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Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity

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Abstract

The ability of the human immune system to respond to vaccination declines with age. We identified an age-associated defect in T cell receptor (TCR)-induced ERK phosphorylation in naïve $CD4^+$ T cells ($P<0.0001$) while other signals, such as ZAP70 and PLC-γ1 phosphorylation were not impaired. The defective ERK signaling was caused by the dual specific phosphatase (DUSP) 6 whose protein levels increased with age ($r = 0.68$, $P < 0.0001$) due to a decline in repression by miR-181a ($r = -0.59$, $P < 0.0001$). Reconstitution of miR-181a lowered DUSP6 levels in naïve $CD4^+$ T cells in elderly individuals. DUSP6 repression with miR-181a or specific siRNA, and DUSP6 inhibition with the allosteric inhibitor (*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one improved $CD4^+$ T cell responses as seen by increased expression of activation markers, improved proliferation and supported preferential T_H1 differentiation. DUSP6 is a potential intervention target for restoring T cell responses in the elderly, which may augment the effectiveness of vaccination.

With increasing age, the ability of the human immune system to protect against new antigenic challenges or to control chronic infections declines[1,2]. More than 90% of all influenza-related deaths in the United States occur in the elderly[3] where the mortality and morbidity with infections is high, and the response to vaccinations is diminished[4,5].

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AUTHOR CONTRIBUTIONS

GL, MY, WL, CMV and JJG designed experiments and analyzed data. GL, MY and WL generated data. MT provided BCI and advice in the design of the DUSP6 inhibition experiments. EK recruited and clinically phenotyped healthy elderly individuals. GL and JJG wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

There are many putative mechanisms that can explain age-related decrements in adaptive immunity. Declining thymic function has frequently been implicated[6–8], but in spite of thymic demise, declines in naïve CD4$^+$ T cell compartment sizes with age are subtle[9]. Moreover, TCR diversity within the naïve CD4$^+$ T cell compartment in 60–65 year-old individuals is not different from that in 20–30 year-old individuals and the diversity only contracts late in life[10,11]. Thus, individuals maintain a diverse repertoire of naïve CD4$^+$ T cells which should endow them with the competence to respond to antigenic challenges or vaccinations into their eighth decade of life.

In mice, an increasing lifespan of naïve CD4$^+$ T cells with age has been associated with the emergence of functional defects[12]. TCR signaling is substantially and globally impaired, preferentially affecting the ability of naïve CD4$^+$ T cells to produce interleukin (IL)-2. Human CD4$^+$ naïve T cells are also affected by age, in particular homeostatic proliferation results in a substantial cumulative telomeric loss that impairs proliferative responses[13,14]. In contrast to mice, global age-dependent changes in human TCR signaling can generally be attributed to expanded effector cell populations that have lost CD27 and CD28 and gained numerous regulatory cell surface molecules[15]. Major signaling defects, such as the lack of calcium influx observed in mouse naïve CD4$^+$ T cells, are not seen in humans and any signaling alterations are subtle and dependent on signal strength[16].

Over the last decade, signal threshold calibration after TCR stimulation has been increasingly defined, and many settings have been identified where signal strength determines remarkably different functional outcomes, ranging from positive and negative selection in thymic development to recognition of agonistic and antagonistic peptides in peripheral T cells and differentiation into effector cell lineages[17]. Among the molecular components calibrating TCR-induced signal strength, feedback loops involving ERK activity play a critical role[18]. TCR threshold calibration is most evident in the differentiation from thymocytes that are selected on self-antigenic peptides, to mature T cells that are no longer responsive to self. Increased expression of several phosphatases including the dual specific phosphatases (DUSP)5 and 6 has been implicated in this loss of TCR sensitivity[19,20]. DUSPs represent a family of phosphatases that dephosphorylate phosphothreonine and phosphotyrosine residues on MAPK and that are pivotal regulators of MAPK activities[21,22].

Here we report that naïve CD4$^+$ T cells from elderly individuals have a reduced signaling potential of the ERK pathway. The underlying mechanism is an age-related decline in miR-181a expression and associated rise in levels of DUSP6. The higher levels of DUSP6 raise the threshold for productive T cell activation by dampening the initial ERK signal after TCR stimulation. Accordingly, lower fractions of naïve CD4$^+$ T cells are activated in the elderly, in particular to low affinity stimuli. Our studies suggest that DUSP6 inhibition may be a potential intervention to improve T cell responses in the elderly.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

ONLINE METHODS

Cells

We obtained peripheral blood from 357 individuals (aged 20–85 years). Subjects with acute diseases, current or previous history of immune-mediated diseases or cancer except limited basal cell carcinoma or chronic diseases not controlled on oral medications were excluded. In all experiments and for each age group, we included an approximate equal number of male and female subjects. The study was approved by the Emory and Stanford Institutional Review Boards, and all participants gave written informed consent. To isolate naïve $CD4^+$ T cells from PBMC, memory T cells and $CD14^+$ monocytes were depleted by anti-CD45RO and anti-CD14 magnetic microbeads (Miltenyi Biotec Inc.). $CD4^+$ cells were then positively isolated. Purity was >95%. Memory $CD4^+$ T cells were isolated by depleting PBMC of $CD45RA^+$ and $CD14^+$ cells followed by positive selection for CD4. In some experiments, $CD4^+$ T cells were purified from whole blood or PBMC with a $CD4^+$ T cell enrichment cocktail kit (STEMCELL Technologies Inc.) followed by positive selection of naïve T cells with anti-CD45RA magnetic microbeads. Dendritic cells (DCs) were generated from $CD14^+$ monocytes by culture with 800 U ml^{-1} GM-CSF and 1,000 U ml^{-1} IL-4 (R&D Systems) for six days, and matured with 1,100 U ml^{-1} TNF-α (R&D Systems) and 1 μg ml^{-1} PGE2 (Sigma) for 24 h.

T cell–DC coculture

Naïve $CD4^+$ T cells (25×10^3 cells) labeled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) were cultured with 0.5×10^3 DC and indicated concentration of toxic shock syndrome toxin 1 (TSST-1, Toxin Technology). Cell and TSST-1 concentrations were optimized to minimize alloreactive responses (Supplementary Fig. 1) and activate approximately 90% of $V\beta2^+$ cells in young individuals. We determined the fraction of $V\beta2^+$ or $V\beta2^-$ $CD4^+$ naïve T cells that had divided on day 4 after stimulation. CD69 and CD25 expression in $V\beta2^+$ $CD4^+$ T cells was examined at 6, 12, 24, and 36 h postactivation by staining with anti-CD69 PE-Cy7 and anti-CD25-APC (all from BD Biosciences). In some experiments, we used $CD4^+$ T cells transfected with miR-181a precursor or DUSP6 siRNA and determined proliferative response by ^{3}H-thymidine incorporation and IL-2 production.

Signaling studies

T cells negatively isolated by Human T Cell Enrichment Cocktail (STEMCELL Technologies Inc.) were stimulated with anti-CD3 (1 μg ml^{-1}) cross-linking or PMA (0.5 ng ml^{-1}) at 37°C, fixed in BD Cytofix buffer, permeabilized by BD Perm Buffer III (for ERK and PLC-γ1) or II (for ZAP70), and stained with the following antibodies: anti-CD3-APC-Cy7, anti-CD4-PerCP, anti-CD8-PE, anti-CD45RA-PE-Cy7, and Alexa Fluor 647-conjugated anti-phospho-ERK1/2 (pT202/pY204), anti-phospho-ZAP70 (pY319)/SYK (pY352) or anti-phospho-PLC-γ1 (pY783) (all from BD Biosciences). Phosphorylation was analyzed on an LSRII flow cytometer (BD Biosciences) with FACS Diva software.

