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Forum Review

Role of Redox Imbalance in the Molecular Mechanisms Responsible for Immunosenescence

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ABSTRACT

The elderly suffer impairments to their immune system, evidenced by higher susceptibility to infections, cancer, and many diseases believed to be autoimmune in nature. A dysregulated overexpression of many proinflammatory cytokines also occurs with aging, as does the synthesis of enzymes that control expression of inflammatory lipid mediators and reactive oxygen species. An inappropriate activation of redox-controlled transcription factors, like nuclear factor-κB, occurs in many tissues from aged donors, and has been linked to excesses in cellular oxidative stress. Recently, the peroxisome proliferator-activated receptor-α (PPARα) has been evaluated for its effects on inflammatory and adaptive immune processes. PPARα provides redox-balancing influences on various lymphoid cell types and their inducible responses. We recently discovered that PPARα transiently suppresses the transcription of γ-interferon (IFNγ) by inhibiting the induction of T-bet. We now report that PPARα expression in CD4+ T cells is affected by the aging process. Lower PPARα levels are present in aged CD4+ T cells, and appear responsible for the suppressed interleukin-2 and exaggerated IFNγ responses by these cells. Restoration of PPARα, T-bet, interleukin-2, and IFNγ responses was found in T cells from aged animals supplemented with vitamin E, suggesting that interventions that focus on restoring redox balance might benefit the ailing aged immune system. *Antioxid. Redox Signal.* 5, 537–548.

INTRODUCTION

Competence of the adaptive immune system declines with age

DECLINES IN THE ABILITY to generate protective immune responses following antigen insults represents one of the most dramatic and consequence-bearing conditions that occur with old age. Dysfunctional humoral and cell-mediated immune responses occur with age, and these aberrations have been implicated in the increased incidence of infectious diseases, hyporesponsiveness to vaccination, and the etiology of numerous chronic degenerative diseases that commonly afflict the elderly (36–38, 64). Such diseases include, but are not limited to, cancer, arthritis, type 2 diabetes, and many other autoimmune-type diseases.

T cells from aged individuals demonstrate impaired responses to antigen and mitogen stimulation, with both CD4+ and CD8+ T cells being adversely affected (30, 40, 41). Compromised proliferative responses following stimulation, and a dramatic change in their induced capacities to produce various cytokines, represent well studied phenomena that separate the responses elicited by young and old T cells. Production of the T-cell growth factor interleukin (IL)-2 becomes compromised with aging, whereas inducible production of other cytokines, like IL-4, IL-10, and γ-interferon (IFNγ), has been reported by many laboratories to be overproduced by activated T cells from aged donors (13, 19, 51, 67). Similar types of abnormalities in both proliferative and cytokine responses have been observed in CD4+ and CD8+ T cells isolated from aged mice and elderly human volunteers. It bears noting, however, that some inconsistencies with regards to the specific

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abnormalities in inducible cytokine synthesis do exist in the literature, and may be due to differences in experimental stimuli used, the phenotypes of responding cells, the organs from which the T cells were isolated, or host species differences.

Although major attention has been focused on T cells when attempting to explain the mechanisms responsible for adaptive immunosenescence, studies have also experimentally demonstrated that some adverse changes also occur with aging in B-cell and dendritic cell (DC) populations (8, 17, 48, 60, 61, 79). Whether, how, and by what mechanisms the aging process affects the physiology of these cell types are not well understood, but could be contributing to the declines in immune function and other immune system dysregulations that occur with the aging condition.

Synthesis of numerous proinflammatory cytokines and enzymes that synthesize inflammation-inducing signaling molecules becomes dysregulated with aging

