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Age-Dependent Signature of Metallothionein Expression in Primary CD4 T Cell Responses Is Due to Sustained Zinc Signaling

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Abstract

The ability to mount adaptive immune responses to vaccinations and viral infections declines with increasing age. To identify mechanisms leading to immunosenescence, primary CD4 T cell responses were examined in 60- to 75-year-old individuals lacking overt functional defects. Transcriptome analysis indicated a selective defect in zinc homeostasis. CD4 T cell activation was associated with zinc influx via the zinc transporter Zip6, leading to increased free cytoplasmic zinc and activation of negative feedback loops, including the induction of zinc-binding metallothioneins. In young adults, activation-induced cytoplasmic zinc concentrations declined after 2 days to below prestimulation levels. In contrast, activated naïve CD4 T cells from older individuals failed to downregulate cytoplasmic zinc, resulting in excessive induction of metallothioneins. Activation-induced metallothioneins regulated the redox state in activated T cells and accounted for an increased proliferation of old CD4 T cells, suggesting that regulation of T cell zinc homeostasis functions as a compensatory mechanism to preserve the replicative potential of naïve CD4 T cells with age.

Introduction

With increasing age, the ability of the immune system to protect against new antigenic challenges or to control chronic infections erodes. The epidemiology of infectious diseases such as influenza infections[1,2] or reactivation of herpes zoster[3] has been informative in defining the timeframe of this age-dependent decline in immunocompetence. Already between the ages of 50 and 70 years, incidence rates and morbidity of infectious diseases start to increase. In the subsequent decades of life, immune failure accelerates and is a major contribution to morbidity and mortality in the elderly.[4,5]

The focus of immunosenescence research has been predominantly on the adaptive immune system, given that antiviral and vaccine responses are affected.[6–8] To initiate an immune response, T cells need to interact with functional antigen-presenting cells to become activated. Dendritic cell (DC) function appears to be rather well maintained with age,[9] focusing attention on T cells. The quality of the immune response is highly dependent on the activation-induced clonal expansion of antigen-specific T cells and their differentiation into effector cells. Defects in activation, clonal expansion, and differentiation all have the potential to impact the immune response negatively. Due to constant turnover, T cells are at risk for replicative senescence. Age-dependent erosion of telomeric length in T cell chromosome attests to this cumulative replicative stress.[4] Defects in proximal T cell receptor signaling have been reported,[10–12] but mostly in mice and in the very elderly.

Of all age-dependent effects, changes in T cell development and homeostasis are most striking. Thymic production of new T cells dwindles[13–15]; after the ages of 40–50 years, thymic activity in humans is too low to rebuild a T cell repertoire, and virtually the entire T cell supply is generated from existing naïve and memory T cells.[16] The lack of influx of new naïve T cells can be expected to lead eventually to a progressive loss of naïve T cells and contraction of T cell receptor diversity.[17–21] Unexpectedly, CD4 T cell homeostatic mechanisms are very robust, and receptor diversity is maintained well into the seventh decade of life. Between the ages of 70 and 75 years, naïve CD4 T cells endure an abrupt and dramatic contraction in diversity, suggesting a period of in-

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creased cell death.[17] The age of 75 appears to be a watershed for human naïve CD4 T cells, after which an intact naïve T cell compartment no longer exists and naïve CD4 T cell responses are severely compromised.

In the current study, we set out to identify early abnormal response patterns in naïve CD4 T cells that occur at an age before the system deteriorates. Here, we report on a novel activation pathway that is selectively affected by age. Naïve CD4 T cells from 60- to 75-year-old healthy individuals compared to young adults responded to stimulation with a sustained increase in cytoplasmic zinc. The activation-induced zinc influx resulted in an overexpression of metallothioneins (MT), which increased the cytoplasmic reduction potential and the reservoir of MT-bound zinc.

Materials and Methods

Subjects

Peripheral blood mononuclear cells were obtained from 98 volunteers, ages 20–75 years. The protocol was approved by the Emory University Institutional Review Board, and all participants gave informed consent. Individuals with a current or previous history of cancer or any chronic inflammatory disease, chronic obstructive pulmonary disease, or any poorly controlled disease (advanced atherosclerotic disease, congestive heart failure, diabetes mellitus, hypertension) were excluded. All subjects were fully ambulatory and did not have any evidence of an acute disease at the time of blood draw.

Fluorescence-activated cell sorting analysis

Antibodies used for fluorescence-activated cell sorting (FACS) analysis included fluorescein isothiocyanate (FITC)-anti-CD45RA, phycoerythrin (PE)-anti-CD45RO, peridinin chlorophyll protein (PerCP)-anti-CD4, APC- or APC-Cy7-anti-CD3, antigen-presenting cell (APC)-anti-CD25, PE-Cy7-anti-CD69 (all from BD Biosciences, San Jose, CA), FITC- or PE-anti-T cell receptor (TCR) Vβ2 (Beckman Coulter, Inc. Fullerton, CA), PE-anti-CCR7 (R&D Systems, Minneapolis MN), and anti-MT1/2 Ab (UC1MT; Abcam Inc. Cambridge, MA) labeled with Alexa Fluor® 488 Zenon mouse immunoglobulin G_1 (IgG_1) labeling kit (Invitrogen, Carlsbad, CA). All samples were acquired using LSRII (BD Biosciences). To stain for intracellular metalloproteinase (MT),[22] cells were permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences). Data were analyzed by using FACS DIVA software (BD Biosciences) or FlowJo (Tree Star, Inc. Ashland, OR). Naïve CD4 T cells were defined as $CD4^+CD45RA^+CCR7^+$. The frequency of $CD4^+CD45RA^+$ T cells that lacked CCR7 or CD28 expression and likely represented CD45RA reverts was very low, even in the elderly. For most studies, $CD4^+CD45RA^+$ cells were therefore taken as naïve CD4 T cells. Cell recovery from cultures was determined by flow-based cell counting. A total of 5×10^4 microparticles (Spherotech, Libertyville, IL) were added to each sample tube before FACS analysis. Cells were acquired by LSRII (BD Biosciences) until 5×10^3 microparticles were acquired.

Cell isolation

CD4 T cells were negatively enriched from whole blood using human $CD4^+$ T cell enrichment cocktail (RosetteSep; StemCell Technologies, Vancouver, Canada). $CD4^+$ naïve T cells were further isolated by positive selection with anti-CD45RA magnetic beads (Miltenyi Biotec, Auburn, CA). In some experiments, CD4 T cells were positively isolated from peripheral blood mononuclear cells using anti-CD4 magnetic beads (Miltenyi Biotec). Myeloid dendritic cells (mDC) were generated from monocytes isolated with anti-CD14 magnetic beads (Miltenyi Biotec). Cells were cultured in RPMI-10% fetal calf serum (FCS) supplemented with 800 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 U/mL interleukin (IL)-4 (both from R&D Systems). At day 6, nonadherent immature DC were stimulated with 1100 U/mL tumor necrosis factor-α (TNF-α; R&D Systems) and 1 μg/mL prostaglandin E2 (PGE2; Sigma, St. Louis, MO) for 24 h. In selected experiments, medium was supplemented with $ZnCl_2$ (Sigma) to the indicated zinc concentrations.