Real-time polymerase chain reaction

We quantified *DUSP6, SHP-2, PTPN22, c-Myc* and *Cyclin D1* transcripts by SYBR qPCR using the following primers: *DUSP6:* 5'-CAGTGGTGCTCTACGACGAG-3' and 5'-GCAATGCAGGGAGAACTCGGC; *SHP-2*: 5'-GAAGTGGAGAGAGGAAAGAG-3' and 5'-GTCCGAAAGTGGTATTGCCAGA-3'; *PTPN22*: 5'-TTCTCTGTATCCTGTGAAGCTG-3' and 5'-CTGTCATCCTCTTGGTAACAACGT-3'; *c-Myc*: 5'-GCAGGGCTTCTCAGAGGCTTG-3' and 5'-GCCTCTCGCTGGAATTACTACAG-3'; *Cyclin D1*: 5'-ACTTGCATGTTCGTGGCTTCT-3' and 5'-CTTCACATCTGTGGCACAGAG-3'; *β-actin*: 5'-ATGGCCACGGCTGCTTCCAGC-3' and 5'-CATGGTGGTGCCGCCAGACAG-3'; 18s rRNA: 5'-GTTGAACCCCATTCGTGATG-3' and 5'-GCCTCACTAAACCATCCAA-3'. Transcript numbers were normalized to β-*actin* (for *DUSP6, SHP-2* and *PTPN22*) or 18s rRNA (for *c-Myc* and *Cyclin D1*) transcripts.

Western blotting

We lysed purified total $CD4^+$naïve $CD4^+$ and memory $CD4^+$ T cells in lysis buffer (Cell Signaling Technology, Inc.). Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with antibody to DUSP6 (Santa Cruz Biotechnology, Inc.), antibody to PTPN22 (gift from Dr. A. C. Chan, Genentech, Inc.) and antibody to SHP-2 (Cell Signaling Technology, Inc). In additional experiments (e.g. Supplementary Figs. 3 and 4), an antibody to DUSP6 from OriGene Technologies, Inc. was used. Membranes were developed with horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology, Inc.) and Immobilon Western chemiluminescence detection system (Millipore).

miRNA quantification

We assayed miR-181a and miR-142 expression levels using a mirVana™ qRT-PCR miRNA detection kit (Applied Biosystems). Briefly, 25 ng of RNA from isolated T cells was reverse-transcribed in 10 μl at 37°C for 30 min using a miRNA- or U6-specific oligonucleotide. miRNAs were then quantified by SYBR qPCR in 25 μl under the following conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s.

Transfection

To overexpress or silence miR-181a, we transfected total or $CD4^+$ T cells with miR-181a precursor or negative control (Applied Biosystems); miR-181a inhibitor or miRNA inhibitor negative control (Thermo Fisher Scientific) using Nucleofector® Kit (Amaxa, Lonza Walkersville, Inc.). To silence DUSP6 expression, we transfected $CD4^+$ T cells with SMARTpool DUSP6 (3 μg) or negative control siRNA (Thermo Fisher Scientific). SMARTpool DUSP6 siRNA included the sequences 5'-GAACUGUGGUGUCUUGGUA-3', 5'-UGGCUUACCUUAUGCAGAA-3', 5'-GACUGUGGCUUACCUUAUG-3' and 5'-GCGACUGGAACGAGAAUAC-3'. DUSP6 silencing effects were confirmed by a second DUSP6 siRNA (5'-UGCAUAUAGAGGAGCCAAAGAGAGA-3', OriGene Technologies). Forty-eight hours after transfection, cells were harvested; DUSP6 transcription and protein expression were quantified, and cells were assayed for their response to CD3/CD28 cross-

linking or stimulation with mDC and TSST-1. To force overexpression of DUSP6 in $CD4^+$ T cells, *DUSP6* cDNA was inserted into vector pIRES2-AcGFP1 (Clontech Laboratories, Inc.). Four micrograms of the pIRES2-DUSP6 or the empty control plasmid were used for each transfection.

DUSP6 inhibition

PBMC were incubated with the DUSP6 inhibitor (*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one (BCI) at 37°C for 1 h. Cells were washed three times and then stimulated by cross-linking with anti-CD3/CD28 antibody (1 μg ml^{-1}). After 24 h, cells were harvested and CD69, CD25 and IL-2 expression were analyzed on an LSRII flow cytometer.

T cell differentiation

$CD4^+$ naïve T cells were transfected with DUSP6 (3 μg) or negative control siRNA. Forty-eight hours after transfection, cells were stimulated with plate-bound anti-CD3 (1 μg ml^{-1}) and anti-CD28 antibody (1 μg ml^{-1}) alone (nonpolarizing control) or in the presence of IL-12 (20 ng ml^{-1}PeproTech, Inc.) and IL-4−neutralizing antibody (10 μg ml^{-1}) for T_H1 or IL-4 (25 ng ml^{-1}) and anti-IFN-γ antibody (10 μg ml^{-1}) (all from R &D Systems) for T_H2 polarization. Cells were collected on day 7, stimulated with PMA (50 ng ml^{-1}Sigma) and ionomycin (500 ng ml^{-1}Sigma) in the presence of brefeldin for 5 h, and analyzed for cytokine production using anti-CD4-PerCP, anti-IFN-γ-FITC and anti-IL-4-PE (all from BD Biosciences).

Statistical analysis

Statistical comparisons were performed by two-way ANOVA using SigmaStat 3.0 software. Age, DUSP6 and miR-181a expression were compared by Pearson's correlation. DUSP6 inhibition experiments with BCI were analyzed by repeated measure ANOVA. TCR-induced expression of activation markers and gene expression after miR-181a overexpression were compared by paired *t* test.

RESULTS

Decreased sensitivity to T cell receptor stimulation with age

The frequency of naïve $CD4^+$ T cells changes with age but this decline does not fully explain the defective T cell response suggesting T cell intrinsic defects. We probed T cell function under relatively physiological conditions by stimulating purified naïve $CD4^+$ T cells from healthy individuals of different age groups with the superantigen toxic shock syndrome toxin (TSST)-1 presented by myeloid dendritic cells (mDCs) from young adults (Supplementary Fig. 1). TSST-1 is known to stimulate all $Vβ2^+$ and a small fraction of $Vβ2^-$ T cells, the latter only at higher concentrations suggesting lower affinity. The number of dendritic cells (DCs) in the system was adjusted so that an alloreactive response in the absence of TSST-1 was not detectable. In initial kinetic experiments, we determined the fraction of $Vβ2^+$ naïve $CD4^+$ T cells that were stimulated to express CD69 and CD25. Expression of these activation markers in elderly naïve $CD4^+$ T cells was delayed, suggesting that longer stimulation is needed for elderly T cells to build up the required

signaling strength (Fig. 1a). To examine this possibility in more detail, we determined the fraction of naïve $CD4^+$ T cells that were activated in eleven 20–35 year-old and eleven 70–85 year-old individuals using decreasing doses of superantigen (Fig. 1b). At high doses, approximately 90% of $V\beta2^+$ T cells entered proliferation independent of age. With decreasing doses, a difference became apparent. Dose-response curves were shifted for the elderly towards higher doses of antigen by a factor of approximately ten. In subsequent experiments with naïve $CD4^+$ T cells from thirty-five 20–35 and seventeen 70–85 year-old individuals at a low TSST-1 concentration of 0.04 ng ml^{-1}a significantly lower number of naïve $CD4^+$ T cells responded to stimulation in the elderly individuals (Fig. 1c). The difference was more pronounced for $V\beta2^-$ naïve $CD4^+$ T cells ($P < 0.0001$) than for $V\beta2^+$ cells ($P = 0.002$). Taken together, these data suggest that TCR activation thresholds are increased in naïve $CD4^+$ T cells from elderly individuals, and productive activation requires higher antigen doses or higher affinity antigens.

