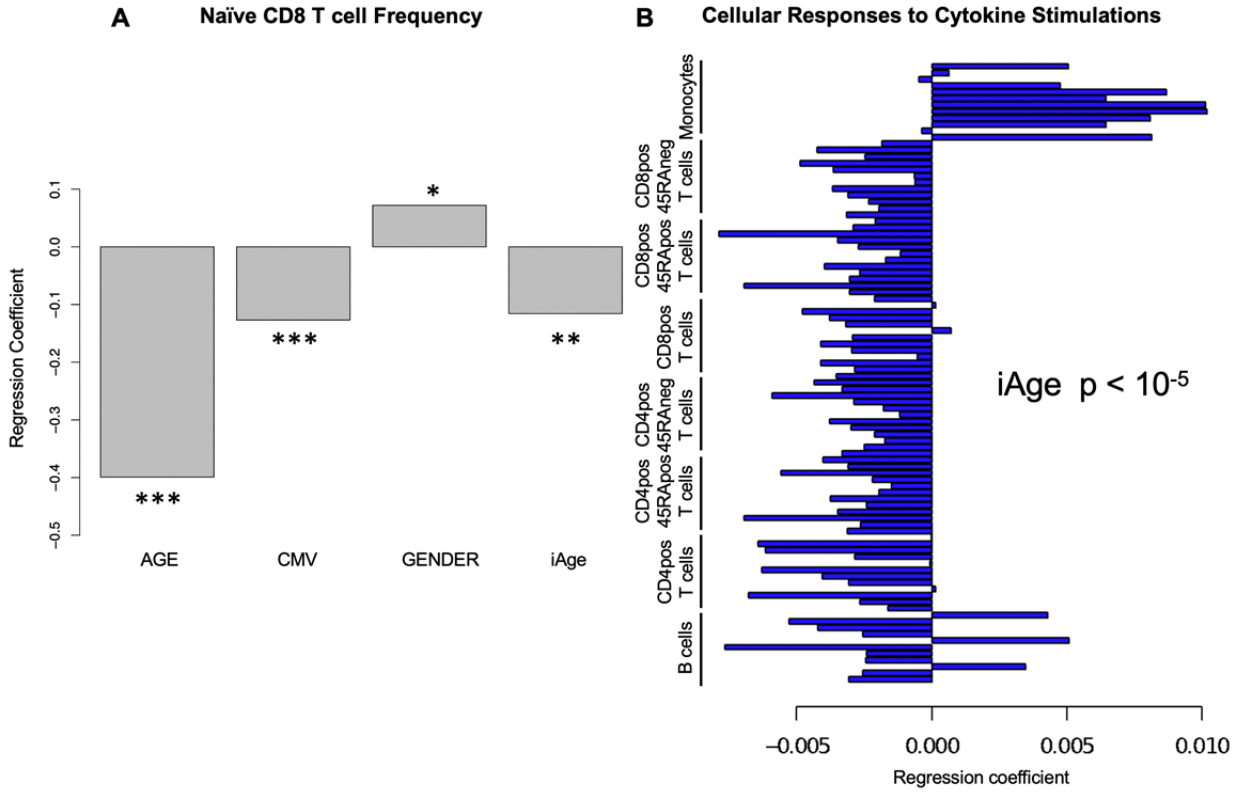

Figure 2. The inflammatory clock of aging correlates with immunosenescence. A hallmark of immunosenescence (naïve CD8 (+) T cells) was used to examine the potential contribution of iAge to this condition. In a multiple regression model, iAge was significantly correlated with the frequency of naïve CD8 (+) T cells to a similar extent to CMV positivity (**A**). Chronological age was the strongest contributor ($P < 10^{-15}$), followed by CMV ($P < 10^{-5}$), iAge ($P < 10^{-3}$) and gender ($P = 0.012$). Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05. The activation of multiple pathways was measured using the phospho-flow method in B cells, CD4 (+) T cells (the CD45RA (+) and CD45RA (-) subsets), CD8 (+) T cells (the CD45RA (+) and CD45RA (-) subsets) and in monocytes. The assay performed correspond to phospho-flow whereby peripheral blood mononuclear cells are plated *ex vivo* and activated with a variety of cytokine stimuli to measure phosphorylation event in STAT proteins (specifically STAT1, STAT3 and 5). iAge is consistently negatively correlated with B cell and T cell responses to cytokine stimuli and positively correlated with monocyte responses (**B**) ($P < 10^{-5}$ by self-contained test of modified Fisher's combined probability).