Reports by a number of laboratories over the past decade have described pronounced age-related abnormalities in the mechanisms that serve to regulate the synthesis of many proinflammatory cytokines (10, 14, 31, 34). It is now well accepted in humans that aging is associated with increased levels of circulating proinflammatory cytokines and other inflammatory components in the blood, with elevated concentrations of tumor necrosis factor-α (TNFα), IL-6, and various acute-phase proteins being commonly observed (9, 10, 16, 28, 31, 81). We demonstrated over 10 years ago that cells within secondary lymphoid tissues from aged mice were constitutively producing the cytokine IL-6, a situation not found normally in similar tissues from mature adult controls (21, 32). In the lymphoid organs from aged mice, myeloid lineage cells showed the highest degree of dysregulation in cytokine production. In addition to IL-6, we also have observed the abnormal overproduction of TNFα, IL-12, migration inhibitory factor, cyclooxygenase-2, and inducible nitric oxide synthase (iNOS) in lymphoid tissues from aged donors (70, 74). Collectively, the cytokines and enzymes whose production becomes dysregulated with old age exert highly pleiotropic activities and, either individually or collectively, alter the physiology of many organs, tissues, and cell types throughout the body.

When under normal regulation, the various species of cytokines that become dysregulated with aging are involved in the host's inflammatory response, an innate immune process that plays an important role in host defense against infectious agents (37, 55, 69). When not properly controlled, however, chronic inflammatory responses turn pathologic, and play facilitory roles in a diverse array of acute and chronic disease conditions (10, 14). Influences by these proinflammatory cytokines have been directly implicated in the pathogenesis of such conditions as Alzheimer's disease, Parkinson's disease, atherosclerosis, arthritis, type 2 diabetes, and osteoporosis (10, 14, 34, 55, 60).

The cytokines whose production becomes dysregulated with aging also have a number of direct influences on cells of the adaptive immune system. DCs, the major antigen presenting cells of the adaptive immune system, undergo "maturation" when exposed to TNFα (4, 62). DC maturation is a process that becomes irreversibly triggered in immature DCs subsequent to influences by a proinflammatory environment. The process of DC maturation greatly alters the physiologic and functional properties of DCs, and also regulates their life span (62, 63, 75). If naive $CD4^+$ T cells are exposed to IL-6 during antigen activation, they are forced to differentiate down a Th-2 pathway (71). The chronic exposure of B cells to IL-6 can also stimulate their sustained proliferation, and has additionally been implicated in the pathophysiology of B-cell lymphoma development (45, 53, 78). Consequently, the chronic low-grade inflammatory activity that occurs in the elderly may ultimately be responsible for many aspects of immunosenescence, and altering host susceptibility to many serious age-related diseases, and for an increased mortality risk (10, 14, 34, 55).

Redox-controlled transcription factor nuclear factor-κB (NFκB) is abnormally activated with aging

One common factor that exists between all of the dysregulated proinflammatory cytokines and enzymes that are age-related is their molecular regulation by the redox-sensitive transcription factor NFκB. NFκB actually represents a small family of transcription factors that control key reactions in inflammatory, acute-phase, and immune responses. Its active form is either a hetero- or homodimeric protein complex, derived from a list of at least five well characterized protein subunits (*e.g.,* p50, p52, p65, c-Rel, and RelB). Each of the NFκB protein subunits has a common N-terminal region that contains sequences important for DNA binding, protein dimerization, and nuclear localization (3, 80). A third inhibitory subunit, inhibitor of κB (IκB), physically associates with the NFκB dimer while it is in the cytosolic compartment and inhibits the dimer's movement into the nucleus. Following activation of an IκB-specific protein kinase pathway, IκB becomes phosphorylated, ubiquinated, and is eventually degraded through proteosome-dependent proteolysis. This allows the now active NFκB dimer to translocate to the nucleus, interact with promoter regions of genes containing an appropriate DNA-binding motif, and ultimately transactivate target gene expression (3, 80). Alternatively, through protein–protein interactions, active NFκB dimers can physically associate with other classes of transcription factors, causing a subsequent repression of gene transcription by gene targets controlled by both interacting partners. The IκB-specific protein kinase pathway can be activated by diverse stimuli, including reactive oxygen species (ROS), mitogenic proteins, inflammatory cytokines, and various bacterial products (72, 80).

It was first reported that the process of aging is associated with the progressive increase in the nuclear level of NFκB in rat liver (76). This initial observation has been extended in various rodent models of aging to show similar age-dependent rises of NFκB activity in the hearts, brains, kidneys, vascular smooth muscle, and gastric mucosa of aged animals (42, 43, 52, 82, 83). We previously reported that normal murine lymphoid tissues also show an age-associated rise in NFκB activity, with both lymphoid (B and T cells) and myeloid lineage cell types showing a similar affected phenotype (74).

