Epigenetic upregulation of FKBP5 by aging and stress contributes to NF-κB–driven inflammation and cardiovascular risk

Anthony S. Zannas[a,b,c,d,1], Meiwen Jia[a], Kathrin Hafner[a], Jens Baumert[e], Tobias Wiechmann[a], Julius C. Pape[a], Janine Arloth[a,f], Maik Ködel[a], Silvia Martinelli[a], Maria Roitman[a], Simone Röh[a], Andreas Haehle[g], Rebecca T. Emeny[h], Stella Iurato[a], Tania Carrillo-Roa[a], Jari Lahti[i,j], Katri Räikkönen[i], Johan G. Eriksson[k,l], Amanda J. Drake[m], Melanie Waldenberger[e], Simone Wahl[e], Sonja Kunze[e], Susanne Lucae[a], Bekh Bradley[n,o], Christian Gieger[e], Felix Hausch[g], Alicia K. Smith[o,p], Kerry J. Ressler[o,q,r], Bertram Müller-Myhsok[a,s,t], Karl-Heinz Ladwig[e,u], Theo Rein[a], Nils C. Gassen[a,v], and Elisabeth B. Binder[a,o,1]

[a]Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, 80804 Munich, Germany; [b]Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514; [c]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514; [d]Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710; [e]Institute of Epidemiology, Helmholtz Zentrum München - German Research Centre for Environmental Health, D-85764 Neuherberg, Germany; [f]Institute of Computational Biology, Helmholtz Zentrum München, D-85764 Neuherberg, Germany; [g]Department of Chemistry, Institute of Organic Chemistry and Biochemistry, Technische Universität Darmstadt, 64289 Darmstadt, Germany; [h]Geisel School of Medicine, Dartmouth College, Lebanon, NH 03755; [i]Department of Psychology and Logopedics, University of Helsinki, 00100 Helsinki, Finland; [j]Turku Institute for Advanced Studies, University of Turku, 20500 Turku, Finland; [k]Department of General Practice and Primary Health Care, Helsinki University Hospital, University of Helsinki, 00100 Helsinki, Finland; [l]Folkhälsan Research Center, 00290 Helsinki, Finland; [m]University/British Heart Foundation Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, United Kingdom; [n]Atlanta Veterans Affairs Medical Center, Decatur, GA 30033; [o]Department of Psychiatry and Behavioral Sciences, Emory University Medical School, Atlanta, GA 30322; [p]Department of Gynecology and Obstetrics, Emory University Medical School, Atlanta, GA 30322; [q]Division of Depression & Anxiety Disorders, McLean Hospital, Belmont, MA 02478; [r]Department of Psychiatry, Harvard Medical School, Boston, MA 02478; [s]Munich Cluster of Systems Biology, SyNergy, 80539 Munich, Germany; [t]Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, United Kingdom; [u]Department of Psychosomatic Medicine and Psychotherapy, Klinikum rechts der Isar, Technische Universität München, 80333 München, Germany; and [v]Department of Psychiatry and Psychotherapy, University Hospital Bonn, 53105 Bonn, Germany

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Aging and psychosocial stress are associated with increased inflammation and disease risk, but the underlying molecular mechanisms are unclear. Because both aging and stress are also associated with lasting epigenetic changes, a plausible hypothesis is that stress along the lifespan could confer disease risk through epigenetic effects on molecules involved in inflammatory processes. Here, by combining large-scale analyses in human cohorts with experiments in cells, we report that FKBP5, a protein implicated in stress physiology, contributes to these relations. Across independent human cohorts (total *n* > 3,000), aging synergized with stress-related phenotypes, measured with childhood trauma and major depression questionnaires, to epigenetically up-regulate *FKBP5* expression. These age/stress-related epigenetic effects were recapitulated in a cellular model of replicative senescence, whereby we exposed replicating human fibroblasts to stress (glucocorticoid) hormones. Unbiased genome-wide analyses in human blood linked higher *FKBP5* mRNA with a proinflammatory profile and altered NF-κB–related gene networks. Accordingly, experiments in immune cells showed that higher *FKBP5* promotes inflammation by strengthening the interactions of NF-κB regulatory kinases, whereas opposing FKBP5 either by genetic deletion (CRISPR/Cas9-mediated) or selective pharmacological inhibition prevented the effects on NF-κB. Further, the age/stress-related epigenetic signature enhanced *FKBP5* response to NF-κB through a positive feedback loop and was present in individuals with a history of acute myocardial infarction, a disease state linked to peripheral inflammation. These findings suggest that aging/stress-driven FKBP5–NF-κB signaling mediates inflammation, potentially contributing to cardiovascular risk, and may thus point to novel biomarker and treatment possibilities.

aging | epigenetics | FKBP5 | inflammation | psychosocial stress

Aging is the single most important risk factor for several disease phenotypes that are leading causes of morbidity and mortality (1). However, individuals of the same age exhibit substantial variability in their risk of developing aging-related disease (2). Among important factors influencing disease risk, studies show that psychosocial stressors, such as childhood trauma, as well as stress-related psychiatric disorders, including major depressive disorder (MDD), increase risk for aging-related diseases, most notably cardiovascular syndromes (3–7).

Significance

Diseases of the aging are the leading cause of morbidity and mortality. Elucidating the molecular mechanisms through which modifiable factors, such as psychosocial stress, confer risk for aging-related disease can have profound implications. By combining studies in humans with experiments in cells, we show that aging and stress synergize to epigenetically upregulate FKBP5, a protein implicated in stress physiology. Higher FKBP5 promotes inflammation by activating the master immune regulator NF-κB, whereas opposing FKBP5, either genetically or pharmacologically, prevents the effects on NF-κB. Further, the aging/stress-related epigenetic signature of *FKBP5* is associated with history of myocardial infarction, a disease linked to inflammation. These findings provide molecular insights into stress-related disease, pointing to biomarker and treatment possibilities.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE72680, GSE58137, and GSE128235).

[1]To whom correspondence may be addressed. Email: aszannas@gmail.com or binder@psych.mpg.de.

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Studies further suggest that aging and stress-related phenotypes may together confer disease risk by increasing peripheral inflammation (5, 8–11), but the underlying mechanisms are poorly understood.

Mechanistically, the effects of stress on inflammation and disease risk could be driven by stress-responsive molecules able to modulate immune function. A plausible such molecule to examine is the FK506-binding protein 51 (FKBP51/FKBP5), a protein cochaperone that is acutely induced by stress and can influence biological processes through protein–protein interactions (12–19). Interestingly, FKBP5 up-regulation has been observed not only with stress exposure and glucocorticoid stimulation but also in the aging brain (20, 21) and in some disease phenotypes (15, 17, 20). However, it is unknown whether aging regulates FKBP5 in the immune system and how this effect, if present, could shape risk for cardiovascular disease. Both aging and stress can have lasting effects on the epigenome (22–25), and *FKBP5* transcription can be regulated by epigenetic mechanisms (26–28); thus, a plausible hypothesis is that stress exposure along the lifespan could epigenetically deregulate *FKBP5* in immune cells, potentially contributing to peripheral inflammation and disease risk.

Here we address these questions by combining genome-wide analyses in human cohorts with mechanistic investigations in cells. Convergent findings support a model whereby aging and stress-related phenotypes synergize to decrease DNA methylation at selected enhancer-related *FKBP5* sites, epigenetically up-regulating *FKBP5* in whole blood and in distinct immune cell subtypes. Higher *FKBP5* in turn promotes NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)-driven peripheral inflammation. Accordingly, the age/stress-related *FKBP5* epigenetic signature is present in individuals with a history of acute myocardial infarction (MI), a disease state linked to peripheral inflammation. We further find that the cellular effects of stress on NF-κB are prevented by either CRISPR/Cas9 deletion of the *FKBP5* gene or a selective FKBP5 antagonist, suggesting FKBP5–NF-κB signaling as a tractable treatment candidate. Together these findings provide molecular insights into mechanisms linking aging and stress with peripheral inflammation and cardiovascular risk, thereby pointing to biomarker and intervention possibilities.

Results

FKBP5 DNA Methylation Decreases Along the Lifespan at Selected Cytosine–Guanine Dinucleotides. DNA methylation at cytosine–guanine dinucleotides (CpGs) can change with age (24), an effect moderated in part by environmental factors (29), including psychosocial stress (23). These epigenetic changes can in turn contribute to disease risk by affecting the expression of molecules regulated by the age-related genomic sites, thus altering cell and tissue function (25, 30). Therefore, to gain insights into the mechanisms through which stress contributes to disease risk, it is relevant to examine the effects of stress on age-related CpGs.