In vitro *primary CD4 T cell responses*

Purified $CD4^+CD45RA^+$ T cells were labeled with 5 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). A total of 25×10^3 labeled cells were stimulated with 0.5×10^3 mDC pulsed with 0.04 ng/mL toxic shock syndrome toxin-1 (TSST-1; Toxin Technology, Inc, Sarasota, FL) in a 96-well round plate. In pilot experiments, DC and TSST-1 concentrations were determined that did not yield detectable alloreactive and suboptimal TSST-1-specific proliferative responses. RNA was obtained at days 0, 3, and 10. Cultures were harvested on day 4, and CFSE fluorescence on $CD4^+V\beta2^+$ T cells was determined by flow cytometry on LSRII. Propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) was used to exclude dead cells. Data were analyzed to determine the fraction of $CD4^+V\beta2^+$ naïve T cells that had entered the cell cycle and started dividing, as well as the mean number of divisions.

Microarray analysis

Total RNA was extracted from cultures at days 0, 3, and 10 after stimulation using an RNeasy Mini Kit (Qiagen, Valencia, CA). Antisense RNA (aRNA) was synthesized using a modified Affymetrix protocol (Affymetrix, Santa Clara, CA; http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, reverse transcriptase (RT) was primed by 20 pmol oligo(dT)-T7 primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAG-GCGG-$(dT)_{24}$-3′) with 40 U SuperScriptIII (Invitrogen, Carlsbad, CA), 2 mM dithiothreitol (DTT), 100 μM dNTP in first-strand buffer at 45°C for 1 h. Immediately following RT, second-strand cDNA was synthesized in a 75-μL reaction containing 100 μM dNTP, 20 U of *Escherichia coli* DNA polymerase I (Invitrogen), 10 U of *E. coli* DNA ligase (Invitrogen), 1 U of RNase H (Invitrogen), and second-strand buffer (Invitrogen). After incubation at 15ºC for 2 h, the resulting double-stranded cDNA template was polished with 10 U of T4 DNA polymerase (Invitrogen) and purified using Phase Lock Gels (Eppendorf, Hamburg, Germany). Biotin-labeled aRNA was obtained with the BioArray HighYield RNA Transcript Labeling Kit (T7) (Enzo, Farmingdale, NY) in a 40 μL *in vitro* transcription reaction containing 1 μg of template cDNA at 37°C for 8 h. The resulting biotinylated aRNA was purified with an RNeasy Mini Kit. Affymetrix GeneChips

(U133A) hybridization was performed at the Institute for Systems Biology (Seattle, WA).

Quantitative RT-PCR

CD4 naïve T cells were activated with DC/TSST-1; total RNA was isolated from cultures at days 0 and 3 after stimulation using TRIzol (Invitrogen, Carlsbad, CA). cDNA templates were synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics GmbH, Mannheim, Germany). Quantitative (q) PCR was performed on an Mx3000 (Stratagene, Cedar Creek, TX) with the following primer sets; MT2A, 5′-AAAGGGGCGTCGGACAA-3′ and 5′-AACGGTCACGGTCAGGGTT-3′, with an annealing temperature at 55°C; MT1X, 5′-GCTTCTCCTTGCCTCGAA-3′ and 5′-CAGCTGCACTTGTCTGACGT-3′ with an annealing temperature at 55°C; 18S, 5′-AGGAATTCCCAGTAAGTGCG-3′ and 5′-GCCTCACTAAACCATCCAA-3′ with an annealing temperature at 63°C. The level of gene expression was calculated by interpolation with a standard curve. cDNA copy numbers were expressed relative to 2×10^5 18S rRNA copies.

Gene silencing

CD4 T cells were stimulated with anti-CD3/anti-CD28 beads (Invitrogen). The cells were transfected on day 1 or 2 after activation with MT2A-specific (target sequence, CCCGCTCCCAGATGTAAAGAA) or Zip6-specific small interfering RNA (siRNA) (target sequences, CTGGTTGATATGGTACCTGAA and AAGGCTTATCAAGTGGTTTAA) (all from Qiagen) using the Amaxa Nucleofector kit (AMAXA GmbH, Germany). AllStars Negative Control siRNA (Qiagen) was used as control.

Cytoplasmic zinc measurements

CD4 T cells were labeled with 2 μM FluoZin3 AM ester (Invitrogen) in phenol red-free RPMI supplemented with 0.02% Pluronic F-127 (Invitrogen) at 37°C for 30 min as described by the manufacturer. Cells were washed two times and stimulated with TSST-1-pulsed mDC. At indicated time points, the cells were harvested, stained with PE-anti-TCR Vβ2, PerCP-anti-CD4, and APC-anti-CD25, and examined on an LSRII flow cytometer.

PhosFlow

MT2A-silenced CD4 T cells were stimulated with 50 ng/mL TNF-α (R&D Systems Inc, Minneapolis, MN) for 10 min or 2 mM H_2O_2 for 15 min. The cells were immediately fixed with Cytofix buffer (BD Biosciences) at 37°C for 10 min. The pellet was resuspended with permeabilizing buffer II (BD Biosciences) and incubated for 30 min on ice. The cells were stained with Alexa Fluor® 647-conjugated anti-nuclear factor-κB (NF-κB) (Clone K10-895.12.50, BD Biosciences) at room temperature for 30 min. Stained cells were analyzed using LSRII flow cytometry.

Determination of T cell superoxide production

T cell superoxide anion formation was determined using electron paramagnetic spin resonance as described recently.[23] Spin probe 1-hydroxy-4-methoxy-2,2,6,6-TMH was used. Following culture in different zinc concentrations, CD4 T cells were centrifuged, medium was removed, and cells were resuspended in a chelexed Krebs/HEPES. TMH (0.5 Mm) was added immediately prior to adding cells to a capillary placed in an electron spin resonance (ESR) chamber. The accumulation of oxidized spin probe adduct was monitored by ESR timescan using the following settings: field sweep, 60 G; center field 3495 G and static field 3475.00 G; microwave frequency, 9.82 GHz; microwave power, 19.9 mW; modulation amplitude, 5 G; conversion time, 327.68 msec; time constant, 5242.88 msec; 512 points resolution; and receiver gain, 1×10^4. Data were analyzed using WinEPR software.