Age-associated T cell receptor signaling defects

To probe early signaling events after TCR stimulation, we used phospho-epitope analysis by flow cytometry. CD3 cross-linking induced equal phosphorylation of ZAP70 when naïve and memory $CD4^+$ T cells from fifteen 20–35 year-old and fifteen 70–85 year-old adults were compared, suggesting that initial signaling is intact and not affected by age (Fig. 1d). Also, PLC-γ1 phosphorylation which is just upstream of IP3 generation and calcium flux was not age-dependent (Fig. 1d) documenting that human T cells do not exhibit the signature of defective calcium signaling that is characteristic for immune aging in mice[23]. In contrast, naïve $CD4^+$ T cells from young adults were significantly more effective in phosphorylating ERK; results from 20 young and 20 old individuals are shown ($P < 0.0001$, Fig. 1d). A similar difference in ERK phosphorylation was not seen for memory $CD4^+$ T cells. Studies examining PMA-induced ERK phosphorylation indicated that the defect affects the ERK pathway distal of Ras/Raf activation. ERK phosphorylation due to PMA-induced PI3-kinase activation was significantly different between the two age groups (Fig. 1e).

Age-associated overexpression of DUSP6

One major feedback loop that controls the activation of the ERK pathway in T cells and that attenuates TCR signaling involves DUSP6, a cytoplasmic phosphatase with substrate specificity for phosphorylated ERK. Increased Dusp6 protein expression during T cell development has been implicated in the reduced sensitivity of mature mouse T cells to respond to self-antigens compared to thymocytes[19]. Given the selective decrease in TCR-induced ERK activation, we explored whether human expression levels of DUSP6 increase with aging. DUSP6 was more abundant in $CD4^+$ T cells from 70–85 year-old adults compared to young adults as determined by Western blots (Fig. 2a, $P = 0.01$). This increase was entirely due to the naïve $CD4^+$ population ($P = 0.008$); we did not see a difference for memory $CD4^+$ T cells (Fig. 2b). To determine the relationship between age and DUSP6 expression in more detail, we examined naïve and memory $CD4^+$ T cells from 60 additional individuals with similar numbers of individuals for each age decade between 20 and 80 years. DUSP6 protein expression in naïve $CD4^+$ T cells linearly increased throughout the

entire age range (r = 0.68, $P < 0.0001$, Fig. 2c, left panel), while it did not exhibit a significant age dependency in $CD4^+$ memory T cells (r = 0.25, n.s., Fig. 2c, right panel).

Loss in miR-181a accounts for increased DUSP6 expression

Increased DUSP6 protein expression with age was not reflected at the transcript level. *DUSP6* transcript numbers in total $CD4^+$ T cells from twenty 20–35 and twenty 70–85 year-old individuals were similar as determined by qPCR (Fig. 3a). Memory $CD4^+$ T cells tended to have lower transcript levels than naïve $CD4^+$ T cells, both in the young and the old (Fig. 3a, right). In mouse studies by Li et al, Dusp6 was one of several phosphatases controlled by miR-181a[19]. We therefore determined whether expression of miR-181a in $CD4^+$ T cells change with age. Comparison of $CD4^+$ T cells from twenty-one 20–35 and twenty-one 70–85 year-old individuals showed a threefold decline (Fig. 3b, $P = 0.0005$) which was mostly attributable to the naïve population (Fig. 3c, $P = 0.0008$). Memory $CD4^+$ T cells have lower miR-181a than naïve $CD4^+$ T cells in the young ($P = 0.004$) and only show a small further decrease with age. In contrast, miR-142, examined as a system control, did not change with age (Fig. 3b). The decline in miR-181a expression in naïve $CD4^+$ T cells is progressive throughout adult life. miR-181a expression was assessed in a cohort of 58 individuals, in part overlapping with those shown in Fig. 3c. Levels declined in naïve (Fig. 3d, left panel, r = −0.59, $P < 0.0001$), but only insignificantly in memory $CD4^+$ T cells (Fig. 3d, right panel, r = −0.19, $P = 0.36$).

Comparison of miR-181a and DUSP6 protein expression yielded a significant negative correlation (Fig. 4a, r = −0.51, $P < 0.0001$) that was only partially explained by their age associations. When adjusted for age, correlations were maintained for young (23–37 years) and middle-aged (37–55 years) adults supporting the notion that miR-181a is an important regulator of human DUSP6 as originally described in the mouse system. We did not find a correlation for individuals older than 55 years, possibly indicating that miR-181a were too low to influence DUSP6 expression (Fig. 4b). To confirm whether the decrease in miR-181a is responsible for increased DUSP6 expression, we transfected $CD4^+$ T cells from elderly individuals with miR-181a precursor and determined DUSP6 protein expression by Western blot. A representative experiment shows a reduced DUSP6 band intensity in $CD4^+$ T cells that were transfected with miR-181a precursor (Fig. 4c). Importantly, elderly $CD4^+$ T cells transfected with the miR-181a precursor expressed increased activation markers after CD3 stimulation compared to control-transfected T cells. An increased IL-2 production by restoring miR-181a levels was reversed by parallel forced overexpression of DUSP6 (Fig. 4d). Activation-induced expression of CD69 was enhanced by either increasing miR-181a or silencing DUSP6. Double transfection with DUSP6 siRNA and miR-181a precursor only facilitated a small additional increase in CD69 expression (Fig. 4e). Conversely, miR-181a silencing reduced activation-induced CD69 expression compared to control-transfected cells (Fig. 4f). Co-transfection with DUSP6 siRNA reversed the effect of transfection with miR-181a inhibitor alone. In summary, these experiments are consistent with the interpretation that miR-181a regulates human TCR threshold by controlling DUSP6 expression. The minor synergistic action between DUSP6 silencing and miR-181a overexpression is likely due to incomplete silencing in the single transfected cells. Furthermore, PTPN22 and SHP-2, which are also targeted by miR-181a in the mouse, were

not influenced by age in human T cells (Fig. 4g). The effect of miR-181a on DUSP5, which is also regulated by miR-181a in the mouse, was undetermined because the expression levels in human T cells were too low to be detected with the available antibody (data not shown).

Increased miR-181a expression restores T cell responses in the elderly

To determine whether restoration of miR-181a levels improves T cell activation in the elderly, T cells from eleven 20–35 and eleven 70–85 year-old individuals were transfected with miR-181a precursor or miRNA precursor negative control. ERK phosphorylation after anti-CD3 cross-linking was determined in gated naïve and memory $CD4^+$ T cells by PhosphoFlow (Fig. 5a). Consistent with the data shown in Figure 1, activation-induced ERK phosphorylation was reduced in elderly naïve, but not memory $CD4^+$ T cells. Overexpression of miR-181a improved the ERK response significantly in elderly naïve $CD4^+$ T cells ($P = 0.002$) to approximately the same response level that is seen in the young adult. A lesser increase was seen in naïve $CD4^+$ T cells from young adults which still reached significance ($P = 0.03$). In contrast, the ERK response in $CD4^+$ memory T cells was not influenced by miR-181a overexpression in the young adult and only slightly improved in the elderly. Increased ERK responses were functionally important. Upon TCR stimulation, T cells from elderly individuals expressed increased transcript numbers of *c-Myc* ($P = 0.008$) and *Cyclin D1* ($P = 0.01$) when transfected with miR-181a (Fig. 5b). In parallel, activation-induced expression of IL-2 ($P = 0.02$) and CD25 ($P = 0.004$) in naïve $CD4^+$ T cells improved (Fig. 5c). Also, miR181a overexpression augmented proliferative responses to CD3 cross-linking (Fig. 5d) and stimulation with DC and TSST-1 (Fig. 5e).