To identify such age-related *FKBP5* CpGs, we used Illumina HumanMethylation450 BeadChip (450K) data from three independent cohorts with broad age range and documented stress-related phenotypes: the Grady Trauma Project (GTP; $n = 393$, age range 18 to 77 y); the Cooperative Health Research in the Region of Augsburg F4 community study (KORA; $n = 1{,}727$, age range 32 to 81 y); and the Max Planck Institute of Psychiatry cohort (MPIP; $n = 537$, age range 18 to 87 y) (demographics in Dataset S1). These analyses included all available CpGs covered by the 450K within or in close proximity (10 kb upstream or downstream) to the *FKBP5* locus (chromosome 6p21.31). After controlling for potential confounders, including age, sex, population stratification, and blood cell proportions in the GTP, KORA, and MPIP, as wells as for smoking and other available cohort-specific covariates (see *SI Appendix, Supplementary Methods* for additional details), and after false discovery rate (FDR) correction for multiple comparisons, two CpGs (cg20813374 and cg00130530) showed consistent and robust age-related decrease in methylation across all cohorts (detailed statistics in Dataset S2). These two age-related sites lie in close proximity to each other proximally upstream of the *FKBP5* transcription start site (TSS; −462 bp for cg20813374 and −484 bp for cg00130530; UCSC Genome Browser; Dataset S2) and show significant pairwise correlations in all cohorts (GTP: $r = 0.83$, $P < 2.2 \times 10^{-16}$; KORA: $r = 0.61$, $P < 2.2 \times 10^{-16}$; MPIP: $r = 0.37$, $P < 2.2 \times 10^{-16}$). The association of age with average methylation of the two CpGs is depicted in Fig. 1*A* and *SI Appendix*, Fig. S1*A*. To validate this finding with a non-hybridization-based DNA methylation method, we performed targeted bisulfite sequencing with the Illumina MiSeq in a smaller sample of female subjects, again observing robust pairwise correlation of the two CpGs ($r = 0.62$, $P = 5.7 \times 10^{-10}$) and significantly lower average methylation of the two sites with increasing age ($n = 77$, $P = 1.9 \times 10^{-2}$; *SI Appendix*, Fig. S2). Given the close proximity and consistent pairwise correlations between the two age-related *FKBP5* CpGs, all subsequent analyses examined the average methylation level of these two sites.

Age-Related Decrease in _FKBP5_ Methylation Is Not Confounded by Blood Cell Type Heterogeneity and Occurs in Purified Immune Cell Subtypes. Peripheral blood cell counts change along the lifespan (31), raising the possibility that heterogeneity in blood cell type composition could be confounding our results (32), despite the consistent inverse relation we observed between aging and *FKBP5* methylation after adjustment for calculated cell types in the regression models (Fig. 1*A* and Dataset S2). To rule out this possibility, we first performed a series of sensitivity analyses in our cohorts. The inverse relation between age and methylation of the two *FKBP5* CpGs (cg20813374 and cg00130530) was consistent across the GTP, KORA, and MPIP cohorts (all P values $<10^{-7}$; Fig. 1*A*), and there was no consistent relation between calculated blood cell subtypes (the potential confounder) and either age or *FKBP5* methylation levels (our variables of interest), suggesting that strong confounding by cell subtypes was not present (Dataset S3). This was further validated using an additional dataset of male and female subjects ($n = 213$) with both 450K data and differential complete blood counts; methylation of the age-related CpGs did not significantly correlate with any of the counted blood cell types (Dataset S3) and was again robustly associated with age after adjustment for sex and all cell types ($\beta = -0.0077$, $SE = 0.0009$, $P = 5.2 \times 10^{-15}$).

To further understand how aging influences *FKBP5* methylation in specific immune cell types, we analyzed publicly available DNA methylation data in whole blood, as well as FACS-sorted CD4 T cells and neutrophils, from male subjects with a broad age range (33). We again observed an inverse relation between age and methylation of the two *FKBP5* CpGs in whole blood ($n = 184$, $r = -0.30$, $P = 3.6 \times 10^{-5}$). Importantly, the same effect size was present in purified CD4 T cells ($n = 46$, $r = -0.32$, $P = 3.3 \times 10^{-2}$), whereas this effect was in the same direction but nonsignificant in purified neutrophils ($n = 48$, $r = -0.20$, $P = 1.7 \times 10^{-1}$; *SI Appendix*, Fig. S3). Together, these findings show that increasing age is associated with lower *FKBP5* methylation in T cell (and likely other distinct immune cell) subtypes and that this effect is not solely the result of age-related changes in blood cell type composition.

Early Life Stress and Depressive Phenotypes Accelerate the Age-Related Decrease in _FKBP5_ Methylation. *FKBP5* responds to stress and glucocorticoids and can undergo decrease in DNA methylation at distinct CpGs (26–28, 34, 35). Therefore, it is plausible that higher stress burden throughout life could induce lasting epigenetic changes, potentially accelerating decrease in methylation of the two age-related *FKBP5* CpGs. To investigate this hypothesis, we examined the combined effects of age and stress-related phenotypes

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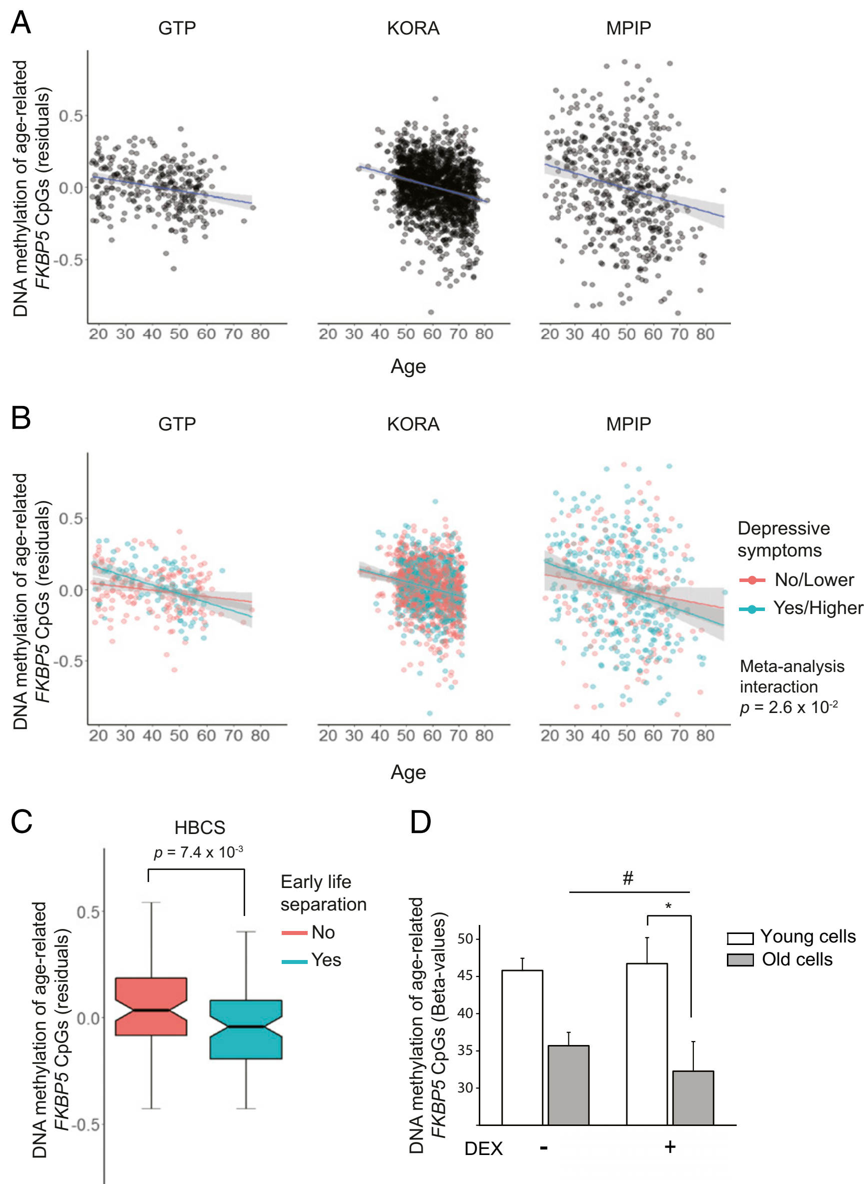