Figure 3

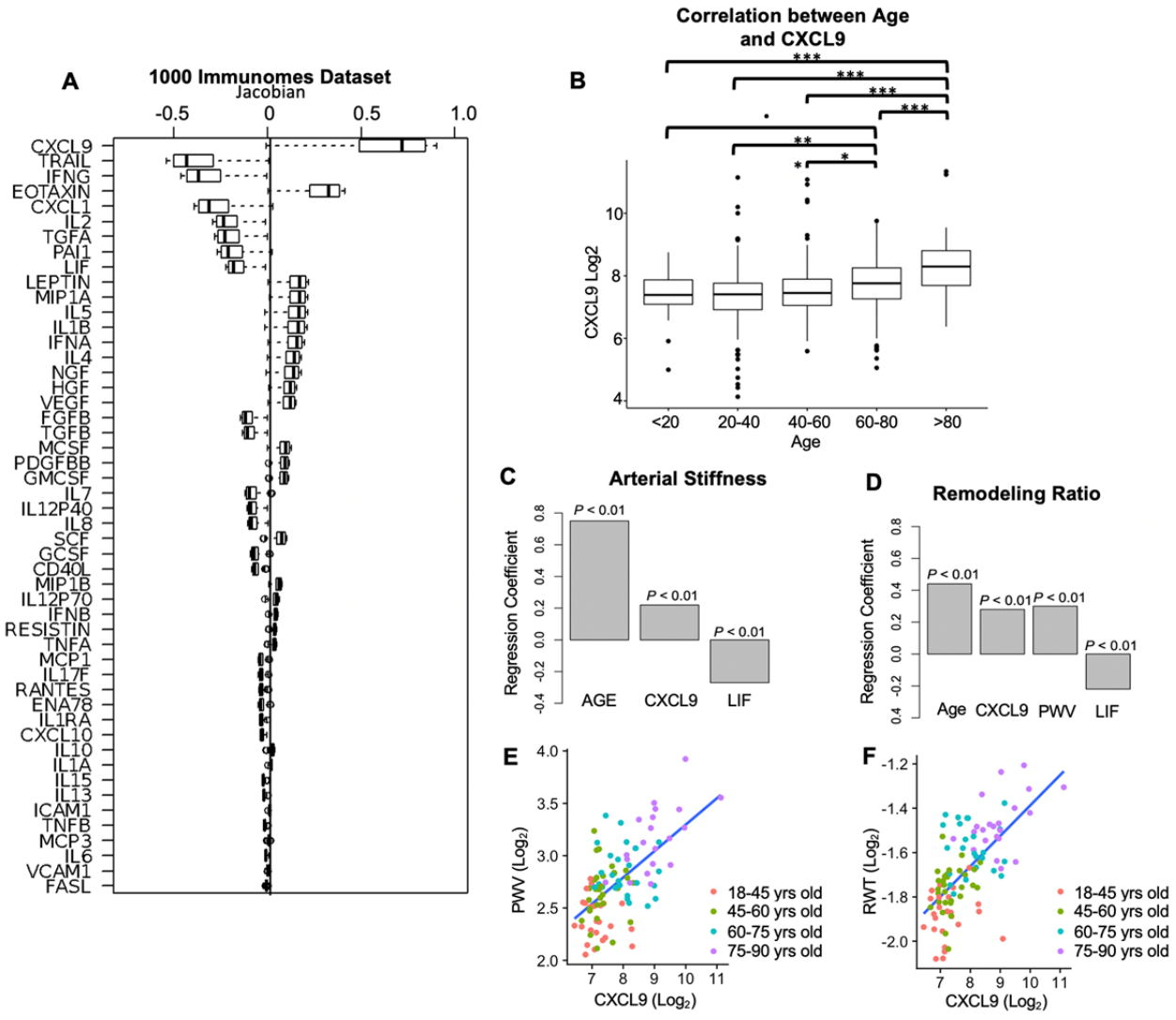

Figure 3. CXCL9 is a major contributor to iAge. The decomposition of the inflammatory score was conducted by estimating the most variable jacobians (first-order partial derivative of the inflammatory clock) **(A)**. Both positive and negative contributors to the inflammatory clock are observed. The top 15 most variable jacobians are CXCL9, EOTAXIN, Mip-1α, LEPTIN, IL-1β, IL-5, IFN-α and IL-4 (positive contributors), and TRAIL, IFN-γ, CXCL1, IL-2, TGF-α, PAI-1 and LIF (negative contributors). Significant differences in the levels of CXCL9 were observed between age groups ($P < 0.001$, by one-way ANOVA test) (**B**). The pairwise differences between groups were evaluated with the Tuckey Honest Significant Differences Test. Significant differences were shown for older age groups (60-80yrs and >80yrs) and younger age groups (<20yrs, 20-40yrs, 40-60yrs). Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05 '.': < 0.1. (**B**) In a validation study, 97 healthy adults (aged 25-90) well matched for cardiovascular risk

factors, were selected from a total of 151 recruited subjects. Cardiovascular age was estimated using aortic pulse wave velocity (PWV), a measure of vascular stiffness and relative wall thickness (RWT), a measure of ventricular remodeling. After adjusting for age, sex, BMI, heart rate, systolic blood pressure, fasting glucose and total cholesterol to HDL ratio, positive correlations were obtained between CXCL9 and PWV ($R = 0.22$) and RWT ($R = 0.3$) ($P < 0.01$), and negative correlations were observed between LIF and PWV ($R = -0.27$), and RWT ($R = -0.22$) (**C** and **D**, respectively). Direct comparisons between CXCL9 and these two cardiovascular aging phenotypes (PWV and RWT) are depicted (**E** and **F**, respectively). No other variable included in the models had high co-linearity as suggested by variance inflation factors (VIF) < 3 for each factor.