Data analysis

The Principal Component Analysis (PCA) projections were obtained considering the whole-transcriptome data on custom-made R scripts (http://www.r-project.org). To select probes as significantly differentially expressed, a normal random variable was fitted to each probe's $\log_2$ normalized expression level individually estimating mean and variance from all replicates of each age group and time point. Given such normal variables, simple probabilities were calculated for the question of interest. For instance, the overexpression question was formalized mathematically as: $P_i = \Pr(A_i > B_i)$, where A_i is the normal variable fitted using the results from the probe i in the 60- to 75-year-old group at day 3 and B_i is the same but using the 20- to 35-year-old group. Probes for which the probability value P_i was greater than a cutoff were considered significant.

Pearson correlation was used to determine the linear relationship between gene expression in the two age groups and between MT expression and proliferative responses. One-way analysis of variance (ANOVA) was used to compare the expression of CD69 and CD25 after stimulation. All other comparisons were done by Mann–Whitney U test or *t*-test, if appropriate (SPSS 15.0, Chicago, IL).

Online supplemental material

Genes differentially expressed between the two age groups after T cell stimulation are provided as Supplemental Table 1. The results of the gene expression arrays for genes involved in cell cycle regulation are shown as a heat plot in Supplemental Fig. 1.

Results

Influence of age on naïve CD4 T cell activation and proliferation

The population of $CD4^+$ naïve T cells only gradually declines with age at a rate of 0.27% per year, and even a 70-year-old individual has an average of 46.6% naïve CD4 T cells.[24] These naïve CD4 T cells are competent to respond to activation stimuli. Naïve CD4 T cells from individuals of different ages were stimulated with TSST-1-pulsed mDC from a young donor. Figure 1A shows an analysis of the activation markers CD69 and CD25. No difference was found when individuals in the age groups between 20–35 and 60–75 years were compared. Also, cell cycle entry upon stimulation was not influenced by age up to the age of 75 years, consistent with the results on early activation markers (Fig. 1B).

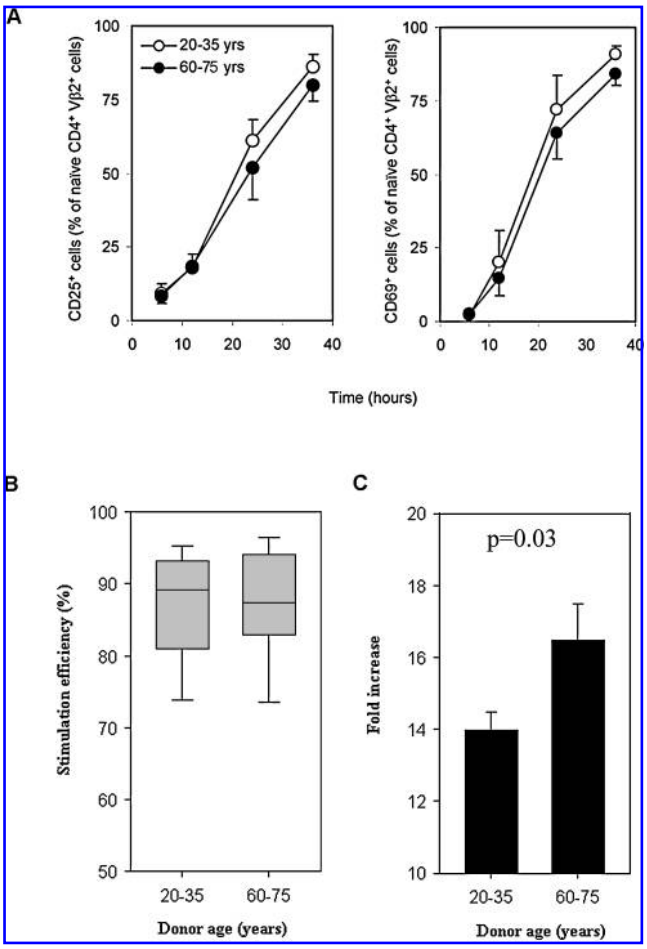

FIG. 1. Age affects naïve CD4 T cell proliferation independent of early activation events. (**A**) Purified $CD4^+CD45RA^+$ T cells were stimulated with TSST-1-loaded mDC and the induction of early activation markers CD25 and CD69 on TCR $V\beta2^+$-gated cells was determined by flow cytometry. Results shown as mean ± SD do not show a difference between the two age groups (20–35 [open circles] and 60–75 years [solid circles]). (**B**) CFSE-labeled $CD4^+CD45RA^+$ T cells were stimulated with TSST-1-loaded mDC for 4 days. The percentages of $V\beta2^+CD4^+$ T cells that had entered the cell cycle and started to proliferate are shown as box plots displaying medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers. Again, no age-dependent difference is seen. (**C**) Naïve CD4 T cells from the 60- to 75-year-old individuals ($n = 15$) underwent a higher number of cell divisions within the 4-day culture than the young adults ($n = 15$, $p = 0.03$. Results are shown for proliferating $V\beta2^+$ T cells on day 4 after stimulation as mean ± SD of fold increases.

Contrary to expectations, naïve CD4 T cells from the older age group proliferated faster and cell numbers increased 16.4-fold in the first 4 days after stimulation compared to 13.9-fold in young adults ($p = 0.03$; Fig. 1C).

Influence of age on activation-induced gene transcription

To identify age-dependent fingerprints of activation-induced transcription, we performed gene expression arrays. Because early T cell activation events appeared to be intact, we focused on time points after stimulation when proliferative responses peaked and T cells differentiated. Purified naïve CD4 T cells from 6 healthy young adults and from 6 healthy individuals aged 60–75 years were stimulated with TSST-1-loaded DC derived from a young adult. RNA was harvested at days 0, 3, and 10 and analyzed using Affymetrix U133A GeneChip arrays. In the PCA projections, whole-transcriptome expression values of both cohorts moved in concert following stimulation (Fig. 2A). Comparison of the activation-induced changes in whole-transcriptome expression values on day 3 further documented an intact T cell response; fold changes in expression values in the two age groups correlated highly ($r = 0.976$, $p < 10^{11}$; Fig. 2B). The similarity was also evident when cytokine and cytokine receptor genes that are traditionally viewed as T cell activation markers were compared ($r = 0.981$, $p < 10^{11}$; Fig. 2C). There was no significant difference for any of the cytokine or cytokine receptor genes. In particular, receptor-ligand pairs that have been implicated in regulating T cell growth, such as IL-2 or the IL-2 receptor α- and β-chains were not differentially expressed between the two age groups. Genes commonly associated with cell cycle function did also not show an age-dependent biased expression (Supplemental Fig. 1).