Fig. 1. Aging and stress are together associated with decreased DNA methylation at selected *FKBP5* CpGs. (*A*) Methylation decreases at selected *FKBP5* CpGs along the human lifespan (GTP: $\beta_{age} = -0.0045$, SE = 0.0008, $P = 8 \times 10^{-8}$; KORA: $\beta_{age} = -0.0055$, SE = 0.0005, $P < 2 \times 10^{-16}$; MPIP: $\beta_{age} = -0.0064$, SE = 0.0012, $P = 7 \times 10^{-8}$; total n = 2,523). (*B*) Depressive phenotypes are associated with accelerated age-related decrease in *FKBP5* methylation (total n = 2,249, meta-analysis interaction $P = 2.6 \times 10^{-2}$, heterogeneity $P = 2.7 \times 10^{-1}$). Statistics per cohort: GTP: interaction $P = 1.9 \times 10^{-2}$, β_{age} for moderate/severe depression = −0.0075 (SE = 0.0014) vs. β_{age} for no/mild depression = −0.0032 (SE = 0.0011); KORA: interaction $P = 6.3 \times 10^{-1}$, β_{age} for higher levels of depression = −0.0063 (SE = 0.0011) vs. β_{age} for lower levels of depression = −0.0047 (SE = 0.0007); MPIP: interaction $P = 1.9 \times 10^{-1}$, β_{age} for depressed = −0.0077 (SE = 0.0015) vs. β_{age} for nondepressed = −0.0044 (SE = 0.0019). (*C*) Early life separation is associated with lower methylation of the age-related *FKBP5* CpGs in the HBCS ($\beta_{separation} = -0.0932$, SE = 0.0343, $P = 7.4 \times 10^{-3}$, mean DNA methylation difference = 1.4%). The *y* axis in *A*, *B*, and *C* depicts the residuals of the average DNA methylation levels (M-values) of the two age-related *FKBP5* CpGs (cg20813374 and cg00130530) and reported statistics are after adjustment for all covariates for each cohort (*SI Appendix, Supplementary Methods*); for a more intuitive visualization, selected panels are also depicted as percent DNA methylation (Beta-values) in *SI Appendix*, Fig. S1. (*D*) In vitro aging and exposure to the stress hormone (glucocorticoid) receptor agonist DEX additively decrease methylation at the age-related *FKBP5* CpGs in the IMR-90 fibroblast model of replicative senescence ($F_{1,6} = 6.3$, interaction $P = 4.6 \times 10^{-2}$, n = 4 replicates per group). Statistical comparisons were performed with two-way mixed-design ANOVA (per experimental design), using replicative age as the between-subject and DEX treatment as the within-subject factor. Statistically significant effects were followed with Bonferroni-corrected pairwise comparisons, shown as follows: $^{*}P < 5 \times 10^{-2}$, statistically significant pairwise comparisons for young vs. old replicative age; $^{\#}P < 5 \times 10^{-2}$, statistically significant pairwise comparison for vehicle vs. DEX-treated old cells. Error bars depict the SE around the group mean. The *y* axis in *D* depicts the average percent DNA methylation of the two *FKBP5* CpGs.

on average methylation of the two CpGs. As information on current depressive symptoms was available in all three cohorts, we first investigated this phenotype. After adjusting for all covariates (*SI Appendix, Supplementary Methods*), depressive phenotypes consistently accelerated the age-related decrease in *FKBP5* methylation (total n = 2,249, meta-analysis interaction $P = 2.6 \times 10^{-2}$; Fig. 1*B*). This association remained significant after further adjusting for education, as a measure of socioeconomic status, in all cohorts (meta-analysis interaction $P = 3.7 \times 10^{-2}$). Because early life trauma is among the strongest risk factors for developing MDD (5), we further examined whether the effect of depression on age-related decrease in *FKBP5* methylation is moderated by childhood trauma

severity as measured with the childhood trauma questionnaire (CTQ) in the GTP. This stratified analysis yielded a significant age–depression interaction in the higher-CTQ (interaction $P = 4.6 \times 10^{-2}$) but not the lower-CTQ group (interaction $P = 3.3 \times 10^{-1}$) and no main effect of childhood trauma severity ($P = 3.7 \times 10^{-1}$). Finally, to examine whether exposure to a severe and prolonged early childhood stressor itself is associated with lasting decrease in methylation of the age-related CpGs, we compared elderly individuals that had prolonged early life separation from their parents with sex- and age-matched nonseparated controls in a fourth cohort, the Helsinki Birth Cohort Study (n = 160, age range 58 to 69 y, demographics in Dataset S1). In this cohort, early life separation was associated with reduced methylation of the age-related CpGs ($P = 7.4 \times 10^{-3}$; Fig. 1C and *SI Appendix*, Fig. S1*B*).

These findings suggest that childhood trauma and depressive phenotypes together accelerate the age-related decrease in *FKBP5* methylation in peripheral blood.

The Effects of Both Aging and Stress on *FKBP5* Methylation Are Recapitulated in Vitro. The findings presented above identify two *FKBP5* CpGs (cg20813374 and cg00130530) that show a consistent association of lower methylation levels with aging and stress-related phenotypes; however, these findings are inherently limited by the use of human subjects where experimental manipulation is not feasible. To experimentally support these associations, we used a cellular model of replicative senescence (IMR-90 fibroblasts) to test whether replicative aging and stress–which is commonly modeled in the dish with the stress (glucocorticoid) hormone receptor agonist dexamethasone (DEX) (26, 36)–influences *FKBP5* methylation at these sites. Population doubling level (PDL) was calculated as previously (37), and *FKBP5* methylation was measured with targeted bisulfite pyrosequencing and compared between cells of young (PDL = 22) and old (PDL = 42) replicative age treated for 7 d with either vehicle (DMSO) or 100 nM DEX. In accordance with our in vivo findings, in vitro aging and DEX additively decreased DNA methylation at the two *FKBP5* CpGs (interaction $P = 4.6 \times 10^{-2}$, DNA methylation decrease in old vs. young cells = 10.1%, and additional methylation decrease in old cells treated with DEX vs. vehicle = 3.4%; Fig. 1*D*). Together with our observations in human cohorts (Fig. 1 *A*–*C*), these convergent findings show that aging and stress may influence *FKBP5* methylation across different cohorts, distinct cell types, and contexts.

Decreased Methylation at the Age/Stress-Related *FKBP5* CpGs Is Associated with *FKBP5* Up-Regulation in Peripheral Blood. DNA methylation patterns can shape gene expression, thereby contributing to cellular function and phenotypic expression (38, 39). The age/stress-related *FKBP5* CpGs identified above lie proximally (<500 bp) upstream of the TSS for all highly expressed isoforms of *FKBP5* and are intronic only for the minimally expressed variant 2 of the gene (UCSC Genome Browser, UCSC Gens Track; GTEx portal). Integrative analysis of chromatin states using ChromHMM (40) showed that the two CpGs colocalize with signatures that are consistent with either enhancers or flanking active TSS in a large number of cell types (Dataset S4). In immune cells, the CpGs are commonly mapped to either an enhancer or flanking active TSS (*SI Appendix*, Fig. S4). Further, in most cell types the two sites show intermediate levels of methylation and colocalize with H3K4me1 and H3K27me3 signatures (Roadmap Epigenome Browser; shown for immune cell proxy in *SI Appendix*, Fig. S5). This landscape is most consistent with a poised enhancer (41) that upon transcription factor binding could interact and regulate the downstream *FKBP5* TSS.

To examine whether DNA methylation of these CpGs influences gene transcription, we used *FKBP5* mRNA data measured in the GTP cohort with Illumina HumanHT-12 Expression BeadChip arrays (n = 355). DNA methylation levels of the age/stress-related sites were inversely associated with *FKBP5* mRNA levels ($P = 1.6 \times 10^{-2}$; Fig. 2*A*). We found similar negative correlations in publicly available data from breast tissue samples of control female subjects (n = 84, r = −0.26, $P = 1.6 \times 10^{-2}$; *SI Appendix*, Fig. S6) (42). Since *FKBP5* transcription is robustly induced by glucocorticoids (28, 43) and given that the CpGs are located in predicted poised enhancers (*SI Appendix*, Fig. S5), we speculated that methylation at the age/stress-related *FKBP5* CpGs could moderate the effect of cortisol on *FKBP5* levels. After confirming a robust positive correlation between cortisol and *FKBP5* mRNA (r = 0.41, $P = 1 \times 10^{-12}$), we found that the cortisol–*FKBP5* relationship was significantly stronger in individuals with below- compared with above-median methylation levels in the GTP (interaction $P = 1.4 \times 10^{-3}$; Fig. 2*B*). In addition, the phenotypes associated with lower methylation levels moderated the relationship between cortisol and *FKBP5* mRNA; specifically, this relationship was significantly stronger in older subjects as defined with a median split of age (interaction $P = 2.4 \times 10^{-5}$; Fig. 2*C*) and in individuals with higher severity of depression and childhood trauma (interaction $P = 7.3 \times 10^{-5}$; Fig. 2*D*). These findings are in line with previous observations that stressors can induce lasting epigenetic effects on other sites of the *FKBP5* locus (26–28) and suggest that the effects of aging and stress converge at distinct CpGs to epigenetically up-regulate *FKBP5* in human blood.