Figure 4

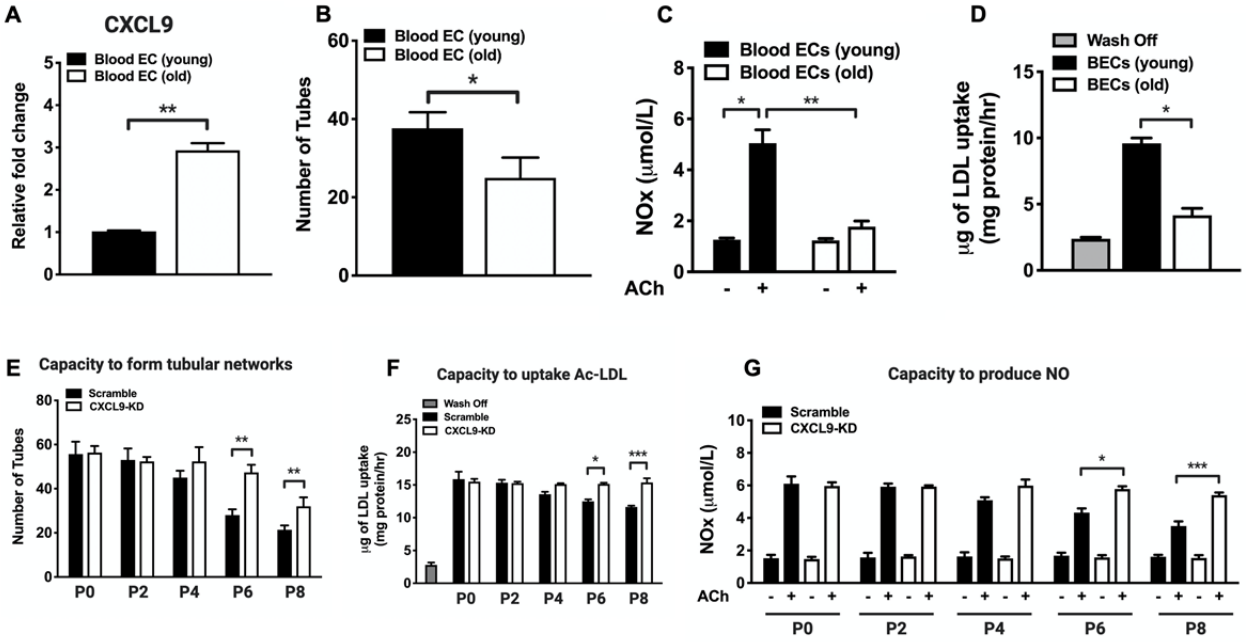

Figure 4. CXCL9 is an important regulator of endothelial cell aging (A) Quantitative PCR data show increased expression of CXCL9 in blood-derived endothelial cells (BECs) of old individuals compared to young. Similarly, significant differences in tube formation capacity are observed in BECs from older and younger subjects **(B).** Quantification of NO production shows impaired capacity of BECs from old individuals to produce NO when compared to young in response to acetylcholine (Ach) (**C**). Quantification of LDL uptake show impaired capacity of BECs from old individuals to uptake Ac-LDL when compared to young (**D)**. Quantification of the number of tubes, LDL uptake and NO production in response to Ach in scramble and CXCL9-KD iPSC-ECs shows a significant improvement in these aging phenotypes in endothelial cells at passage 6 and 8 with silencing of the CXCL9 gene **(E-G).** Scramble = hiPSCs infected with lentivirus carrying nonsense-sequence shRNA. CXCL9-KD = hiPSCs infected with lentivirus carrying sequence-specific short hairpin RNA to knockdown expression of CXCL9. Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05