We hypothesized the presence of a linkage between the well accepted age-associated increases in cellular oxidative stress, the condition that underscores the oxidative stress theory of aging, and the constitutive elevations in NFκB present

in numerous tissues from aged donors, including lymphoid tissues. The dysregulated overexpression of the numerous bioactive proinflammatory cytokines, whose chronic presence has been linked to many diseases of the elderly, would then represent a major consequence of oxidative stress-induced activation of NFκB and other redox-sensitive transcription factors. We were able to experimentally test our hypothesis in a murine model of aging, where the presence of active NFκB and the dysregulated production of various proinflammatory cytokines and enzymes were evaluated in aged mice and in aged mice given vitamin E supplementation (70, 74). The vitamin E-supplemented animals were found to be normal, with minimal active NFκB in their lymphoid organs and an absence of constitutive cytokine and proinflammatory enzyme presence. Further studies have now demonstrated that aged animals supplemented with vitamin E regain near-normal levels of immunocompetence (29).

Nuclear hormone receptor peroxisome proliferator-activated receptor-α (PPARα) is involved in the control of inflammatory responses

Two additional groups of aged mice were included in our experiments using vitamin E supplementation with aged mice (70, 74). Aged animals were provided oral supplementation with low doses of the steroid dehydroepiandrosterone (DHEA) or with low doses of the chemical, WY14,643. Both of these chemical agents are known activators of PPARα, a nuclear hormone receptor that is structurally related to the classical steroid hormone receptors (*e.g.,* glucocorticoid, estrogen, androgen, etc.) (15, 27). We evaluated these chemical supplements for their antiinflammatory activities because we had previously demonstrated that DHEA treatment of aged mice was able to effectively restore normal T-cell cytokine profiles and normal immune responsiveness when administered in low doses to aged mice (2, 19, 21).

PPARα becomes a transactivation competent transcription factor following its ligand activation and heterodimerization with the 9-*cis*-retinoic acid receptor (27). Its major known transactivation functions are to control synthesis of the peroxisomal proteins involved in peroxisomal fatty acid metabolism, as well as to control a number of proteins important for the mitochondrial β-oxidation of fatty acids (11, 27, 39). Activated PPARα can also up-regulate the synthesis of IκB and, like many of the other nuclear hormone receptor members, is able to effectively "cross-couple" with active NFκB and a number of other active transcription factors and inhibit their activities (22–24). Our studies determined that treatment of aged mice with DHEA or WY14,643 was effective at suppressing active NFκB in their lymphoid tissues, and also correcting the abnormal expression of the various proinflammatory molecules that are overproduced with aging (70, 74). When similar experiments were conducted in aged mice lacking a functional PPARα protein (PPARα−/− mice), DHEA and WY14,643 supplementation was found to be ineffective, whereas vitamin E treatment remained capable of reducing lymphoid tissue levels of active NFκB and effectively eliminating the constitutive expression of proinflammatory cytokines in these animals. Therefore, the ability of DHEA or WY14,643 to reverse some aspects of the aged phenotype seems to be absolutely dependent on the presence of functional PPARα protein (70).

Besides its regulatory influence over the activity of NFκB in lymphoid cells from aged donors, PPARα and the other two known PPAR isoforms that exist in most eukaryotic cells (PPARγ and PPARβ), are now appreciated to exert pronounced regulatory influences over NFκB activities in a variety of distinct cell types (7, 25). In addition, activated PPARs can alter the activities of numerous unrelated transcription factors involved in both inflammatory and immune responses (for reviews, see 6, 11, 15, 27). Consequently, this subset of nuclear hormone receptors is now considered important in the maintenance of physiological homeostasis of many distinct tissue and cell types. They carry out these functions in diverse ways biochemically, through their influences in fatty acid metabolism and glucose utilization, as well as through their involvement in the physiologic suppression of the dysregulated chronic inflammatory responses that occur under conditions of redox imbalance. Dysregulations in the cellular activities by PPARs are now being repeatedly implicated as important etiologic factors in a wide variety of clinical conditions and chronic disease states. A number of excellent reviews have recently been published that focus attention on the roles played by the PPARs in physiology and inflammation control (6, 11, 15, 20, 25, 27, 39, 50).