***FKBP5* Up-Regulation Promotes NF-κB–Related Peripheral Inflammation and Chemotaxis.** To examine potential functional effects of *FKBP5* up-regulation in an unbiased manner, we used genome-wide gene expression data from peripheral blood in the GTP cohort (n = 355) to identify genes that are coregulated with *FKBP5*. After FDR correction for multiple comparisons (FDR-adjusted P < 0.05), *FKBP5* correlated significantly with a total of 3,275 genes (Dataset S5). Using these transcripts as input and the unique array genes expressed above background in blood (except *FKBP5*) as reference (9,538 genes), we performed pathway and disease association analysis in WebGestalt. The strongest enrichment was observed for inflammation and was conferred by a total of 123 inflammation-related genes (FDR-adjusted $P = 8.1 \times 10^{-6}$; Fig. 3*A* and Dataset S6). Notably, *FKBP5* showed robust positive relation with a host of proinflammatory genes, such as interleukin and toll-like receptors (Dataset S7). Furthermore, *FKBP5* levels were positively associated with the granulocyte proportion (r = 0.22, $P = 5.8 \times 10^{-5}$) and the granulocyte-to-lymphocyte ratio (r = 0.31, $P = 7.4 \times 10^{-9}$; *SI Appendix*, Fig. S7), an inflammation marker that is associated with increased cardiovascular risk and mortality (44, 45), but not with the proportions of CD4 T cells (r = −0.05, $P = 3.6 \times 10^{-1}$). These associations suggest that FKBP5-related inflammation could be driven by enhanced chemotaxis of granulocytes and other proinflammatory cells. As plausible mediator of this effect, we focused on interleukin-8 (IL-8), a major chemokine that recruits and activates granulocytes and other proinflammatory cells (46). Although FKBP5 down-regulation has been found to suppress IL-8 production in melanoma cells (19), no studies have examined whether FKBP5 up-regulation influences IL-8 secretion by immune cells. To address this possibility, we overexpressed *FKBP5* in Jurkat cells, a human T cell line that allowed efficient and reproducible transfection with *FKBP5* expression vectors (∼3.2-fold induction; Fig. 3*B*), and measured their potential to secrete IL-8. *FKBP5* overexpression nearly doubled IL-8 secretion upon immune stimulation ($P = 4.4 \times 10^{-7}$; Fig. 3*C*), supporting that increased *FKBP5* in T cells could drive chemotaxis of proinflammatory cells.

To further examine whether the effects of FKBP5 on the immune system may be driven by distinct transcription factors, we performed transcription factor target analysis in the GTP cohort using the same input and reference gene sets (3,275/9,538). The strongest enrichment was observed for NF-κB (FDR-adjusted

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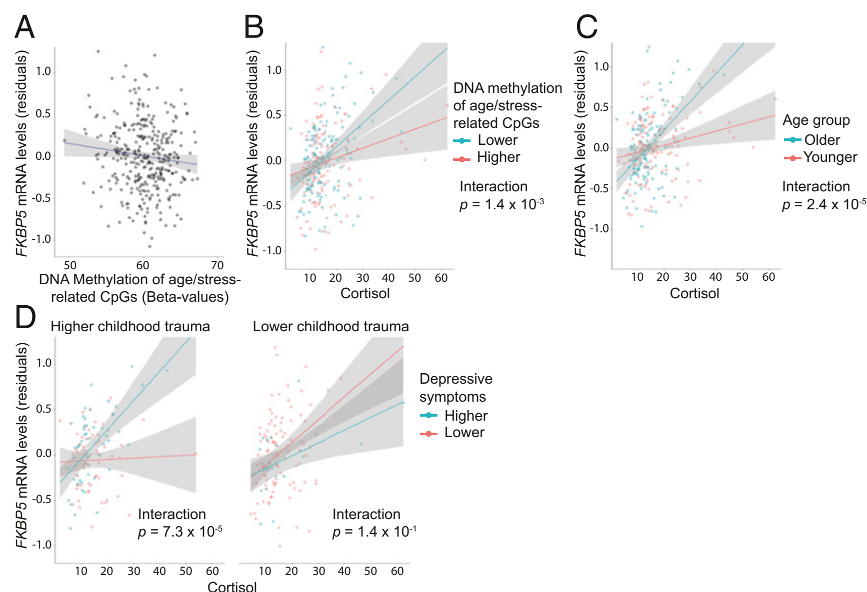

Fig. 2. Aging and stress-related phenotypes are associated with epigenetic up-regulation of *FKBP5* in peripheral blood in the GTP (n = 355). (*A*) *FKBP5* mRNA levels are negatively associated with average methylation of the age/stress-related CpGs ($\beta = -0.3835$, SE = 0.1585, $P = 1.6 \times 10^{-2}$). (*B* and *C*) The cortisol–*FKBP5* relationship is stronger at lower methylation levels of the age-related *FKBP5* CpGs: interaction $P = 1.4 \times 10^{-3}$, $\beta_{cortisol}$ for lower methylation = 0.0299 (SE = 0.0044) vs. $\beta_{cortisol}$ for higher methylation = 0.0069 (SE = 0.0039). The cortisol–*FKBP5* relationship is stronger in older ages: interaction $P = 2.4 \times 10^{-5}$, $\beta_{cortisol}$ for older subjects = 0.0376 (SE = 0.0050) vs. $\beta_{cortisol}$ for younger subjects = 0.0075 (SE = 0.0035). (*D*) Higher levels of depressive symptoms are associated with stronger cortisol–*FKBP5* relationship in subjects with higher levels of childhood trauma (cortisol–depression interaction $P = 7.3 \times 10^{-5}$) but not in subjects with lower levels of childhood trauma (cortisol–depression interaction $P = 1.4 \times 10^{-1}$). In all panels, *FKBP5* mRNA residuals are after controlling for all covariates (*SI Appendix*, *Supplementary Methods*).

$P = 4.5 \times 10^{-3}$; Fig. 3*A* and Dataset S8), a master immune regulator that has been linked to FKBP5 (13, 17), and this was driven by a total of 75 NF-κB gene targets (Fig. 3*A* and Dataset S9). To experimentally confirm that FKBP5 up-regulation promotes NF-κB signaling in immune cells, we performed dual-luciferase reporter assays comparing NF-κB activity between Jurkat cells overexpressing *FKBP5* and cells transfected with control vector. *FKBP5* overexpression led to increased NF-κB activity in response to immune stimulation ($P = 5.5 \times 10^{-3}$; Fig. 3*D*). Together these findings support that FKBP5 up-regulation in immune cells promotes NF-κB–dependent peripheral inflammation accompanied by the release of proinflammatory cytokines, such as IL-8. Therefore, our subsequent analyses sought to better characterize the mechanisms through which FKBP5 impacts the NF-κB pathway.

Changes in *FKBP5* Levels Are Associated with Extensive Alterations in the NF-κB Coexpression Network. To determine the network-level effects of FKBP5 deregulation on NF-κB signaling, we used the gene expression data in the GTP cohort (n = 355) to calculate the pairwise correlations between genes encoding molecules that directly interact within the NF-κB pathway, as defined in the KEGG Pathway Database. These pairwise correlations were adjusted for the expression levels of all other genes in the pathway and compared between subjects above vs. below the median split for *FKBP5* expression levels. As shown arithmetically in Dataset S10 and schematically in Fig. 3*E*, several partial pairwise correlations within the NF-κB pathway differed between the two groups, but the strongest and only significant effect after multiple test correction was noted for the *MAP3K14–CHUK* pair ($r_{lower\ FKBP5} = 0.13$ vs. $r_{higher\ FKBP5} = -0.28$, FDR-adjusted $P = 1.1 \times 10^{-2}$, permutation $P = 2.6 \times 10^{-3}$). This effect remained robust after controlling for sex, age, cortisol, and Houseman-calculated blood cell proportions (FDR-adjusted $P = 1.3 \times 10^{-4}$, permutation $P = 7.1 \times 10^{-3}$), indicating that the effects of *FKBP5* on NF-κB signaling are not confounded by cortisol levels or blood cell composition.