Figure 5

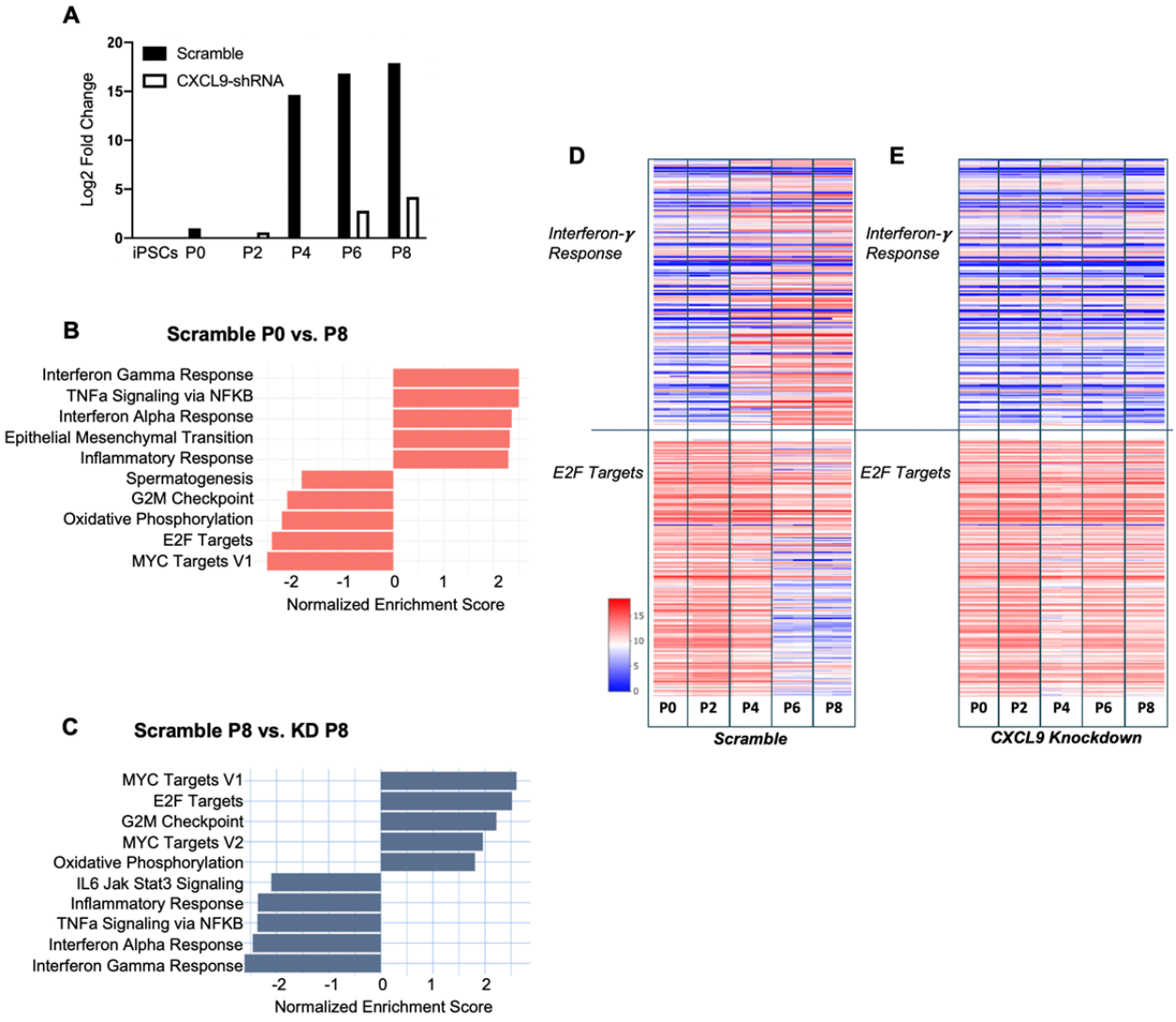

Figure 5. Early cellular senescence and loss of angiogenesis capacity in iPSC-derived aging endothelia is reversed by silencing CXCL9. Pathway enrichment analysis and tube network formation of Scramble vs. CXCL9-KD were analyzed. hiPSCs infected with lentivirus carrying nonsense-sequence shRNA (Scramble) and hiPSCs infected with lentivirus carrying sequence-specific short hairpin RNA to knockdown expression of CXCL9 (CXCL9-KD) were both induced to endothelial cells (see Methods). RNA-seq analysis was conducted on cells at passage 0, 2, 4, 6, 8 for both conditions. CXCL9 mRNA in Scramble was highly upregulated as early as passage 4, while CXCL9 mRNA expression in CXCL9-KD did not significantly change with in vitro cellular aging (**A**). **(B)** shows pathway enrichment comparing Scramble at passage 0 and passage 8. Upregulated inflammatory pathways and downregulated proliferation pathways are depicted (P8 vs P0). Comparing Scramble at P8 with CXCL9-KD at P8 shows that silencing of CXCL9 leads to a complete reversal of the early endothelial cell senescence phenotype (**C**). An example of inflammatory pathway (IFN-γ) and an example of proliferation pathway (E2F Targets) in shown in (**D**). Relative expression of genes in the hallmark pathways for Scramble at passage 0, 2, 4, 6, 8 (S0, S2, S4, S6, S8) are shown. **(E)** shows an example of inflammatory pathway (IFN-γ) and an example of proliferation pathway (E2F Targets) for CXCL9-KD at

passage 0, 2, 4, 6, 8 (KD0, KD2, KD4, KD6, KD8). Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05