A detailed analysis identified 967 probes that had a probability of at least 80% being differentially expressed at day 3 while being not different at day 0. Of these, 34 were higher and 68 probes lower in the elderly by a factor of more than 1.5 (Supplemental Table 1); for the remainder, expression differences, albeit statistically significant, were small. Gene ontology (GO) enrichment analysis of the 34 overexpressed probes using the DAVID Bioinformatics Resource tool[25] identified zinc ion binding ($p = 3.3 \times 10^{-2}$), copper ion binding ($p = 1.1 \times 10^{-4}$), and cadmium ion binding ($p = 4.7 \times 10^{-5}$) as significantly associated GO terms. Similarly, the functional annotation analysis pointed to chelation as a common denominator of overexpressed genes ($p = 9.3 \times 10^{-8}$). Six probes identifying the four metallothionein genes MT1F, MT1G, MT1X, and MT2A were entirely responsible for the clustering. Additional members of the metallothionein gene family, including MT1H, MT1H-like, MT1E, and MT1M, were also overexpressed in T cells from the elderly on day 3 with probabilities of 0.76–0.79 just below the chosen cutoff. MT3 and MT4 were not expressed in T cells. Expression patterns of MT family members showed a uniform kinetic behavior. Expression was low before, increased with stimulation in both age groups, and then declined again. MT were more induced in the elderly population, as illustrated in Fig. 2D showing the Affymetrix data as ratios of MT expression in the two age groups at the different time points.

The influence of age on the activation-dependent induction of MT was confirmed in independent cohorts of healthy individuals. Representative kinetic studies of MT1X transcription after T cell stimulation by qPCR are shown in Fig. 3A. Stimulation with TSST-1-pulsed DC induced MT expression peaking at day 3 in young as well as old CD4 naïve T cells and the subsequent studies therefore focused on day 3. Figure 3B compares the induction in young and elderly adults on day 3. Upon stimulation, MT2A and MT1X transcripts increased 57-fold and 33-fold, respectively, in the 60- to 75-year-old adults, whereas induction was about three- to five-fold less in young adults ($p = 0.03$ and $p = 0.006$, re-

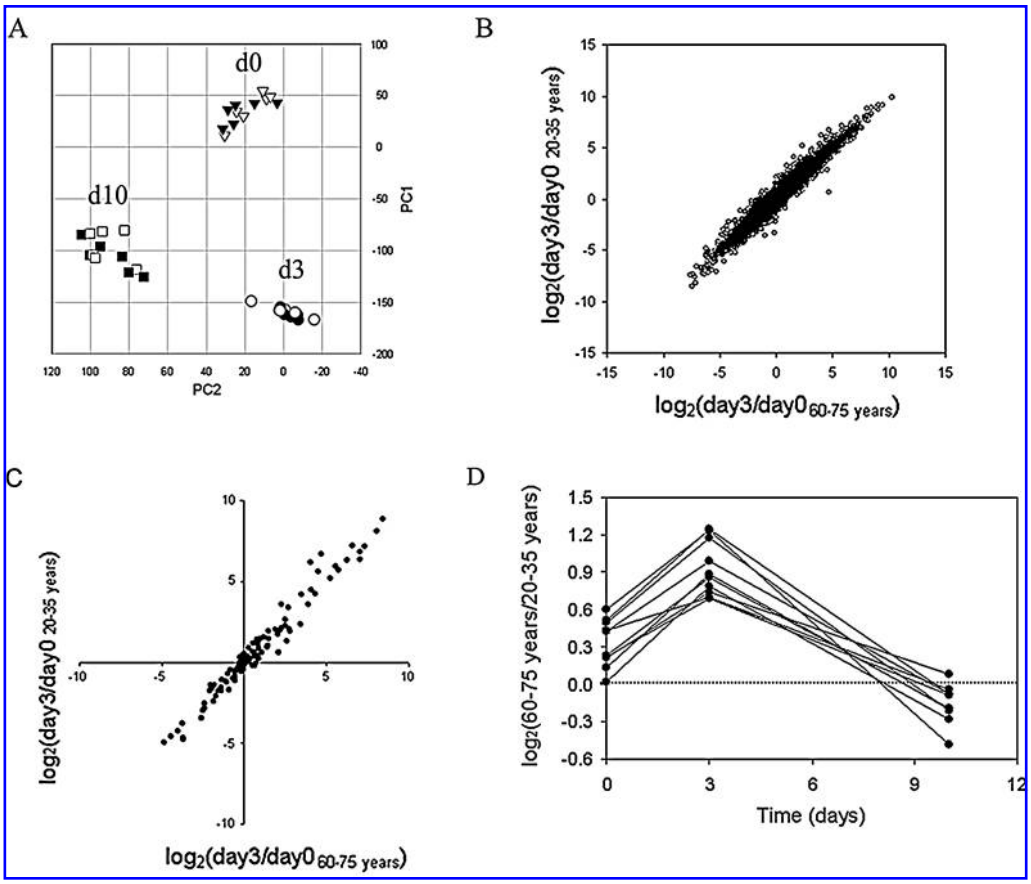

FIG. 2. Age causes a selective transcriptional fingerprint in activated naïve $CD4^+$ T cells. $CD4^+CD45RA^+$ T cells from 6 individuals 20–35 years old (*closed symbols*) and 6 individuals 60 to 75 years old (*open symbols*) were stimulated as described in Fig. 1A. (**A**) Gene expression analysis was performed on days 0, 3, and 10. PCA projection did not show an age-dependent discrimination. (**B**) Activation-induced change in whole-transcriptome expression values correlated highly between the two age groups ($r = 0.976$, $p < 10^{-11}$). Results are shown as the mean $\log_2$ ratio for 6 individuals in each age group. (**C**) Separate analysis of cytokine and cytokine receptor gene expression ($r = 0.981$, $p < 10^{-11}$) confirmed that activation-induced gene expression was intact. (**D**) Probes differentially expressed were analyzed using DAVID's Gene Ontology enrichment tools. Metal binding was the GO term most significantly associated with genes overexpressed on day 3 in the 60- to 75-year-old individuals. Metallothioneins (MT) were entirely responsible for the annotation clustering. The dynamics of the expression of MT family members is shown as the $\log_2$ ratio of the mean expression values in the two age groups. MT3 and MT4, which were not expressed in T cells at any time point, were omitted.

spectively). No difference in expression levels was seen before stimulation (data not shown). This increased transcription was reflected at the protein level (Fig. 3C; $p = 0.007$).