FKBP5 Up-Regulation Promotes NF-κB Signaling by Strengthening the Interaction of Key Regulatory Kinases. Since FKBP5 is involved in scaffolding of regulatory protein complexes, it could enhance NF-κB signaling by influencing protein–protein interactions between regulators of the NF-κB pathway. Intriguingly, *MAP3K14* and *CHUK*, the transcript pair most influenced by *FKBP5* levels (Fig. 3*E*), respectively encode the NF-kappa-B-inducing kinase (NIK) and the antagonist of nuclear factor kappa-B kinase subunit alpha (IKKα), two key regulatory kinases of the alternative NF-κB pathway. NIK interacts with and phosphorylates IKKα at serine 176 ($pIKK\alpha^{S176}$), thereby activating IKKα and facilitating NF-κB signaling (47, 48).

To examine whether FKBP5 modulates the NIK–IKKα protein complex, we performed a series of protein–protein binding experiments in human Jurkat cells and peripheral blood mononuclear cells (PBMC). These experiments showed binding of FKBP5 with both NIK and IKKα and binding between NIK and IKKα (Fig. 4*A*). We then examined whether glucocorticoid treatment and FKBP5 up-regulation can influence the FKBP5–NIK–IKKα complex. Both cell types were stimulated with DEX that robustly induces *FKBP5* expression (28, 43). After confirming the DEX-induced up-regulation of FKBP5 (∼2.2-fold), we found that DEX treatment significantly increased the binding between FKBP5, NIK, and IKKα in both Jurkat cells and PBMC; this increase was abolished by concomitant treatment with the selective FKBP5 antagonist SAFit1 (49) in both cell types (Fig. 4 *A* and *B*). Accordingly, these effects on protein binding were accompanied by an increase in $pIKK\alpha^{S176}$, whereas $pIKK\alpha^{S176}$ induction was abolished by treatment with SAFit1 (Fig. 4*C*). This effect on $pIKK\alpha^{S176}$ was recapitulated by *FKBP5* overexpression and again blocked by concomitant treatment with SAFit1 in Jurkat cells (Fig. 4*D*). Additionally, in this cell line deletion of the *FKBP5* gene with CRISPR/Cas9 abolished the effect of DEX on $pIKK\alpha^{S176}$ levels but did not influence vehicle-treated cells (*SI Appendix*, Fig. S8), thus mimicking the effects of SAFit1. In line with these functional effects on the NIK–IKKα complex, *FKBP5* overexpression nearly doubled NF-κB activity in Jurkat cells, whereas this effect was again prevented by concomitant treatment with SAFit1 (Fig. 4*E*). As schematically summarized in Fig. 4*F*, these convergent findings show that FKBP5 up-regulation strengthens NIK–IKKα binding, increases $pIKK\alpha^{S176}$, and in effect promotes NF-κB signaling.

NF-κB Signaling Promotes *FKBP5* Expression via an NF-κB Response Element Containing the Age/Stress-Related CpGs. Notably, the above-identified age/stress-related *FKBP5* CpGs flank an NF-κB response element (*SI Appendix*, Fig. S9), raising the possibility that NF-κB signaling could itself modulate *FKBP5* expression in immune cells via this site. To address this possibility, we performed

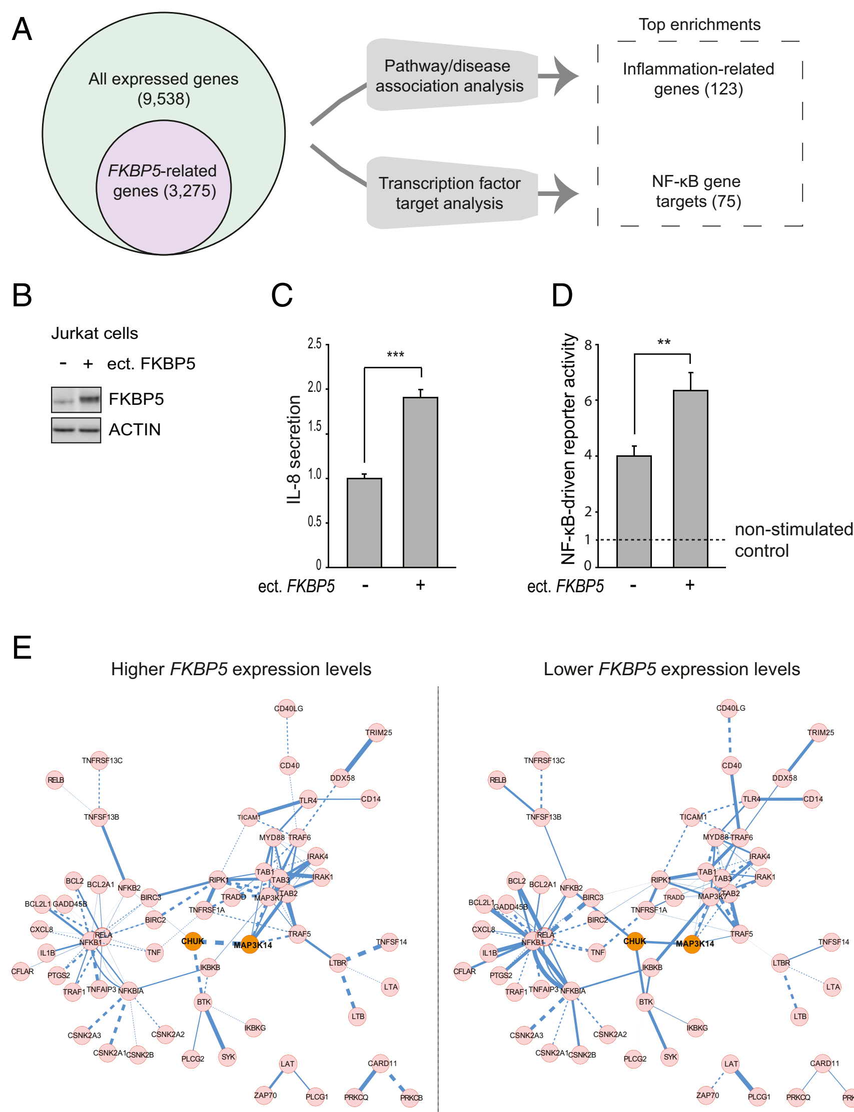

Fig. 3. *FKBP5* up-regulation promotes NF-κB–driven peripheral inflammation. (*A*) *FKBP5*-related genes in peripheral blood show enrichment for inflammation-related genes and NF-κB gene targets. The number of genes for each analysis is shown in parentheses. Statistical details are provided in Datasets S5–S9. (*B*) Western blotting confirming *FKBP5* overexpression in Jurkat T cells transfected with FKBP51-FLAG vs. cells transfected with the control vector. (*C*) *FKBP5* overexpression nearly doubles IL-8 secretion by Jurkat T cells stimulated overnight with 25 ng/mL of phorbol-12-myristate-13-acetate and 375 ng/mL of ionomycin (PMA/I). The bar graph depicts IL-8 secretion in stimulated cell supernatants measured with ELISA from two independent experiments ($t = 8.8$, $P = 4.4 \times 10^{-7}$, $n = 8$ per condition). For each experiment, fold ratios of IL-8 secretion were calculated relative to stimulated cells expressing the control vector. IL-8 was not detectable in nonstimulated cells. (*D*) *FKBP5* overexpression increases NF-κB activity in stimulated Jurkat T cells. The bar graph depicts NF-κB reporter activity in stimulated cells measured with dual-luciferase reporter assays from three independent experiments ($t = 3.2$, $P = 5.5 \times 10^{-3}$, $n = 9$ per condition). For each experiment, fold ratios of NF-κB activity were calculated relative to nonstimulated cells expressing the control vector. (*E*) *FKBP5* expression changes are associated with extensive alterations in the NF-κB coexpression network in peripheral blood. The circles depict genes encoding molecular partners of the NF-κB pathway. Continuous lines (edges) represent positive and dotted lines negative pairwise correlations corrected for expression levels of all other genes in the pathway (details in *SI Appendix, Supplementary Methods*). Edge widths are proportional to the absolute value of the respective correlation coefficient. The gene pair with the most robust difference in correlation between the two groups (*CHUK-MAP3K14*) is highlighted in orange. Statistical details for all gene pairs are provided in Dataset S10. Error bars depict the SE around the group mean. $^{**}P < 10^{-2}$; $^{***}P < 10^{-3}$.

dual-luciferase reporter gene assays using a CpG-free vector (50). We inserted into this vector the *FKBP5* sequence that surrounds the NF-κB response element and includes the two CpG sites of interest but completely lacks any other CpGs (*SI Appendix*, Fig. S9). Immune stimulation induced expression of this reporter construct in monocyte-derived human cell lines (THP-1) (Fig. 5*A*), thus supporting functionality of this response element in immune cells. Furthermore, in vitro DNA methylation of the age/stress-related *FKBP5* CpGs within this reporter construct resulted in statistically significant reduction (~40%) of baseline expression levels and nearly abolished the induction seen with immune stimulation (Fig. 5*A*). To further examine whether these functional effects are mediated by alterations in NF-κB binding, we used a biotinylated oligonucleotide-mediated chromatin immunoprecipitation (ChIP) method (51) (Fig. 5*B* and *SI Appendix*, Fig. S9). After confirming immune stimulation-driven NF-κB binding in THP-1 cells to the enhancer, in vitro DNA methylation essentially abolished NF-κB binding to the age/stress-related enhancer site (Fig. 5 *C* and *D*). Together, these findings demonstrate that NF-κB signaling—which, as we showed above, is promoted by FKBP5 (Figs. 3 and 4)—can in turn trigger *FKBP5* expression in immune cells, thereby forming a positive feedback loop that can potentiate FKBP5–NF-κB signaling.