DUSP6 inhibition augments T cell activation

Given that increased expression of DUSP6 is the major consequence of miR-181a loss in elderly human $CD4^+$ T cells, inhibition of DUSP6 may be sufficient to restore $CD4^+$ T cell activation. When $CD4^+$ T cells were transfected with DUSP6 siRNA, a decline in protein expression was evident after 48 h (Fig. 6a); similar results were observed with two different siRNA sequences. Transfected $CD4^+$ T cells from 60–80 year-old individuals were maintained for 48 h and then stimulated under suboptimal condition with anti-CD3/CD28 antibodies. Silencing of DUSP6 increased induction of CD69 ($P < 0.0001$), CD25 ($P < 0.0001$) and IL-2 ($P < 0.0001$) (Fig. 6b). Increased IL-2 production by silencing of endogenous DUSP6 was overcome by forced overexpression of DUSP6 (Fig. 6c). DUSP6 silencing also increased proliferative responses, in particular at low concentrations of stimulating anti-CD3 monoclonal antibody (Fig. 6d) or to stimulation with TSST-1 and DC (Fig. 6e). Similar results were found with the second DUSP6 siRNA (Supplementary Fig. 5). With optimal doses of anti-CD3 monoclonal antibody, proliferative responses in DUSP6-silenced and control T cells reached the same plateau consistent with the interpretation in Figure 1 that low affinity antigen responses were more affected in the elderly.

Initial TCR signal strength not only determines whether a T cell is activated or not, but also has lasting effects on T cell differentiation. High signal strength favors a T_H1, and low signal strength a T_H2 response. To determine whether DUSP6 activity influences T cell polarization, naïve $CD4^+$ T cells transfected with DUSP6 siRNA were activated and cultured under T_H1 and T_H2 polarizing conditions. DUSP6 silencing supported the

induction of a T_H1 response ($P = 0.003$) while disfavoring the differentiation into T_H2 cells ($P = 0.0006$) compared to control transfected T cells (Fig. 6f).

These results support the notion that DUSP6 is a suitable pharmacological target to improve naïve $CD4^+$ T cell responses, in particular in the elderly. (*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one (BCI) has been shown to be an inhibitor of DUSP6 activity[24]. As BCI binds to an allosteric site and not the catalytic phosphatase domain, its cross-reactivity is limited. We stimulated peripheral blood mononuclear cells (PBMC) from ten 60–85 year-old individuals under suboptimal conditions with anti-CD3/CD28 antibodies in the presence of increasing concentrations of BCI. CD69 and CD25 mean fluorescent intensity (MFI) were quantified as markers of T cell activation. BCI enhanced induction of CD69 expression on naïve ($P < 0.0001$) (Fig. 6g, left panel) in a dose-dependent manner. BCI treatment also enhanced CD25 expression (middle panel) and IL-2 production in naïve $CD4^+$ T cells (right panel).

DISCUSSION

Our studies demonstrate that naïve $CD4^+$ T cells lose TCR sensitivity and signaling strength with age due to reduced expression of miR-181a. Since miR-181a represses DUSP6, DUSP6 protein concentrations increase with age dampening ERK activation. ERK activity is a pivotal regulator of TCR threshold calibration by controlling positive feedback loops in the TCR-induced activation cascade; reduced ERK activity impairs signal strength and activation of individual T cells. Moreover, due to an increased TCR threshold setting, only T cells with higher affinity to antigen are activated leading to a contracted immune response to a given antigenic peptide. Inhibition of DUSP6 emerges as a key intervention to strengthen and broaden naïve $CD4^+$ T cell responses in the elderly.

Declining regenerative capacity with age and inability to maintain a balance between functional T cell subsets and a diverse TCR repertoire have been held responsible for the deteriorating adaptive immune response[11,25,26]. Indeed, homeostasis of $CD8^+$ T cells is not well maintained with age, naïve and central memory $CD8^+$ T cells are lost while terminally differentiated effector T cells accumulate, and clonal $CD8^+$ T cell expansion dominates the repertoire[9,27]. $CD8^+$ T cell oligoclonality and senescence correlate with poor vaccine responses and general mortality and may account for the prolonged viremia in elderly patients infected with influenza[28–30]. In contrast, $CD4^+$ T cell homeostasis is better maintained over life[31]. In spite of thymic demise in mid-adulthood, compartment sizes of naïve and central memory $CD4^+$ T cells are substantial, and expansion of the $CD28^-$ $CD4^+$ T cell population is infrequent and usually related to disease[15]. Nevertheless, adaptive immune responses that rely on $CD4^+$ T cell function, such as the production of antibodies after vaccination, are impaired with age[4].

While signaling studies in mouse systems have been very successful to define age-related defects[32], including a defect in the ERK pathway[33], initial findings with human T cells[34,35] have not been further studied and defects are not well characterized, in part because human age-related defects are more variable and subtle and are difficult to quantify, given the changes in T cell subset distributions with age. Also, transcription of signaling molecules

involved in TCR signaling does not appear to be influenced by age[36]. In the present study we demonstrate a selective dampening of the ERK signaling pathway. This defect was seen in naïve $CD4^+$ T cells and did not involve naïve or memory $CD8^+$ T cells (unpublished observation) or memory $CD4^+$ T cells. The underlying mechanism is a loss in miR-181a and therefore not obvious in transcriptional studies.

The concept that a miRNA expression level acts as a rheostat that controls TCR signaling and accounts for different TCR sensitivities at different developmental stages has been introduced by Li et al[19]. Immature T cell precursor populations such as double-positive thymocytes undergoing selection by the recognition of self-antigen express high levels of miR-181a. The inhibition of this miRNA impairs positive and negative selection. In contrast, mature T_H1 and T_H2 cells expressed low levels of miR-181a decreasing TCR sensitivity. Overexpressing miR-181a increased signaling strength and facilitated responses to partially agonistic or antagonistic peptides. In consensus with these mouse studies, we found a higher expression of miR-181a in naïve $CD4^+$ T cells from young adults compared to memory $CD4^+$ T cells. Cumulative homeostatic proliferation may have induced a similar loss of miR-181a with age in naïve cells as with T cell differentiation. This loss appears to be rather selective because no other features of memory T cells are observed in elderly phenotypically naïve $CD4^+$ T cells. These cells express CD28, CD27 and CCR7 and have a diverse TCR repertoire setting them apart from memory T cells[11,37].

In the mouse, miR-181a modulated TCR sensitivity by inhibiting the protein expression of several phosphatases including Dusp5, Dusp6, SHP-2 and PTPN22[19]. In human $CD4^+$ T cells, only an upregulation of DUSP6 with age was found. Consistent with this finding, forced overexpression of DUSP6 in elderly T cells reversed the improved T cell responsiveness obtained from increasing miR-181a expression. The relative selectivity in humans for this one phosphatase involved in TCR threshold calibration holds the promise that DUSP6 inhibition will significantly improve the immune response in the elderly. A small-molecule DUSP6 inhibitor has recently been described[24] and experiments indeed document that this inhibitor improves responses of elderly T cells *in vitro* (Fig. 6g).