PPARα affects the timing and nature of cytokines produced by activated CD4⁺ T cells

We recently uncovered a new role for PPARα in the regulation of normal CD4⁺ T-cell physiology (49). These studies determined that unliganded PPARα was capable of transiently suppressing the transcription and protein expression of T-bet, a regulatory T-box transcription factor whose activities are essential for initiating IFNγ synthesis in activated CD4⁺ T cells (44, 77). Additionally, T-bet has also been demonstrated to transcriptionally suppress the cytokine IL-2 in these same T-cell types (77). CD4⁺ T cells lacking the influences provided by an endogenous PPARα gene (PPARα−/−) were found to be markedly compromised in their ability to produce IL-2 following activation. Both mRNA and protein expression were negatively affected. In contrast, CD4⁺ T cells from these same PPARα−/− donors initiated transcription of their IFNγ gene at very early time points following activation, and greatly overproduced IFNγ protein (49).

CD4⁺ T cells from aged mice produce much lower quantities of IL-2 and elevated levels of IFNγ following activation, when their responses are compared with those of T cells from mature adult donors (13, 19, 30). This altered cytokine phenotype is similar to what is observed when comparing activities by CD4⁺ T cells from wild-type (WT) and PPARα−/− donors of similar ages (49). The primary objective of the present investigation is to determine whether the dysregulations in cytokine synthesis that are commonly observed in CD4⁺ T cells from aged hosts might be similar in responsible mechanism to those responsible for the altered cytokine synthesis by CD4⁺ T cells from PPARα−/− donors. In this situation, a premature initiation of T-bet synthesis and protein expression is observed in PPARα−/− T cells following activation. Although a number of additional, and presently unresolved, factors may also be involved in the altered control over cytokine synthesis by aged CD4⁺ T cells, our results suggest that age-related declines in PPARα levels in CD4⁺ T cells from aged donors play an influential role in these dysregulated processes.

MATERIALS AND METHODS

Animals and diets

Colonies of PPARα WT (PPARα+/+) and homozygous knockout (PPARα−/−) mice were expanded from breeding pairs obtained from Dr. F.J. Gonzalez (Metabolism Branch, National Institutes of Health, Bethesda, MD, U.S.A.). The derivation and phenotypic characteristics of these animals have previously been reported (56). Six-week-old and 20–24-month-old female C57BL/6 mice were purchased from the National Institute on Aging. BALB/c and D011.10 TCR transgenic mice on the BALB/c background were obtained from the Jackson Laboratories (Bar Harbor, ME, U.S.A.). Female mice at 6–12 weeks of age and female mice 18–22 months of age were used in these experiments. They were aged in our facility. All mice were housed in the University of Utah Animal Resource Center, which routinely monitors for the most prevalent murine pathogens, uses sentinel animals as a means for early detection of murine hepatitis virus, and maintains strict compliance with regulations established by the Animals Welfare Act. In some experiments described within the text, aged mice were provided dietary supplementation with the antioxidant, vitamin E, as previously described (29) for a 3–5 week period before they were killed.

Isolation of CD4⁺ T cells

Spleens from experimental mice were collected following death and the tissue gently dissociated into a single-cell suspension in freshly prepared tissue culture medium. Following washing, the isolated cells were diluted to a concentration of 2.5×10^8 cells/ml. For each 40 µl of cell suspension (10^7 cells), 10 µl of cell separation cocktail was added from the murine CD4⁺ T-cell isolation kit (Miltenyi Biotec, Auburn, CA, U.S.A.). After a 15-min incubation at 4°C, colloidal superparamagnetic microbeads (20 µl/10^7 cells) conjugated to a monoclonal anti-biotin antibody were added to the cell suspension and incubated for an additional 15 min at 4°C. The cell suspension was then loaded onto an AutoMacs (Miltenyi Biotec) and run according to the manufacturer's recommendation. The negatively selected cells (>90% CD4⁺) were collected, washed twice in complete medium, and used as starting cell populations in the experiments described.