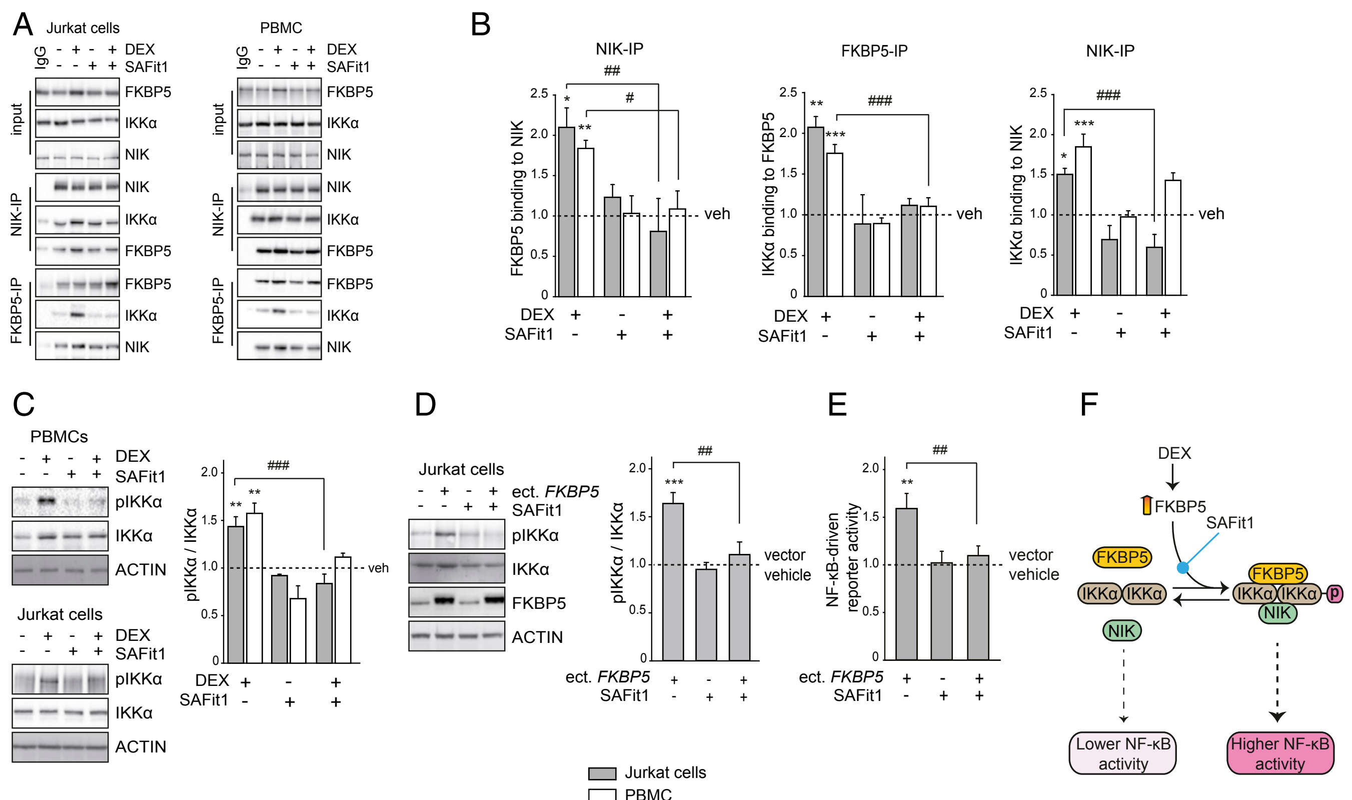

Fig. 4. FKBP5 up-regulation promotes NF-κB signaling by strengthening the binding of key regulatory kinases, and these effects are prevented by selective FKBP5 antagonism. (*A* and *B*) Immunoprecipitation (IP) for either FKBP5 or NIK followed by Western blotting and binding quantification in lysates from Jurkat cells or PBMC treated with the stress hormone (glucocorticoid) receptor agonist DEX, which robustly induces *FKBP5* expression, and/or the selective FKBP5 antagonist SAFit1. IgG: control IP without primary antibody. Jurkat: FKBP5 to NIK binding, DEX × SAFit1 $F_{1,8} = 9.3$, interaction $P = 1.6 \times 10^{-2}$; IKKα to FKBP5 binding, DEX × SAFit1 $F_{1,8} = 4.7$, interaction $P = 6.2 \times 10^{-2}$; IKKα to NIK binding, DEX × SAFit1 $F_{1,8} = 5.8$, interaction $P = 4.3 \times 10^{-2}$. PBMC: FKBP5 to NIK binding, DEX × SAFit1 $F_{1,8} = 5.7$, interaction $P = 4.4 \times 10^{-2}$; IKKα to FKBP5 binding, DEX × SAFit1 $F_{1,8} = 11.2$, interaction $P = 1 \times 10^{-2}$; IKKα to NIK binding, DEX × SAFit1 $F_{1,8} = 3.9$, interaction $P = 8.4 \times 10^{-2}$. $n = 3$ biological replicates per condition. (*C*) Western blotting of Jurkat cell and PBMC lysates ($n = 3$ replicates per condition) showing increase in the functional phosphorylation of IKKα at serine 176 (pIKKα) by DEX that is prevented by SAFit1 (Jurkat: DEX × SAFit1 $F_{1,8} = 12.9$, interaction $P = 7 \times 10^{-3}$; PBMC: DEX × SAFit1 $F_{1,8} = 0.6$, interaction $P = 4.6 \times 10^{-1}$). (*D*) Similar effects are observed when Jurkat cells are transfected with an expression construct encoding FKBP5 (ect. *FKBP5*) and treated with SAFit1 (ect. *FKBP5* × SAFit1 $F_{1,12} = 6.6$, interaction $P = 2.5 \times 10^{-2}$, $n = 4$ replicates per condition). (*E*) *FKBP5* overexpression increases NF-κB activity in Jurkat cells stimulated overnight with 25 ng/mL of phorbol-12-myristate-13-acetate and 375 ng/mL of ionomycin, and this increase is prevented by concomitant treatment with SAFit1 (ect. *FKBP5* × SAFit1 $F_{1,32} = 4.5$, interaction $P = 4.2 \times 10^{-2}$, $n = 9$ replicates per condition). NF-κB reporter activity was measured with dual-luciferase reporter assays in three independent experiments. (*F*) Scheme summarizing the results from protein–protein binding and reporter gene experiments. All treatments with DEX and SAFit1 were performed for 24 h at 100 nM concentration. All data are shown as fold changes compared with the control-vector vehicle-treated cells. All statistical comparisons were performed with two-way ANOVA, using either DEX treatment or *FKBP5* overexpression as the first factor and SAFit1 treatment as the second factor. Statistically significant effects were followed with Bonferroni-corrected pairwise comparisons, shown as follows: $^{*}P < 5 \times 10^{-2}$, $^{**}P < 10^{-2}$, $^{***}P < 10^{-3}$, statistically significant pairwise comparisons for control vs. DEX or ect. *FKBP5*; $^{\#}P < 5 \times 10^{-2}$, $^{\#\#}P < 10^{-2}$, $^{\#\#\#}P < 10^{-3}$, pairwise comparisons for vehicle vs. SAFit1 treatment (shown only for significant interaction terms of the respective two-way ANOVAs). Error bars depict the SE around the group mean.

This positive feedback can thus be accentuated by decreased methylation of the NF-κB–responsive *FKBP5* enhancer, which can occur as a consequence of aging and stress.