The increased susceptibility of the elderly to infection represents a major public health problem. Preventive measures by vaccinations are of critical importance; however, so far they have had only a moderate impact because of reduced immune responsiveness[38]. Improving vaccine responses to overcome age-related immune defects is a challenge but also represents a unique opportunity to attain healthy aging. Approaches have mainly targeted antigen presentation by increasing antigen dose or by using adjuvant. Influenza vaccination using an oil-in-water emulsion adjuvant, MF59, has yielded increased induction of protective antibodies[39–41]. Increasing dosage of of influenza and varicella zoster vaccines[42,43] have improved clinical efficacy consistent with the interpretation that elderly T cells have an increased threshold to respond that can be overcome by increased antigen doses. Our studies suggest that, in addition to enhancing the vaccine, T cells can be directly targeted to improve responsiveness by lowering the TCR activation threshold and modifying early TCR signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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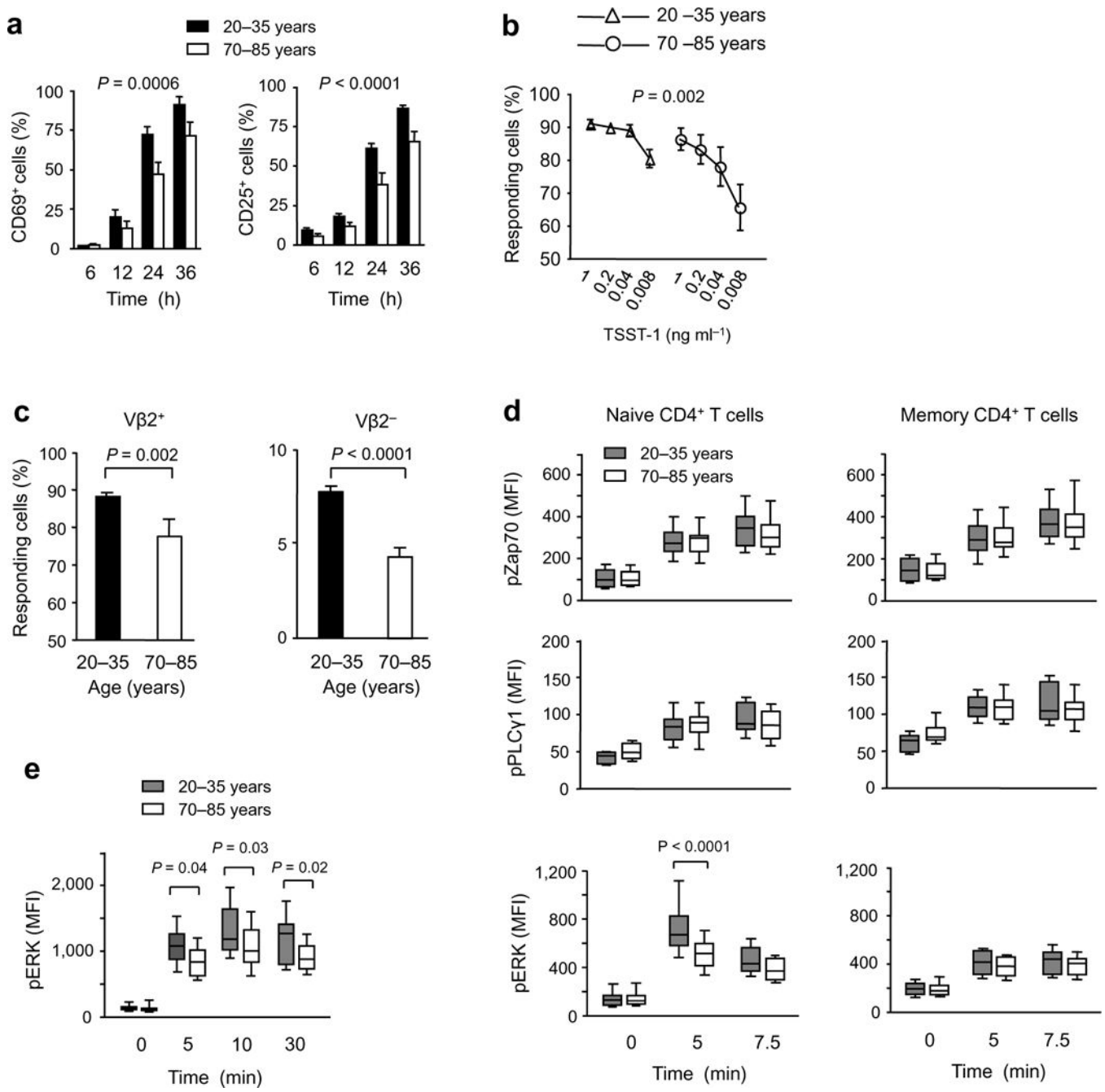

Figure 1. Decreased sensitivity of naïve CD4$^+$ T cells to respond to antigenic stimulation with age

Purified naïve CD4$^+$ T cells from 20–35 and 70–85 year-old individuals were stimulated by the superantigen TSST-1 and mDCs from young adults. (**a**) Expression of CD69 (left panel) and CD25 (right panel) on Vβ2$^+$ CD4$^+$ T cells at indicated time points is shown for six young and six elderly individuals. (**b**) The proportions of Vβ2$^+$ CD4$^+$ T cells that had responded to stimulation to declining TSST-1 concentrations were calculated from CFSE dilution histograms on day 4. Results represent mean ± s.e.m. of 11 young (open triangles) and 11 elderly adults (open circles). (**c**) Naïve CD4$^+$ T cells were stimulated with 0.04 ng ml^{-1} TSST-1 and the frequencies of responding Vβ2$^+$ (high responder to TSST-1) and Vβ2$^-$

(low responder to TSST-1, see Supplementary Fig. 1) $CD4^+$ T cells were determined. Results represent mean ± s.e.m. of 35 young (solid bars) and 17 elderly adults (open bars). (**d**) T cells were stimulated by CD3 cross-linking and examined for ZAP70, PLC-γ1 and ERK phosphorylation by PhosphoFlow. Medians, 25^{th} and 75^{th} percentiles as boxes and 10^{th} and 90^{th} percentiles as whiskers are shown for gated $CD4^+CD45RA^+$ naïve (left) and $CD4^+CD45RA^-$ memory T cells (right panels) from 20−35 (shaded boxes) and 70–85 year-old individuals (open boxes). (**e**) PMA-induced ERK phosphorylation was examined in $CD4^+$ T cells from thirteen 20–35 (shaded boxes) and eleven 70–85 year-old individuals (open boxes) at indicated time points.