Transcriptional control of MT induction

The selectively increased induction of MT family members in T cells of 60- to 75-year-old individuals raised the question of whether transcriptional control mechanisms of MT are different in the elderly. MT promoters are unique in having binding sites for MTF-1, which is regulated by zinc cations. Experiments shown in Fig. 4A documented that activation-induced MT transcription was influenced by external zinc concentrations. Naïve CD4 T cells were stimulated with TSST-1-loaded DC in zinc-supplemented medium, and total MT expression was quantified by flow cytometry. As already shown in Fig. 3, T cell activation upregulated cytoplasmic MT. External zinc concentrations increased MT in activated cells, whereas they hardly influenced cytoplasmic MT in the nonactivated, TSST-1-nonresponsive $V\beta2^-$ T cells in the same culture. To explore whether the age-dependent overexpression of MT is related to different zinc homeostasis, we determined the profile of zinc-inducible genes in CD4 T cells and compared it to the activation-induced gene expression in elderly T cells (Fig. 4B). Naïve CD4 T cells from 6 adults were stimulated with TSST-1-pulsed DC in medium. The zinc concentrations in this medium are entirely due to the serum supplementation and amount to approximately 3 μM zinc. Cultures were either maintained in normal medium or switched to 45 μM zinc-supplemented medium on day 3. The number of genes with a higher expression level in the 45 μM zinc-supplemented medium ($n = 22$ for probability greater than 0.95) included all MT genes that were expressed in T cells. Genes whose transcription was enhanced by the higher zinc concentrations were preferentially induced in naïve T cells of the elderly after TSST-1/DC stimulation. Sixty four percent of highly zinc-inducible genes (14 of 22) were >0.75 probable to be overexpressed in activated naïve

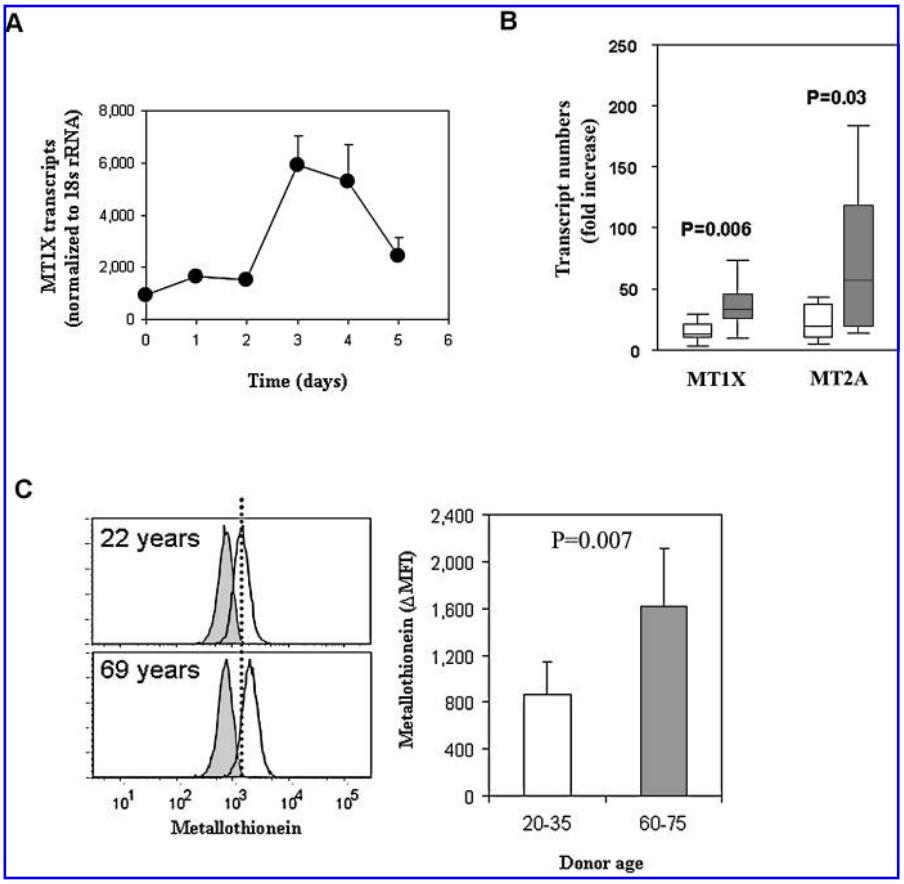

FIG. 3. Overexpression of MT in naive CD4 T cells. $CD4^+CD45RA^+$ T cells were stimulated with TSST-1-pulsed DC. (**A**) T cells were harvested at indicated time points. MT1X transcription was quantified by qPCR. Data are representative of five independent experiments. (**B**) MT1X and MT2A transcription was quantified on days 0 and 3 in twelve 20- to 35- (open boxes) and eleven 60- to 75-year-old individuals (shaded boxes). Data are shown as box plots of fold increase. (**C**) Intracellular MT protein expression was quantified in gated $V\beta2^+CD25^+$ cells on day 4 following stimulation by FACS with Alexa 488-labeled anti-MT antibody. (*Left panel*) One representative histogram (*bold line*, Alexa 488 labeled anti-MT; *gray shaded*, isotype control). Results for seven donors age 20–35 years and five donors age 60 to 75 years are shown as mean ± SD in the right panel.

CD4 T cells from 60- to 75-year-old healthy individuals, compared to 10.9% (2437 of 22261) of the zinc noninducible genes ($p = 5 \times 10^{-9}$ by a two-tailed Fisher exact test). Of interest, the zinc importer solute carrier family 30A1 (ZnT1) was also inducible by zinc and showed a trend toward overexpression in the 60- to 75-year-olds with a probability of 0.75, raising the possibility that a feedback loop maintaining zinc homeostasis is overstimulated in the T cells of this age group.

The influence of age on zinc homeostasis in activated naïve CD4 T cells

To examine the regulation of cytoplasmic zinc in T cells, CD4 T cells were loaded with the zinc-selective fluorescent indicator probe FluoZin3 as described.[26] Cells were then activated with TSST-1-pulsed DC. Cytoplasmic labile zinc in activated $V\beta2^+$ cells increased over the first 48 h after stimulation, but then dipped fairly rapidly over the next 24 to 48 h when the concentrations of MT, the major cytoplasmic scavenger for zinc, increased (Fig. 5A). This decrease was attenuated in T cells from 60- to 75-year-old individuals. The increased cytoplasmic labile zinc concentrations were the more remarkable, as the older individuals had higher MT levels and therefore increased zinc binding capacity.

In addition to MT, several zinc importers (Zip) and exporters (ZnT) control zinc homeostasis. In the gene array, ZnT1 or SCL30A was highly expressed in activated T cells from the older individuals, suggesting that export mechanisms are intact. Therefore, we focused on analyzing Zip importers. Expression of Zip proteins is cell-specific; in preliminary studies, T cells expressed foremost Zip6 after stimulation (data not shown). To examine the role of Zip6 on the activation-induced expression of MT, T cells were stimulated with anti-CD3/anti-CD28 beads and transfected with Zip6-specific or control siRNA on day 1. MT induction as assessed by FACS on day 4 after stimulation was reduced in the Zip6-silenced T cells (Fig. 5B), supporting the hypothesis that the induction of MT expression after T cell stimulation is dependent on zinc influx and that T cells from 60- to 75-year-old individuals have increased zinc influx leading to increased MT expression.