Age/Stress-Related Decrease in *FKBP5* Methylation Is Associated with a History of Acute MI. Proinflammatory states confer risk for cardiovascular disease, most notably acute cardiovascular syndromes (52). Thus, the convergent findings presented above, indicating that lower methylation of the age/stress-related *FKBP5* CpGs up-regulates *FKBP5*, which in effect promotes peripheral inflammation, prompted us to examine whether this lower methylation signature is also associated with higher risk for acute coronary events. To address this possibility, we used data on self-reported history of MI that were available in both the KORA (1,648 subjects without vs. 62 subjects with history of MI) and the MPIP (310 controls vs. 8 cases) cohorts. Given the small number of subjects with MI as compared without MI in our cohorts, we used a statistical approach that resolves case–control imbalances by calculating and controlling for propensity scores for the MI assignment (53). After adjustment for all covariates (*SI Appendix*, *Supplementary Methods*), methylation of the age-related sites was significantly lower in individuals with history of MI in both cohorts (KORA: $\beta = -0.0535$, $P = 7.9 \times 10^{-3}$; MPIP: $\beta = -0.1992$, $P = 1.2 \times 10^{-3}$; Fig. 6*A*). This association remained significant after further controlling for education in both the KORA ($P = 4.7 \times 10^{-3}$) and MPIP ($P = 2.6 \times 10^{-5}$).

Discussion

Aging and stress-related phenotypes are associated with heightened inflammation and cardiovascular risk (5, 8–11), but the underlying molecular mechanisms remain elusive. Here we uncover a role for FKBP5 in these relations. As schematically summarized in Fig. 6*B*, our findings suggest that aging and stress synergistically decrease DNA methylation at selected regulatory *FKBP5* CpGs that moderate the efficiency of an NF-κB–responsive enhancer. Reduced methylation at this site enhances

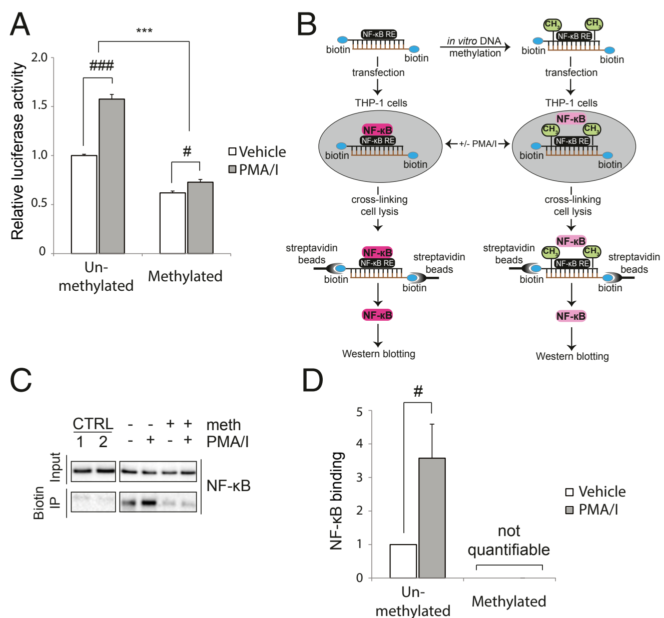

Fig. 5. NF-κB signaling drives *FKBP5* expression via a response element gated by the age/stress-related CpGs. (*A*) Data from dual-luciferase reporter gene assays using a CpG-free luciferase reporter construct, which includes the *FKBP5* sequence surrounding the NF-κB response element and the age/stress related CpGs (insert sequence shown in *SI Appendix*, Fig. S9) but completely lacks other CpGs. This reporter construct was in vitro-methylated and transfected into monocyte-derived human cell lines (THP-1). Cells were then stimulated overnight with 25 ng/mL phorbol-12-myristate-13-acetate and 375 ng/mL ionomycin (PMA/I), a combination that robustly induces NF-κB signaling. Data are derived from two independent experiments (n = 12 replicates per condition). Comparison was performed using two-way ANOVA with methylation and treatment as factors ($F_{1,44} = 59.5$, interaction $P < 10^{-3}$), and statistically significant effects were followed with Bonferroni-corrected pairwise comparisons. (*B*–*D*) The effect of in vitro DNA methylation on PMA/I-induced NF-κB binding to the NF-κB response element was examined using biotinylated oligonucleotide-mediated ChIP in THP-1 cells (oligonucleotide sequence shown in *SI Appendix*, Fig. S9). Schematic summary of the experimental setup is shown in *B*. After ChIP, NF-κB/p65 binding was quantified by Western blotting using antibodies specific for NF-κB (*C*: example blots; *D*: quantifications). CTRL (control) 1: magnetic beads lacking conjugated streptavidin; CTRL 2: cells transfected with nonbiotinylated oligonucleotide. Bar graph shows data derived from four independent experiments ($t = 2.5$, $P = 4.4 \times 10^{-2}$, n = 4 per condition). Statistical t test compared cells carrying the unmethylated probe treated overnight with vehicle or PMA/I. Binding was not quantifiable for cells carrying the methylated probe. Data are always shown as fold changes compared with the vehicle-unmethylated cells. Error bars depict the SE around the group mean. P values for pairwise comparison are shown as follows: $^{***}P < 10^{-3}$, statistically significant pairwise comparisons for methylated vs. unmethylated. $^{\#}P < 5 \times 10^{-2}$; $^{\#\#\#}P < 10^{-3}$, statistically significant pairwise comparisons for vehicle vs. drug treatment.

FKBP5 responses in immune cells, an effect that promotes NF-κB–driven peripheral inflammation, in part through protein–protein interactions between FKBP5 and key regulatory kinases of the NF-κB pathway. NF-κB binding to the *FKBP5* enhancer can in turn stimulate *FKBP5* expression, thereby forming a positive feedback loop of FKBP5–NF-κB signaling that potentially contributes to proinflammatory states and heightened cardiovascular risk. Finally, we find that both CRISPR/Cas9 deletion of *FKBP5* and treatment with a selective FKBP5 antagonist can prevent the cellular effects of stress and FKBP5 up-regulation on NF-κB signaling.

By interrogating all 450K-covered *FKBP5* CpGs for age-related changes in DNA methylation, a biological process thought to contribute to disease states (25), we identified two closely juxtaposed sites at which CpG methylation decreases through an interplay of aging, early life stress, and depressive symptoms. These findings were replicated in both individuals of European descent and African Americans collectively from seven independent human cohorts (total $n > 3{,}000$) with whole-blood DNA methylation data. Further analyses in purified blood cells showed that age-related decrease in *FKBP5* methylation occurs in CD4 T cells but not in neutrophils, suggesting that aging may affect *FKBP5* methylation differentially across immune cell types. Notably, depressive phenotypes had consistent effects on *FKBP5* methylation in all analyses, whereas the impact of early life stress differed across cohorts, directly influencing methylation in the HBCS but only moderating the epigenetic effects of depression in the GTP. This difference may reflect the more homogeneous stressor as well as the older and narrower age composition in the HBCS. Despite this difference, the impact of both early life stress and depression consistently occurred in the same direction, decreasing *FKBP5* methylation in older ages. Together with previous work (22–24, 26–29), these findings suggest that the epigenetic influence of environmental