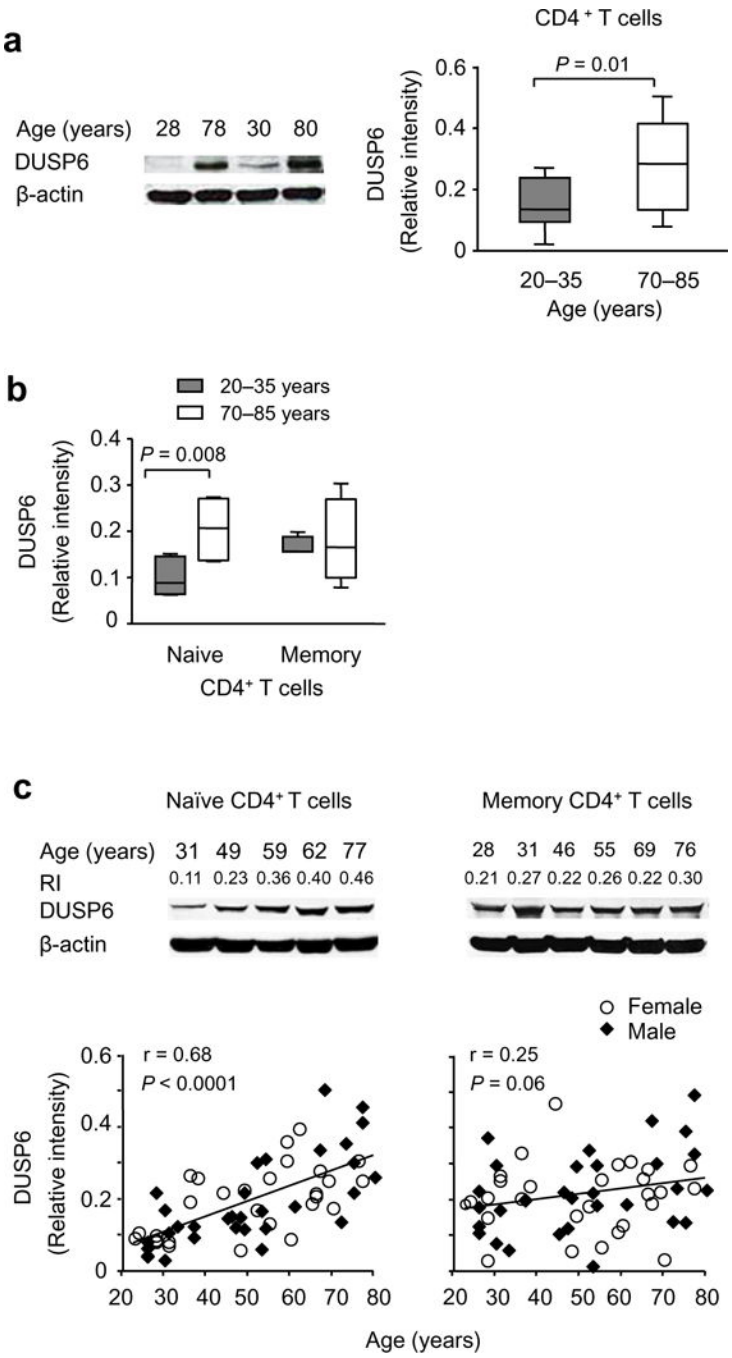

Figure 2. Age-associated expression of DUSP6

(**a**) DUSP6 expression in CD4$^+$ T cells was examined by Western blotting. Representative blots are shown in the left panel. The box plots summarize the results from twelve 20–35 (shaded boxes) and twelve 70–85 year-old individuals (open boxes). (**b**) DUSP6 protein levels in isolated CD4$^+$ naïve and memory T cells for six young (shaded boxes) and six elderly (open boxes) adults are shown. (**c**) DUSP6 protein levels in CD4$^+$ naïve (left) and memory T cells (right) are shown as representative Western blots and as scatter plots of 60 male (diamond) or female (circle) individuals covering the age range from 20 to 80 years.

Full gels are shown in Supplementary Figure 2. DUSP6 protein levels in $CD4^+$ naïve T cells correlated with age ($r = 0.68$, $P < 0.0001$). RI = relative intensity compared to β-actin

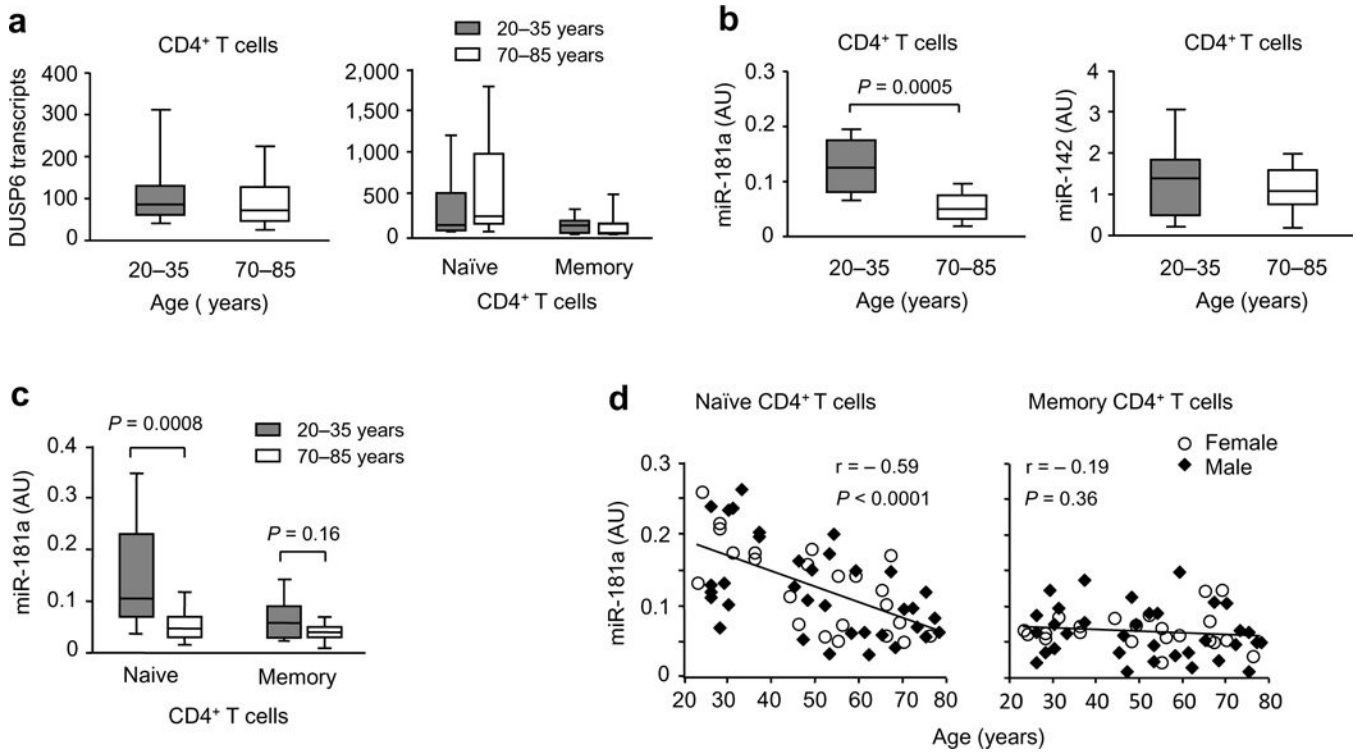

Figure 3. Age-associated expression of miR-181a

(**a**) *DUSP6* transcript levels were quantified in total CD4$^+$ T cells from twenty 20–35 (shaded) and twenty 70–85 year-old adults (open boxes, left panel). Results for isolated naïve and memory T cells are summarized for 23 young (shaded boxes) and 14 elderly individuals (open boxes, right panel). (**b**) CD4$^+$ T cells from twenty-one 20–35 (shaded) and twenty-one 70–85 year-old adults (open boxes) were compared for the expression of miR-181a by qPCR; miR-142 was quantified in the same samples as a control. (**c**) Naïve and memory CD4$^+$ T cell subset analysis was done in 22 young (shaded boxes) and 16 elderly individuals (open boxes); miR-181a expression is shown as box plots. (**d**) miR-181a levels in naïve (left panel, $n = 58$, $r = -0.59$, $P < 0.0001$) and memory CD4$^+$ T cells (right panel) from male (diamonds) and female individuals (circles) are blotted against age.