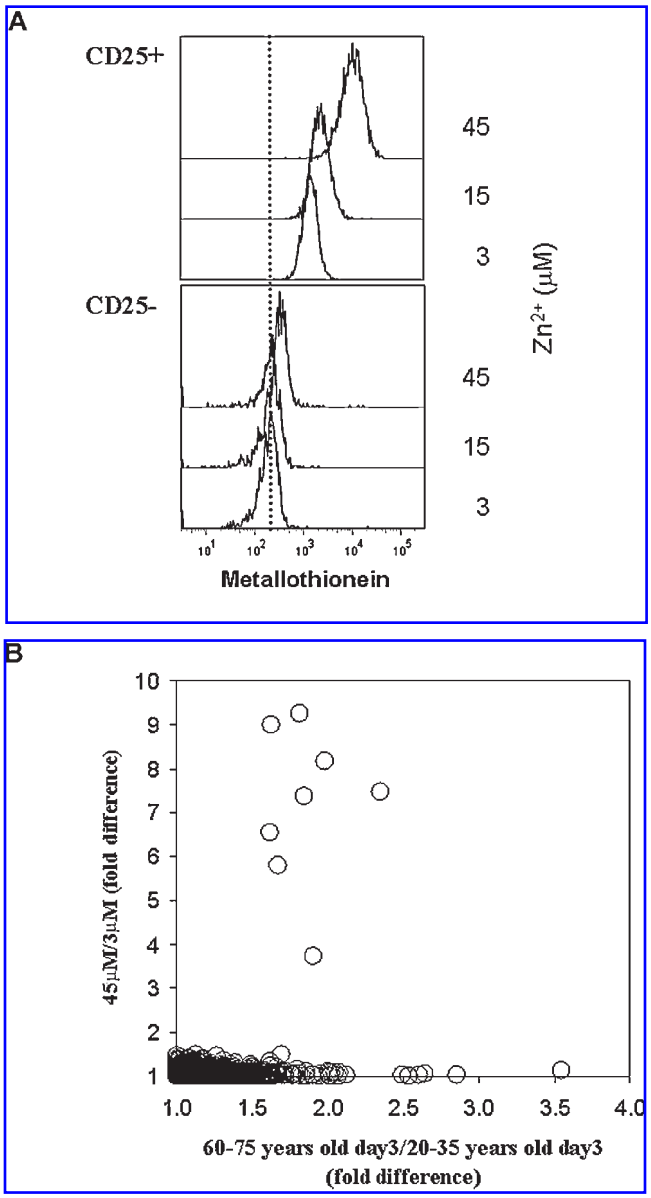

FIG. 4. Zinc-induced gene transcription. (**A**) $CD4^+CD45RA^+$ T cells were stimulated with TSST-1-pulsed DC and cultured in different zinc concentrations. MT expression in activated $V\beta2^+CD25^+$ and nonactivated $CD25^-$ T cells were assessed by flow cytometry on day 4. Histograms representative of seven experiments show the zinc dependence of MT induction in activated T cells. (**B**) $CD4^+CD45RA^+$ T cells were activated and then cultured in 3 μM and 45 μM zinc-containing medium. Zinc-induced gene expression was assessed by Affymetrix gene arrays and compared to the genes preferentially expressed in the 60- to 75-year-old individuals on day 3 after stimulation. Of 22 genes induced by the higher zinc concentrations, 14 were found to be preferentially induced in 60- to 75-year-old individuals with a probability of >0.75 ($p = 5 \times 10^{-9}$).

MT as scavenger of reactive oxygen species

In addition to its function as a reservoir for zinc cations, MT serve as a scavenger system for reactive oxygen species (ROS). To determine the relative contribution of MT in T cells to neutralizing ROS in lieu of the traditional cellular redox systems, T cells were stimulated with TSST-1-pulsed DC in normal and in zinc supplemented medium (3 and 75 μM zinc), and superoxide production was determined by electron paramagnetic spin resonance using the TMH spin probe. Culture in the zinc-supplemented medium significantly reduced cytoplasmic superoxide production ($p = 0.006$; Fig.6A). To confirm that reduced superoxides were due to the induction of MT, T cells were stimulated and then transfected with siRNA for MT2A or control siRNA. MT2A is the most prevalent MT in T cells; MT siRNA reduced MT2A transcript expression by more than 60% and global MT protein expression by approximately 40% (data not

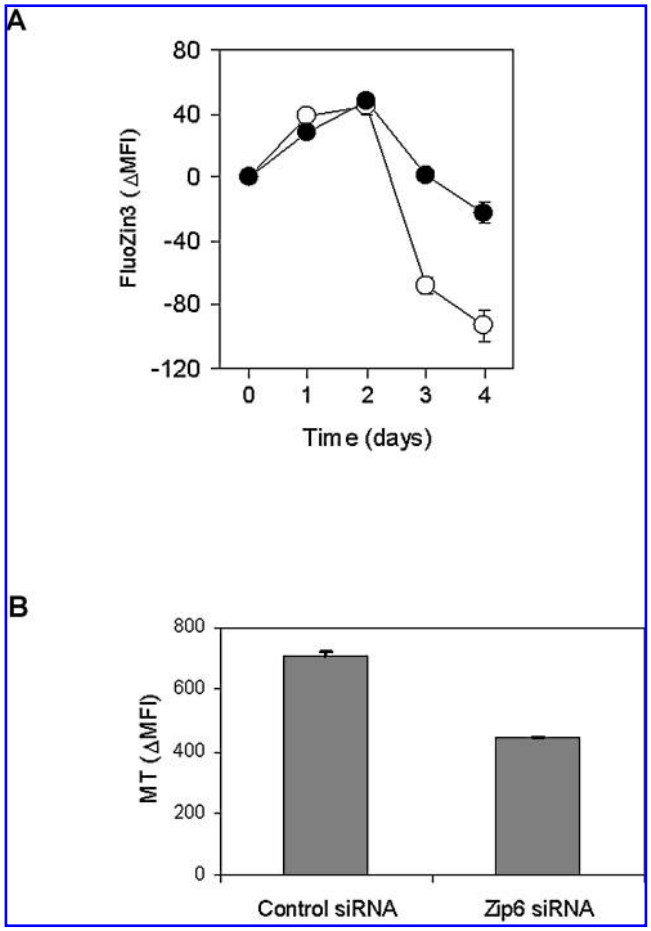

FIG. 5. Influence of age on activation-induced zinc flux. (**A**) CD4 T cells were labeled with 2 μM FluoZin3 and stimulated with TSST-1-loaded DC. FluoZin3 fluorescence intensity indicative of cytoplasmic labile zinc concentrations in $V\beta2^+CD25^+$ cells peaked 2 days after stimulation. Cytoplasmic labile zinc kinetics were compared in T cells in young and old adults; samples were always run in parallel. Results from a 35-year-old (*open circles*) and a 62-year-old individual (*black circles*) are shown as mean ± SD of triplicate wells and are representative of three donor pairs. (**B**) CD4 T cells were stimulated with anti-CD3/anti-CD28-coated beads. After 24 h of stimulation, the cells were transfected with Zip6-specific or control siRNAs and cultured in IL-2-supplemented medium. MT expression was assessed by flow cytometry on day 4 after stimulation. Results are representative of three experiments.