ELISA

Freshly isolated CD4⁺ T cells were activated on multiwell plates that were pretreated with 2 µg/ml anti-CD3. Soluble anti-CD28 (1 µg/ml) was added for costimulation. The cells were activated for various lengths of time at 37°C in an atmosphere of 5% CO_2 in air. Cell culture supernatants were collected for quantitative evaluation of immunoactive IL-2 and IFNγ by ELISA as described previously (7, 73).

FACS analysis

Freshly prepared splenocytes were washed and resuspended in complete RPMI/2% fetal bovine serum (FBS)/0.1% NaN_3 to a concentration of $0.5–1 \times 10^6$ cells/100 µl. Cells were incubated for 15 min at 4°C with anti-FcgR II/III antibody (clone 2.4G2; PharMingen, San Diego, CA, U.S.A.) at a 1:50 dilution to block nonspecific binding of the antibody reagents. After incubation with anti-FcgR II/III antibody, cells were washed twice with RPMI/2% FBS/0.1% NaN_3 and resuspended in 100 µl of RPMI/2% FBS/0.1% NaN_3. Fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal rat and hamster antibodies against various markers, including murine CD4 (clone RM4–5), CD44 (clone IM7), CD62L (clone Mel 14) (PharMingen), or KJ1–26 antibodies, were added individually to the cells at a final concentration of 0.5 µg/ml. Cells were incubated with the antibodies for 20 min at 4°C, washed three times with RPMI/2% FBS/0.1% NaN_3, and then resuspended in 1 ml of phosphate-buffered saline/0.1% NaN_3. Isotype controls included the use of purified rat IgG2a (clone R35–95), rat IgG2b (clone A95–1), and polyclonal hamster IgG (PharMingen). Analysis of cell surface staining with labeled antibodies was performed using a FACScan (Becton–Dickinson, Mountain View, CA, U.S.A.). For each sample, cells were gated according to forward (FSC) and side (SSC) scatter, and 30,000 events were acquired and analyzed using CellQuest 3.1f software.

Quantitative, real-time PCR

Reverse transcription was performed as previously described (70). mRNA was isolated by the method of Chomczynski and Sacchi (12), and PCR was performed in a fluorescence temperature cycler (Light Cycler; Idaho Technology) as fully described elsewhere (65). The Light Cycler monitors the cycle-by-cycle accumulation of fluorescently labeled products. The cycle at which the product is first detected is used as an indicator of relative starting copy. Melting curves were acquired to determine specificity of the PCR (65). PCR products for each of the primer sets were confirmed by running samples on agarose gels. The PCR reaction was carried out in 10 µl final volume containing 3 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 1:30,000 dilution of SYBR Green I, 5 µ*M* (each) primer, 0.05 U of *Taq* polymerase, and 11 ng of TaqStart Antibody. Oligonucleotides used for these analyses are as follows: murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-AGT ATG TCG TGG AGT CTA C-3′ and 5′-CAT ACT TGG CAG GTT TCT C-3′; murine PPARα: 5′-GTG GCT GCT ATA ATT TGC TGT G-3′ and 5′-GAA GGT GTC ATC TGG ATG GGT G-3′; murine T-bet: 5′-GGA TTC TGG GGT TTA CTT CTT-3′ and 5′-TTC TCT GTT TGG CTG GCT GTT-3′; murine IFNγ: 5′-CTT CCT CAT GGC TGT TTC TGG-3′ and 5′-CGA CTC CTT TTC CGC TTC CTG-3′; and murine IL-2: 5′-GTC ACA TTG ACA CTT GTG CTC C-3′ and 5′-AGT CAA ATC CAG AAC ATG CCG-3′. GAPDH transcript levels were used to normalize the amount of cDNA in each sample, and PPARα, T-bet, IFNγ, and IL-2 transcript levels were reported relative to levels found in the control sample.