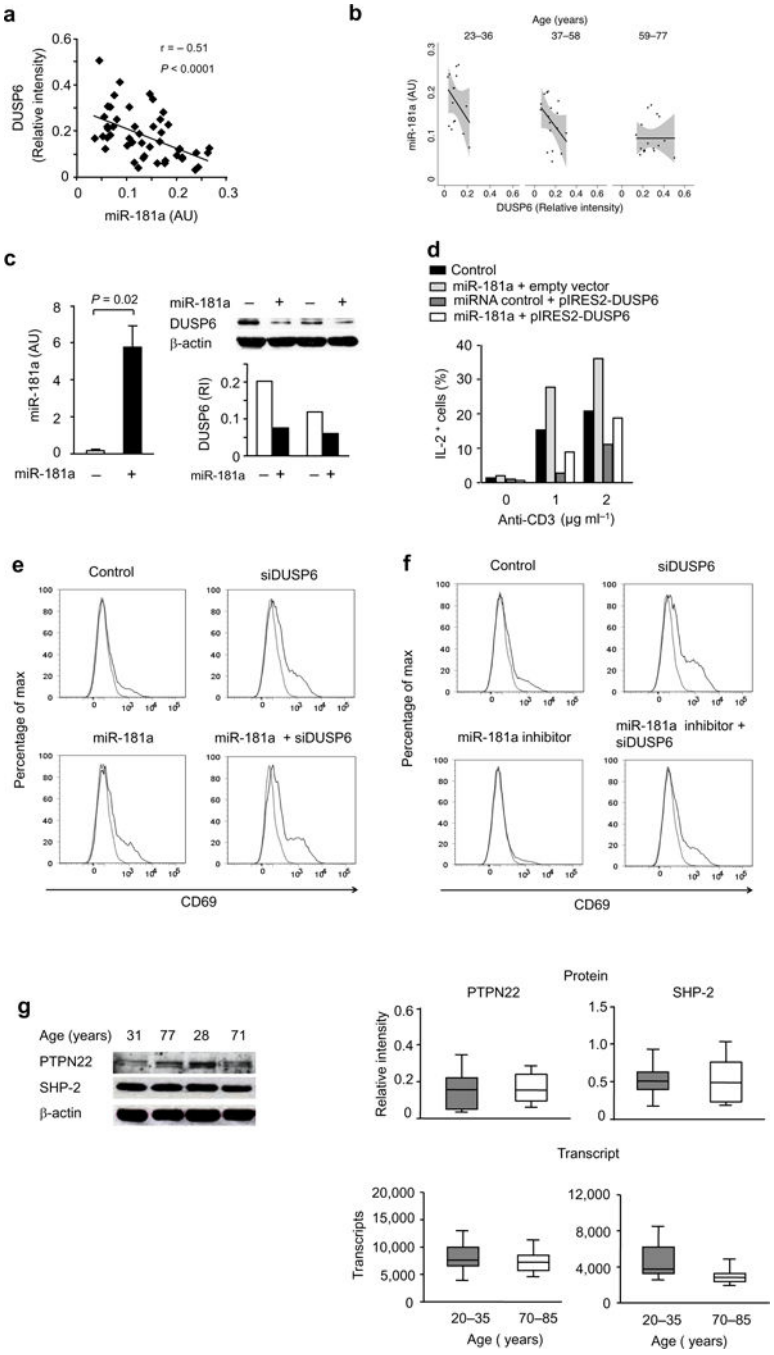

Figure 4. miR-181a controls DUSP6 expression

(**a**) miR-181a expression levels in CD4$^+$ naïve T cells from 49 individuals between 20 and 80 years of age are blotted against DUSP6 protein levels. (**b**) Differential correlations between miR-181a and DUSP6 protein expression in different age groups. (**c**) miR-181a in CD4$^+$ T cells from elderly adults transfected with miR-181a precursor or miRNA precursor negative control was quantified by qPCR (mean ± s.e.m., n = 5, left panel); Western blots of DUSP6 protein expression are representative of five. Full gels are provided in Supplementary Figure 3. (**d**) Frequencies of IL-2 expression after anti-CD3/CD28

stimulation in $CD4^+$ T cells transfected with miR-181a or control siRNA, pIRES2-DUSP6 or control plasmid as indicated were determined. One of two experiments is shown. (**e**) CD69 expression after anti-CD3/CD28 stimulation was determined in $CD4^+$ T cells that were transfected with miR-181a precursor and/or siRNA-DUSP6 as indicated. Transfection with negative control siRNA or precursor miRNA served as control. Dotted lines represent CD69 levels on unstimulated transfected T cells. (**f**) Histograms of stimulation-induced CD69 expression are shown for $CD4^+$ T cells transfected with miR-181a inhibitor and/or siRNA-DUSP6 or the respective negative controls. (**g**) PTPN22 and SHP-2 transcript and protein expression was assessed in $CD4^+$ cells. Representative Western blots, box plots summarizing Western blot results for ten 20–35 and ten 70–85 year-old individuals and transcript numbers for twenty 20–35 (shaded boxes) and twenty 70–85 year-old (open boxes) individuals are shown.

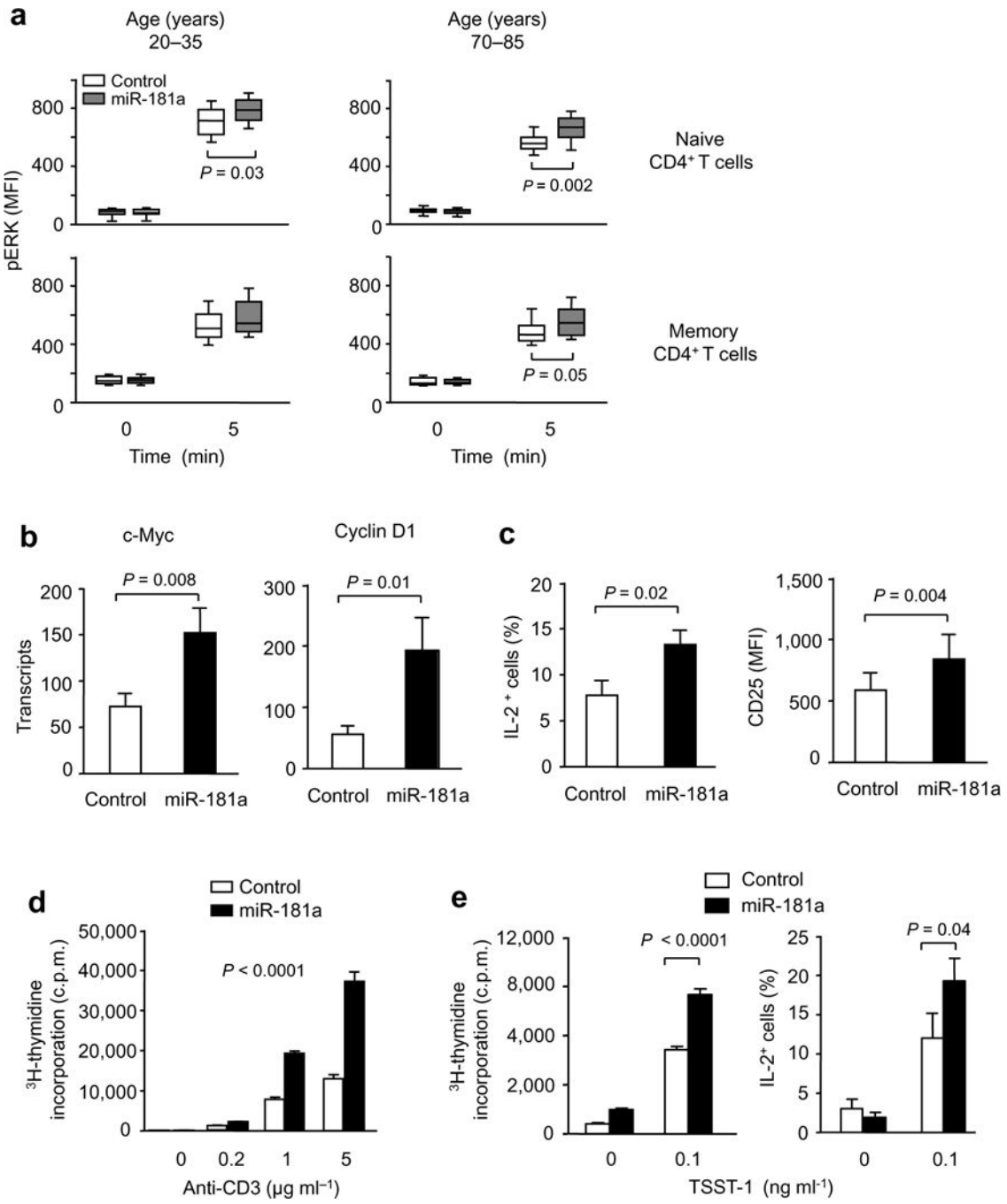

Figure 5. miR-181a overexpression restores T cell activities in elderly CD4$^+$ T cells