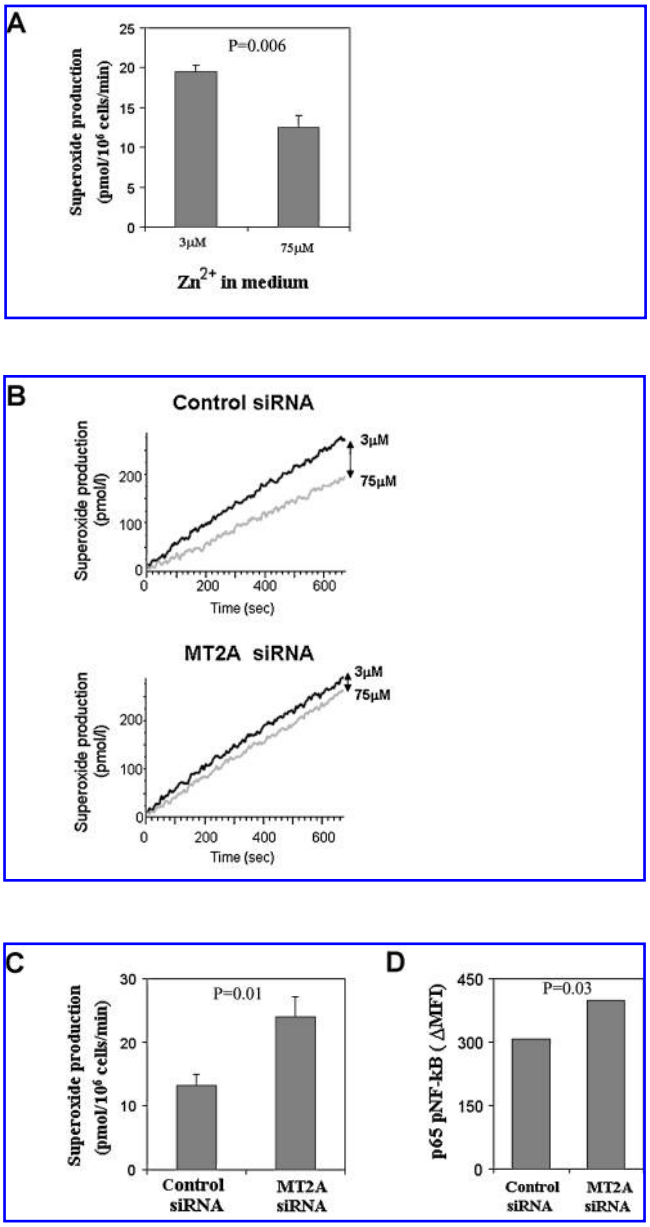

FIG. 6. MT induction decreases reactive oxygen species and dampens the NF-κB pathway. (**A**) CD4 T cells were cultured with DC and TSST-1 in 3 μM and 75 μM Zn^{2+} supplemented medium. Cells were harvested on day 4, and total superoxide production was determined by ESR. The culture in the higher zinc concentrations significantly reduced superoxide production ($p = 0.006$). (**B**) Cells were cultured as in **A** and transfected with MT2A-specific or control siRNA on day 2. A representative experiment depicting the ESR signal reflecting superoxide anion production is shown. (**C**) Superoxide production in CD4 T cells cultured for 4 days in 75 μM Zn^{2+} and transfected with MT2A-targeted or control siRNA is shown as mean ± SEM. The production was significantly increased after MT2A silencing ($p = 0.01$). (**D**) CD4 T cells were stimulated with anti-CD3/anti-CD28 beads for 2 days and transfected with MT2A-specific or control siRNA. At 24 h after transfection, cells were stimulated with 50 ng/mL of TNF-α. NF-κB phosphorylation was quantified after 10 min by PhosFlow. Data are shown as Δ MFI of stimulated and unstimulated cells and are representative of three independent experiments.

shown). MT2A silencing significantly enhanced superoxide production (Fig. 6B, C; $p = 0.01$). ROS formation is known to influence NF-κB signaling, and we therefore hypothesized that MT expression will reduce the NF-κB response. T cells were activated with anti-CD3/anti-CD28-coated beads, transfected with control or MT2A-specific siRNA on day 1, and then probed for NF-κB by PhosFlow after stimulation with 50 ng/mL TNF-α. Representative data are shown in Fig. 6D. Selective inhibition of MT2A induction increased the cytoplasmic concentration of pNF-κB (p65) after stimulation by about 30% ($p = 0.03$).

Functional role of MT in T cell proliferation

MT expression in tumor lines has been correlated with increased proliferative behavior in tumors.[27] To examine the influence of MT induction on T cell proliferation, CD4 T cells were stimulated with anti-CD3/anti-CD28 beads and transfected with control or MT2A-specific siRNA on day 2. Cell recovery and apoptosis rates were determined by FACS on day 3 following the transfection. MT2A silencing reduced T cell expansion by about 30% ($p = 0.02$; Fig. 7A). Figure 7B shows a regression analysis of MT expression and proliferative responses in 12 healthy individuals of different ages. CFSE-labeled naïve CD4 T cells were stimulated with TSST-1-pulsed DC. The number of MT1X and MT2A transcripts was determined by qPCR; the average number of divisions within $V\beta2^+$ cells was calculated from CFSE dilution. The two parameters correlated highly ($r = 0.73$, $p = 0.007$), suggesting that the higher proliferative responses in the 60- to 75-year-old individuals was due to the overexpression of MT.

Discussion

While transcriptionally profiling naïve CD4 T cell responses in the adult, we identified a hereto undefined molecular pathway that is important to T cell activation and that is modified with age. Antigen-mediated stimulation of naïve CD4 T cells transiently increased cytoplasmic labile zinc, at least in part through the zinc importer Zip6. The increase in cytoplasmic labile zinc induces the transcription of MT in a negative feedback loop. MT influence the intracellular milieu through several mechanisms, including their ability to scavenge ROS and to control zinc delivery to metalloproteins. The activity of this pathway is more pronounced with age; naïve CD4 T cells from young adults have a less sustained cytoplasmic labile zinc elevation and consequently lower MT expression after stimulation than older individuals.