Preparation of nuclear extracts and immunoblot analysis

Nuclear extracts were prepared from T cells following treatment for various times with immobilized anti-CD3 and soluble anti-CD28 as described previously (18). In brief, cells were washed twice with ice-cold phosphate-buffered saline containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF), re-

suspended in 250 µl of buffer A (10 m*M* HEPES, pH 7.8, 0.1 m*M* EDTA, 10 m*M* NaCl, 3 m*M* $MgCl_2$, 300 m*M* sucrose, 10 µg/ml aprotinin, 100 µ*M* leupeptin, 1 m*M* dithiothreitol, and 1 m*M* PMSF), and incubated on ice for 10 min. Next, 25 µl of 1% Nonidet P-40 was added and mixed carefully. Cells were then collected by centrifugation at 800 *g* for 1 min at 4°C and washed with 200 µl of buffer A. Nuclei were then resuspended in 50 µl of buffer B (20 m*M* HEPES, pH 7.8, 3 m*M* $MgCl_2$, 420 m*M* NaCl, 0.2 m*M* EDTA, 25% glycerol, 10 µg/ml aprotinin, 100 µ*M* leupeptin, 1 m*M* dithiothreitol, and 1 m*M* PMSF) and incubated for 15 min on ice. Nuclear debris was removed by centrifugation at 16,000 *g* for 1 min.

The supernatant was then removed, and protein content was determined by the Bradford assay (70). Equal amounts of nuclear protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.) as previously described (18). After blocking with 5% nonfat milk in Tris-buffered saline, blots were incubated with anti-T-bet antibody (kindly provided by Dr. Laurie H. Glimcher, Harvard University) for 1 h at room temperature. Membranes were then washed and incubated with goat anti-rabbit horseradish peroxidase conjugate (1:2,000 dilution in Tris-buffered saline with Tween-20) for 45 min at room temperature. After washing, bands were visualized using a chemiluminescence kit according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

RESULTS

PPARα expression is depressed in CD4+ T cells isolated from aged mice

We have previously reported that activated CD4+ T cells isolated from young PPARα−/− mice produce increased levels of IFNγ, but significantly lower levels of IL-2 when compared with parallel populations of activated CD4+ T cells from young WT mice (49). The dysregulated production of IL-2 and IFNγ by the activated PPARα−/− CD4+ T cells from young donors is strikingly similar to the cytokine profile of CD4+ T cells isolated from aged WT donors (13, 19, 51, 67). In addition, we and other investigators have reported that the expression of PPARα declines from normal levels in a number of tissues within aged rodents (47, 70). Based on the similarity of cytokine profiles by activated CD4+ T cells isolated from young PPARα−/− mice and aged WT mice, we questioned whether the expression of PPARα in CD4+ T cells becomes altered with aging. To address this question, PPARα mRNA levels were compared in CD4+ T cells isolated from 2-month- and 24-month-old mice using quantitative RT-PCR (qRT-PCR). As shown in Fig. 1a, the basal levels of PPARα mRNA in resting T cells from aged donors were significantly depressed when compared with the PPARα mRNA levels within T cells from young donors. Similar to what has been reported, an increase in IFNγ protein production was observed in T cells from aged donors following activation (Fig. 1b). Additionally, we found that activated T cells from aged mice produced less IL-2 over a 24-h period post activation when compared with T cells isolated from young donors (Fig. 1c). These