(**a**) T cells from eleven 20–35 (left panels) and eleven 70–85 year-old individuals (right panels) were transfected with miR-181a precursor (shaded) or negative control (open boxes). ERK phosphorylation before and 5 min after anti-CD3 cross-linking was determined in CD4$^+$CD45RA$^+$ naïve and CD4$^+$ CD45RA$^-$ memory T cells by PhosphoFlow. (**b, c**) T cells transfected with miR-181a precursor (solid) or negative control (open bars) and activated by CD3/CD28 stimulation were analyzed for the expression of *c-Myc* and *Cyclin D1* transcripts by qPCR (**b**) and for IL-2 and CD25 expression in CD4$^+$CD45RA$^+$ naïve T

cells by flow cytometry (**c**). qPCR results are shown as mean ± s.e.m. transcript numbers of experiments with T cells from ten 60–85 year-old individuals. MFI of CD25 expression (n = 15) or frequencies of IL-2 positive cells (n = 6) from 60–85 year-old individuals are expressed as mean ± s.e.m. (**d**) Proliferative responses of elderly $CD4^+$ T cells that were transfected with miR-181a precursor (solid bars) or negative control (open bars) are shown as ^{3}H-thymidine incorporation and blotted against the concentration of stimulating anti-CD3/CD28 antibody. One experiment representative of five is shown. (**e**) miR-181a transfected T cells were stimulated with TSST-1 presented by mDC. ^{3}H-thymidine incorporation (mean ±s.e.m. from triplicate cultures of one of three experiments) and frequencies of IL-2−producing cells (mean of three experiments) are shown.

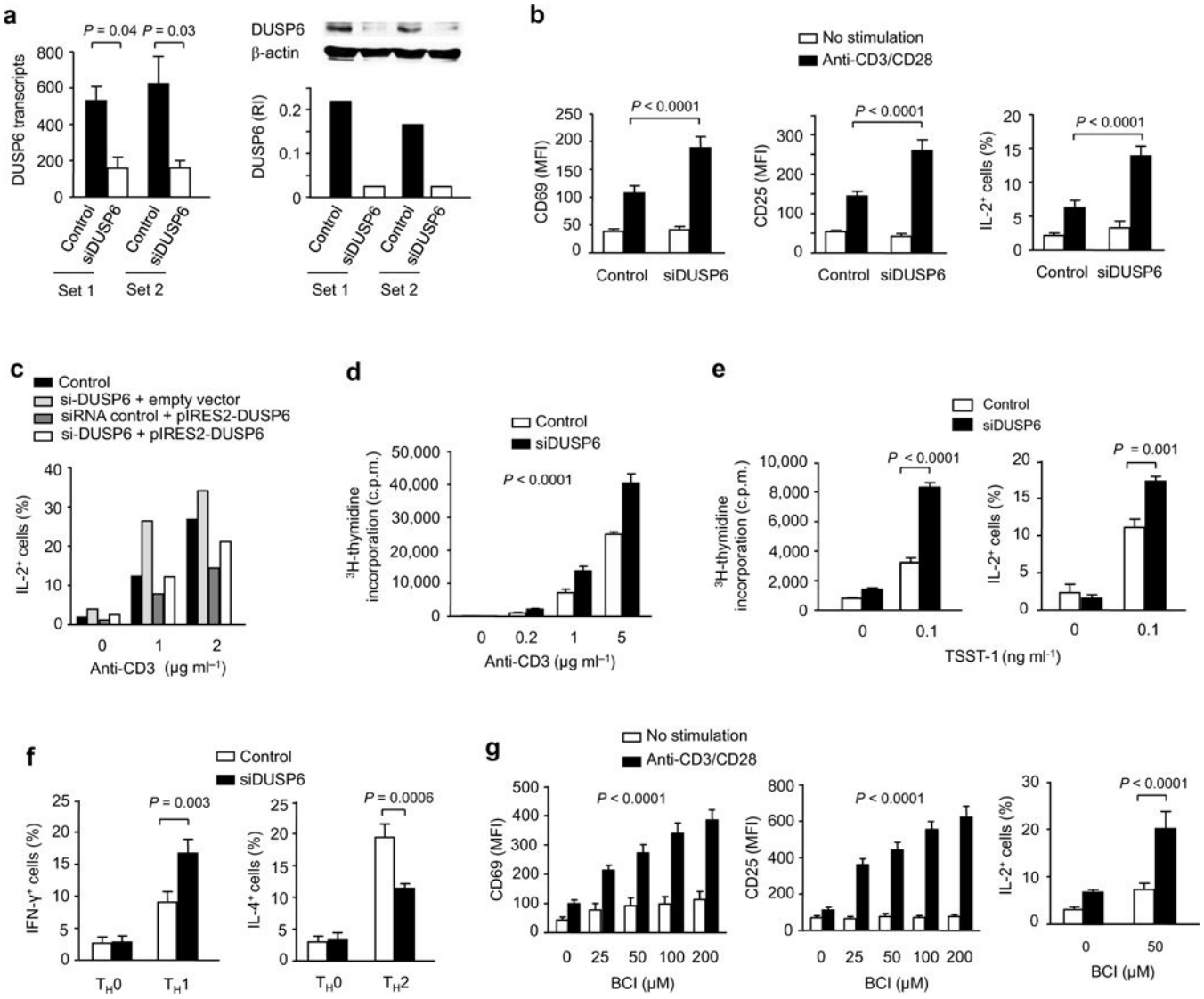

Figure 6. DUSP 6 inhibition augments activation of elderly CD4$^+$ T cells

(**a**) DUSP6 transcripts (mean ±s.e.m., n = 5) and protein (one Western blot representative of three) in CD4$^+$ T cells from elderly adults transfected with DUSP6-specific or control siRNA. Two different sets of siRNA were used. Full gels are shown in Supplementary Figure 4. (**b**) CD69, CD25 and IL-2 expression in naïve CD4$^+$ T cells transfected with siDUSP6 or control siRNA were determined by flow cytometry. MFI of CD69 and CD25 represent mean ± s.e.m. of five, frequencies of IL-2−producing cells mean ± s.e.m. of eight 60–80 year-old individuals. (**c**) IL-2 production was determined in DUSP6-silenced CD4$^+$ T cells reconstituted with a pIRES2-DUSP6 plasmid. One experiment representative of two. (**d**) CD3-induced proliferation of CD4$^+$ T cell from elderly individuals after DUSP6 silencing was measured by ^{3}H-thymidine incorporation. One experiment representative of four. (**e**) Proliferative responses (mean of triplicate cultures) and frequencies of IL-2−producing cells (mean of three experiments) of DUSP6-silenced T cells after stimulation with TSST-1 and DC. (**f**) Generation of IFN-γ− and IL-4−producing cells from

naïve $CD4^+$ T cells transfected with DUSP6-specific (solid bars) or control siRNA (open bars) and cultured under nonpolarizing (T_H0) or T_H1 or T_H2 polarizing conditions. Frequencies of cytokine-producing cells represent mean ± s.e.m. from five 60–80 year-old individuals. (**g**) TCR-induced CD69 and CD25 expression and IL-2 production in cells preincubated with the DUSP6 inhibitor BCI are shown as mean ± s.e.m. from ten 60–85 year-old individuals.