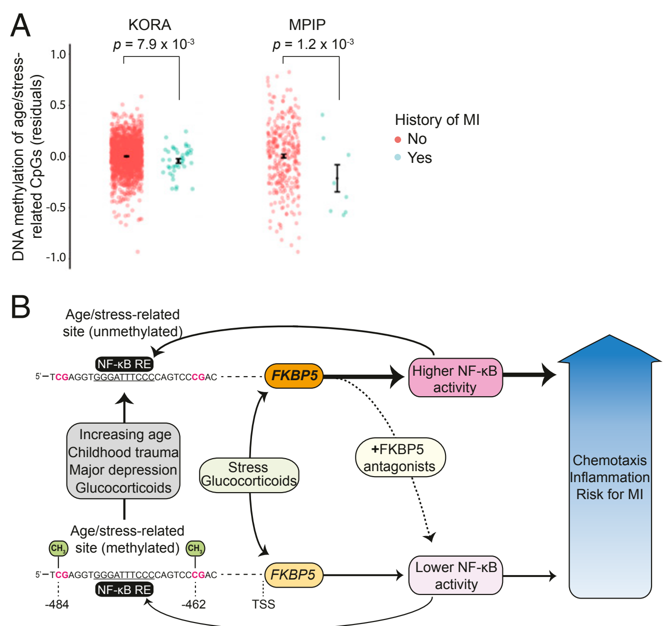

Fig. 6. Association of age/stress-related *FKBP5* decrease in DNA methylation with a history of MI and overall scheme summarizing study findings. (*A*) Age/stress-related decrease in *FKBP5* methylation is associated with a history of MI in two independent cohorts: KORA, n = 1,648 subjects without vs. 62 with history of MI, $\beta_{MI} = -0.0535$, SE = 0.0201, $P = 7.9 \times 10^{-3}$, mean DNA methylation difference = 1.8% and MPIP, n = 310 subjects without vs. 8 with history of MI, $\beta_{MI} = -0.1992$, SE = 0.0611, $P = 1.2 \times 10^{-3}$, mean DNA methylation difference = 5.3%. The y axis depicts average DNA methylation levels of the two age/stress-related *FKBP5* CpGs (cg20813374 and cg00130530), after adjusting for all covariates (*SI Appendix*, *Supplementary Methods*). Error bars depict the SE around the group mean. (*B*) Schematic summary of study's findings showing how aging, childhood trauma, and depressive symptoms interact to decrease *FKBP5* methylation at selected CpGs (cg00130530 and cg20813374) located proximally upstream of the TSS. These epigenetic changes enhance *FKBP5* responses in immune cells, an effect that in turn promotes NF-κB signaling, whereas this is prevented when cells are concomitantly treated with selective FKBP5 antagonists. Notably, NF-κB signaling is not only activated by FKBP5 but it can also trigger *FKBP5* transcription through an NF-κB response element that is flanked and moderated by the age/stress-related CpGs. This forms a positive feedback loop of FKBP5–NF-κB signaling that may be enhanced in individuals with lower methylation at this site. Enhanced *FKBP5* responses and NF-κB activity may in turn promote chemotaxis of proinflammatory cells and peripheral inflammation, potentially contributing to cardiovascular risk.

exposures and life stress may become more evident as life advances. Using an in vitro model of replicative senescence, we further show that replicative aging and stress hormone (glucocorticoid) exposure additively decrease methylation at the same age/stress-related CpGs. Although cellular senescence and glucocorticoid exposure in a dish have limitations as models of in vivo processes, our convergent findings in humans and cells suggest that aging and stress synergistically influence selected *FKBP5* CpGs across different cohorts, distinct cell types, and contexts.

Epigenetic effects involving *FKBP5* have been previously reported to occur in intronic glucocorticoid response elements, possibly as a result of glucocorticoid receptor binding to the DNA (26, 28, 54); here we identified lower methylation levels at two CpGs that include a functional NF-κB response element site and colocalize with a poised enhancer within 500 bp upstream of the *FKBP5* TSS in most immune cells, including CD4 T cells, the cell type that shows age-related decrease in *FKBP5* methylation. This functional annotation and our human cohort and in vitro data together support a model whereby age/stress-related decrease in *FKBP5* methylation can enhance transcription factor binding and consequent *FKBP5* responses in distinct immune cell types. The cross-sectional nature of our cohort data precludes firm conclusions about causal directions, but we speculate that decreased methylation at the age/stress-related *FKBP5* sites may additively result from the combined effects of cellular aging, repeated activation of the enhancer, and stress-induced *FKBP5* transcription along the human lifespan. Although the overall effect size of these DNA methylation changes is modest, small effect sizes in a complex tissue, such as whole blood, likely reflect the mean of variable effects across distinct cell types. This is suggested by the larger age-related decrease in *FKBP5* methylation in T cells than neutrophils in the FACS-sorted dataset (*SI Appendix*, Fig. S3). Another limitation of the 450K array is its sparse coverage of CpGs present in the human genome. Future studies may uncover additional *FKBP5* sites of interest by employing methods that allow more comprehensive coverage, such as targeted bisulfite sequencing (55).

Through a combination of unbiased network analyses in human cohorts and mechanistic investigations in immune cells, we characterized a multilevel positive regulatory feedback between the stress-responsive cochaperone FKBP5 and the NF-κB signaling cascade. More specifically, FKBP5 was found to exert pronounced effects on NF-κB–related gene networks and to promote NF-κB signaling by strengthening the interactions between NIK and IKKα, two key regulatory kinases of the NF-κB pathway. These findings are congruent with previous observations that FKBP5 down-regulation can inhibit NF-κB signaling (13, 15, 17–19) and show that FKBP5 interacts with NIK and mediates the glucocorticoid-driven modulation of the NIK–IKKα regulatory complex in immune cells. Intriguingly, NF-κB can in turn trigger *FKBP5* transcription through an NF-κB response element that is flanked and moderated by the age/stress-related CpGs, thereby forming a positive feedback loop that can potentiate FKBP5–NF-κB signaling, especially in individuals with lower methylation at these *FKBP5* CpGs. Both CRISPR/Cas9 deletion of the *FKBP5* gene and treatment with the selective FKBP5 antagonist SAFit1 prevent the cellular effects of stress, as modeled in vitro by stress hormone treatment, and *FKBP5* overexpression on NF-κB signaling. In contrast, as shown both here and in a previous study (49), SAFit1 does not influence immune function under baseline conditions, suggesting that FKBP5 antagonism may represent a pharmacological intervention that–if targeted at individuals with up-regulated *FKBP5*–could prevent some of the unwarranted age/stress-related alterations in immune function. However, the potential in vivo relevance of the pharmaceutical modulation of FKBP5–NF-κB signaling will need to be tested in future studies.

We also find convergent evidence that FKBP5 promotes inflammation, a biological process tightly linked with NF-κB signaling. This effect may in part result from the enhanced chemotaxis and recruitment of proinflammatory cells, a possibility supported by the positive association of *FKBP5* mRNA levels with the granulocyte-to-lymphocyte ratio and the ability of FKBP5 to augment immune cell secretion of the major chemokine, and NF-κB target, IL-8 by Jurkat cells, which is a T cell line. The latter finding extends a previous study showing that *FKBP5* down-regulation suppresses NF-κB–mediated production of IL-8 in melanoma cells (19). Both IL-8 levels and the granulocyte-to-lymphocyte ratio are inflammatory markers associated with heightened cardiovascular risk and mortality (45, 56, 57). Together these findings suggest that older individuals with higher stress burden, who show exaggerated *FKBP5* responses, are also more prone to developing heightened inflammation and acute cardiovascular risk upon stress exposure. This hypothesis is supported by our observation, in two independent cohorts, that history of MI is associated with decreased methylation at the age/stress-related *FKBP5* CpGs. *FKBP5* up-regulation could thus represent one molecular link for the known association of depression and early life adversity with heightened inflammation and cardiovascular risk (3, 4, 6, 58, 59). Nevertheless, the association of MI with decreased *FKBP5* methylation should be interpreted with caution until further validation in larger, independent studies with more balanced case–control assignment. Furthermore, mechanistic dissection of the potential role of FKBP5 in cardiovascular risk will require longitudinal studies examining the convergent effects of stress and aging in purified immune cell types.

In conclusion, our findings suggest that aging and stress decrease DNA methylation at selected enhancer-related *FKBP5* sites, contributing to epigenetic up-regulation of FKBP5 in immune cells, increased NF-κB–driven peripheral inflammation, and heightened cardiovascular risk. While disease risk is undoubtedly shaped by multiple molecular effectors and mechanisms, the present study offers insights by uncovering a mechanism through which aging and stress confer disease risk at the molecular level. Such molecular insights may help identify biomarkers and novel treatment candidates for stress-related disease and will require orchestrated translational research efforts. Despite these intriguing possibilities, social policies should always strive to ameliorate or, when possible, prevent excessive psychosocial stress in the first place.

Materials and Methods

Given space constraints in the main text, selected methods are provided here and further details can be found in *SI Appendix*, *Supplementary Methods*. The effects of aging and stress-related phenotypes on *FKBP5* DNA methylation were examined in the GTP, KORA, MPIP, and HBCS cohorts (60, 61). CpG methylation across the *FKBP5* locus was measured with the Infinium HumanMethylation450 BeadChip, and identified CpGs were further validated with targeted bisulfite sequencing. CpG annotation was performed with the UCSC Genome Browser and the Roadmap Epigenome Browser. Genome-wide gene expression data were measured in the GTP using Illumina HumanHT-12 v3 and v4 Expression BeadChips (62). Enrichment analysis for disease-associated genes and for gene targets of transcription factors was performed using WebGestalt. For NF-κB coexpression network analyses, pathway data were derived from the KEGG Pathway Database, and gene pair correlation coefficients were adjusted using the R package GeneNet. Cell culture experiments were performed in PBMC from healthy donors, as well as Jurkat, THP-1, or IMR-90 cell lines. *FKBP5* knockout Jurkat cells were generated using CRISPR/Cas9 plasmids containing gRNA that targets human *FKBP5* and a GFP reporter. The functional effect of DNA methylation at the age/stress-related *FKBP5* site was analyzed with dual-luciferase assays using a CpG-free luciferase reporter construct (50). Statistical analyses were performed with Sigma Plot version 13.0 (for experimental data) or R version 3.1.0 (for all other data).

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