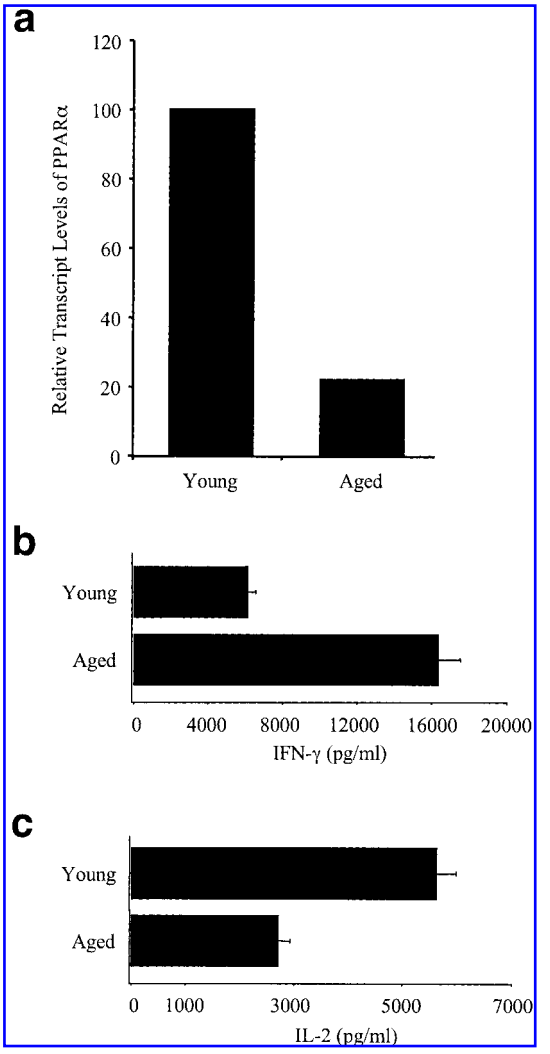

FIG. 1. T cells isolated from aged animals exhibit dysregulated production of activation-induced IL-2 and IFNγ that correlates with decreased basal levels of PPARα expression. (**a**) Freshly isolated splenic T cells from 2- and 24-month-old C57BL/6 mice were analyzed by qRT-PCR for basal levels of PPARα mRNA expression. Splenic T cells isolated from 2- and 24-month-old mice were activated on immobilized anti-CD3 plus soluble anti-CD28. Following a 24-h activation period, the levels of IFNγ (**b**) and IL-2 (**c**) in the culture supernatants were measured by ELISA.

differences in cytokine production were due to kinetic differences in the transcription of the IFNγ and IL-2 genes post activation. The T cells from the aged donors exhibited an earlier initiation of IFNγ gene transcription and an earlier termination of the IL-2 gene transcription post activation compared with T cells isolated from young donors (data not shown).

T-bet is expressed earlier in aged CD4+ T cells following activation

It has been suggested that the altered production of IL-2 and IFNγ by aged CD4+ T cells may represent a consequence of an increase in the percentage of memory T cells that reside

within secondary lymphoid organs of aged individuals (67). Memory T cells, similar to what is observed with aged T cells, exhibit an increase in the activation-induced levels of the effector cytokines, like IFNγ and IL-4, while producing lower levels of IL-2 (67). To address if differences in the memory T-cell populations between young and aged mice contribute to the differences observed in activation-induced cytokine production, we utilized $CD4^+$ T cells isolated from young and aged DO11.10 TCR-transgenic mice. The T cells within these mice have a T-cell receptor (TCR) repertoire that is limited to a specific peptide of ovalbumin (66). Therefore, in the absence of ovalbumin being administered to DO11.10 mice, all $CD4^+$ T cells remain in a naive state throughout their life span.

FACS analysis confirmed that the majority of $CD4^+$ T cells from the spleens of aged BALB/c mice express low levels of CD62L (L-selectin), whereas the majority of $CD4^+$ T cells from the spleens of young adult BALB/c mice express high levels of this cell surface marker. In contrast, the majority of $CD4^+$ T cells from the spleens of young adult and aged D011.10 donors express high levels of CD62L (Fig. 2a). Unfortunately, T lymphocytes from BALB/c strain mice express high levels of CD44 (Pgp-1) constitutively, and cannot be accurately phenotyped as naive or memory cells using this cell surface marker (59). Finding that the majority of $CD4^+$ T cells from D011.10 donors express high levels of CD62L, regardless of donor age, suggests that the naive phenotype has been retained in these TCR transgenic mice throughout the aging process.