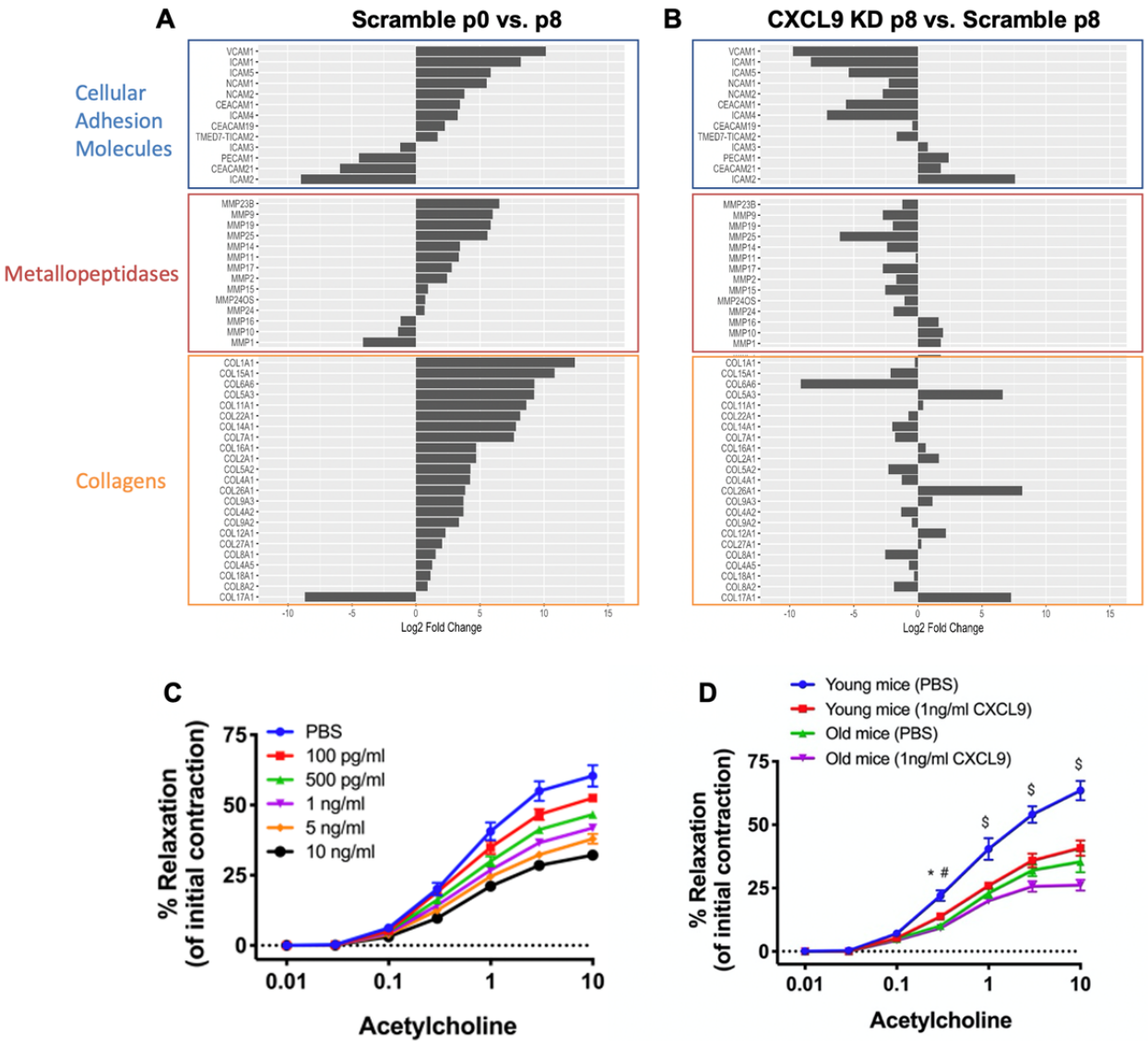

Figure 6. CXCL9 promotes a vascular stiffness gene expression signature in the aging endothelium and impairs endothelial function. The expression levels of hallmark vascular stiffness genes: cellular adhesion molecules (CAMs), metallopeptidases (MMPs), and collagen genes (COLs) were analyzed in Scramble and CXCL9-KD aging cells. In (**A**), CAMs, MMPs and COLs are highly expressed in Scramble passage 8 compared to passage 0. The knock down of CXCL9, completely restores the expression of CAMs and MMPs, but not COLs (**B**). Line graph of percent relaxation of mouse thoracic aortic sections incubated with increasing concentrations of CXCL9 shows impaired vascular reactivity to Acetylcholine, suggesting CXCL9 dampens vascular function (**C**). Similar trend is observed when CXCL9 is given to either young or old mice (**D**). CXCL9 disrupts the relaxation supposedly induced by Acetylcholine. Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05