We focused on the age period between 60 and 75 years because the age of 75 years appears to be a turning point for naïve CD4 cells, after which the repertoire rapidly contracts and naïve cells are increasingly defective.[28] The naïve CD4 T cell response was assessed in an *in vitro* system where T cell priming was simulated by using a superantigen and mDC. Superantigen concentration and the number of mDC were chosen to be suboptimal to unmask putative age-dependent differences. Global T cell responsiveness was intact, as documented in the functional assays as well as in the transcriptome studies, suggesting that TCR-mediated signaling and costimulation were not affected in this age group. Surprisingly, the functional studies documented a higher pro-

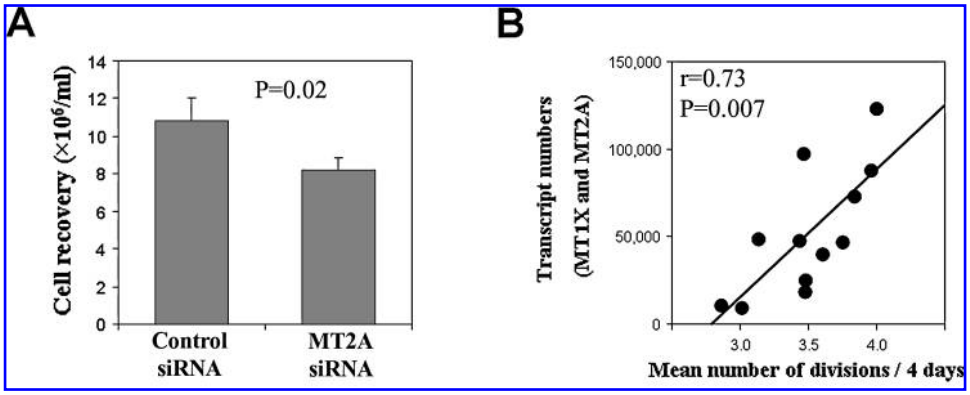

FIG. 7. Induction of MT supports T cell proliferation. (**A**) Purified CD4 T cells were stimulated with anti-CD3/anti-CD28 beads for 2 days and transfected with MT2A-specific or control siRNA. After 24 hours, 10^5 transfected cells/200 μL were expanded in IL-2-supplemented medium. Cell recovery was assessed after 48 h by flow-based cell counting after PI staining. Data are representative of four experiments. (**B**) CFSE-labeled $CD4^+CD45RA^+$ T cells were stimulated with TSST-1-loaded DC. The mean number of divisions was calculated from the CFSE dilution profile on day 4 and correlated with the transcription of MT2A and MT1X on day 3 as quantified by qPCR.

liferative rate in naïve CD4 T cells in the older age group. This finding was counterintuitive because human naïve CD4 T cells with age show evidence of an extensive replicative history with telomeric erosion.[29]

T cell stimulation was associated with an impressive upregulation of the MT family members MT1E, F, G, H, M, X, and MT2A in young adults and more so in individuals aged 60 years and older. In kinetic studies, MT transcriptional activity peaked later than traditional T cell activation markers, raising the possibility of an intermediary mechanism controlling the T cell activation-induced transcription. MT synthesis is primarily controlled by zinc that enables MTF-1 to bind to the multiple metal response elements in the MT promoter.[30,31] In addition to zinc, a number of inflammatory cytokines, including interferon-γ, TNF-α, and IL-6, induce MT transcription. Gene expression profiling did not yield any age-dependent fingerprint in these cytokine genes, and therefore we focused on the hypothesis that age influences cellular zinc homeostasis.

Bioavailable cytoplasmic zinc in stimulated CD4 T cells increased in the first 48–72 h and then slowly returned back to prestimulation levels. This decline was blunted in activated CD4 naïve T cells from 60- to 75-year-old individuals. Cellular components that regulate the cellular zinc homeostasis are MT and zinc transporters.[30,32,33] MT bind up to seven zinc atoms via a total of 20 cysteines. This binding is redox sensitive; increased oxidative or nitrosative stress can reduce the binding capacity of MT and release zinc.[34,35] Data shown in Fig. 6 clearly demonstrate that MT is an important redox system in activated T cells. Therefore, increased production of ROS during T cell activation in the elderly could explain increased zinc bioavailability and MT induction. Alternatively, zinc transport mechanisms may be involved. Two zinc transporter systems can be distinguished.[33,36,37] Members of the ZnT (SLC30A) family lower intracellular zinc by mediating zinc efflux into the extracellular fluid or influx into intracellular vesicles.[37] To the contrary, Zip proteins of the SLC39A family mediate the influx of zinc from extracellular or intracellular sources into the cytoplasm.[36] Effective negative feedback loops with induction of MT or ZnT molecules explain the normalization of cytoplasmic labile zinc in activated T cells, a feedback loop that was obviously even more induced in the elderly. The increased concentration of cytoplasmic labile zinc that we observed in activated T cells was dependent on Zip6 transporter expression. Silencing of Zip6 significantly reduced MT expression after T cell stimulation, confirming that the activation-induced upregulation of MT is due to zinc influx. The increased MT expression in stimulated naïve CD4 T cells of the older individuals could therefore be explained by increased Zip6 activity.

Fluctuations of intracellular zinc after stimulation have only recently received attention.[38] Mast cell activation via the FcεRI receptor induces a zinc wave within minutes, presumably through increased activity of intracellular zinc transporters.[39] In contrast, DC respond to lipopolysaccharide stimulation with a change in the expression profile of zinc transporters and decrease in cytoplasmic labile zinc, which is necessary for DC maturation.[40] In our studies, T cells respond to antigenic stimulation with the activation of the zinc importer Zip6 and the influx of zinc from extracellular sources. The increased intracellular level of labile zinc results in the transcriptional activation of a selected set of genes including MT and ZnT1, which lower the level of cytoplasmic labile zinc again.

Why T cells respond to antigen stimulation with zinc influx and a manifold MT induction and what the functional consequences are of the increased expression in individuals older than 60 years is of particular interest. Zinc is involved in many biological processes.[41–43] The immune system is particularly dependent on zinc,[44] and zinc deficiency has been implicated in the declining immune function with age,[45,46] MT function in zinc homeostasis, protection against exogenous heavy metal toxicity, and intrinsic oxidative damage.[47] The higher zinc influx and the associated induction of MT may represent a protective mechanism for the aging T cell to neutralize the potentially damaging effects of ROS produced during activation and to increase the proliferative potential, which is of utmost importance for the rapid clonal expansion upon antigen recognition that is characteristic of successful T cell im-

munity. Indeed, there was a strict correlation between activation-induced proliferation and MT expression, and silencing of MT expression significantly increased apoptosis and reduced cell recovery.

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Author Disclosure Statement

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