Figure 7

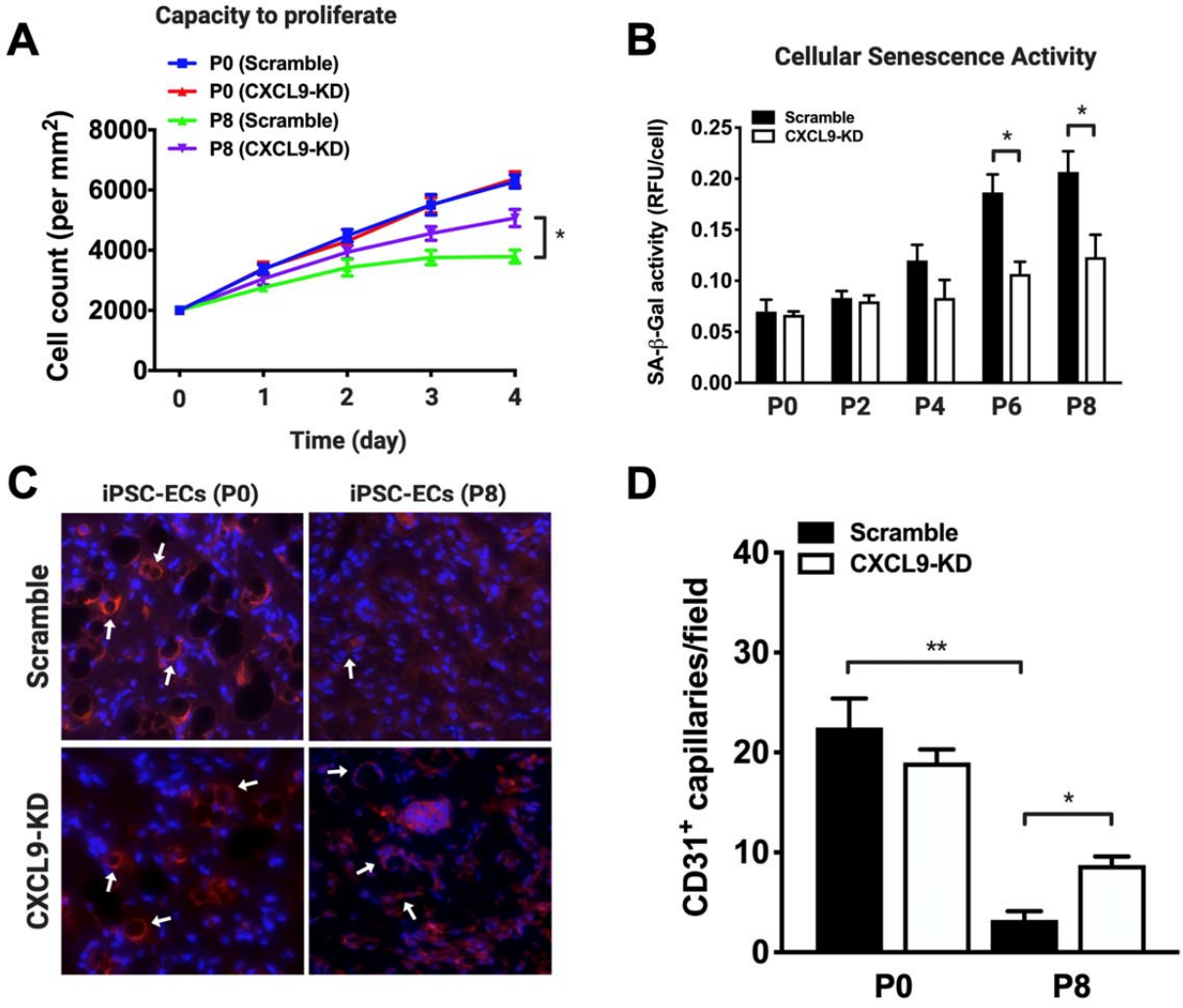

Figure 7. CXCL9 regulates endothelial cell senescence and capillary network formation *in vivo*. (A) Growth curves over 4 days show recovery of cell proliferation in CXCL9-KD iPSC-ECs in later passages when compared to scramble iPSC-ECs. **(B)** Cellular senescence activity assay shows restoration of SA-β-gal activity in CXCL9-KD iPSC-ECs at later passages when compared to scramble iPSC-ECs. **(C)** Representative immunohistochemical images showing CD31+ human capillaries from serially passaged scramble and CXCL9-KD iPSC-ECs. **(D)** Quantification of CD31+ capillaries show improved capacity of late passaged CXCL9-KD iPSC-ECs to form *in vivo* capillary networks. Significance codes: '***': <0.001 '**': <0.01 '*': < 0.05

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