When $CD4^+$ T cells were isolated from 2-month- and 20-month-old DO11.10 mice and evaluated for IL-2 and IFNγ production following cellular activation, it was found that the

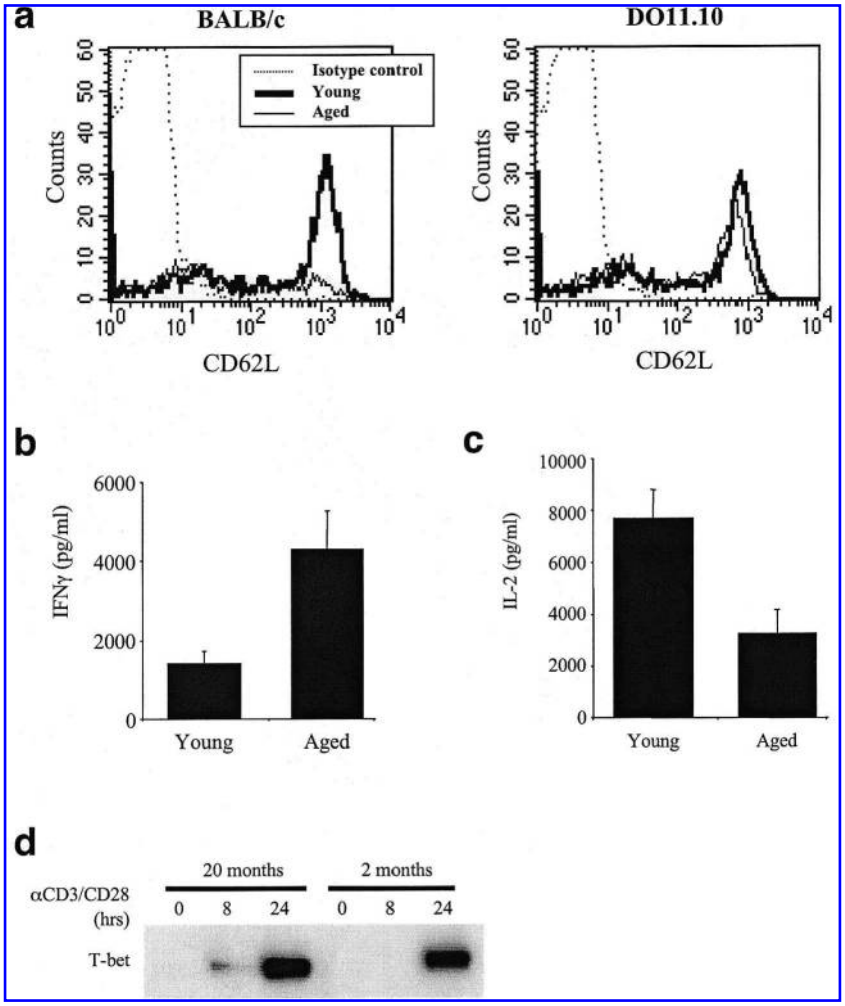

FIG. 2. Kinetic induction of T-bet protein is accelerated in aged DO11.10 T cells. (**a**) $CD4^+$ T cells from young adult and aged BALB/c or D011.10 mice were evaluated for their level of expression of CD62L, as a marker for naive T cells. In the experiments presented, the lymphocytes were gated on the $CD4^+$ T cells. Splenic $CD4^+$ T cells isolated from 2-month- and 20-month-old DO11.10 mice were activated for 24 h with immobilized anti-CD3 and anti-CD28. IFNγ (**b**) and IL-2 (**c**) levels in the culture supernatants were then analyzed for by ELISA. (**d**) T-bet protein was analyzed by western blot on nuclear extracts generated from freshly isolated $CD4^+$ T cells from 2-month- and 20-month-old D011.10 mice, or $CD4^+$ T cells from these two groups that were activated for 8 or 24 h under the same conditions as stated above.

20-month-old T cells markedly overproduced IFNγ and underproduced IL-2 when compared with T cells isolated from the 2-month-old DO11.10 mice (Fig. 2b and c). The T cells from the aged DO11.10 mice also contained a depressed level of PPARα mRNA when compared with T cells isolated from young DO11.10 donors (data not shown). These data suggest that the differences observed in IL-2 and IFNγ production between T cells from young and aged donors is not simply a reflection of the differences in the memory T-cell population known to exist between these two groups of animals. Consequently, other factors must also contribute to the dysregulated cytokine phenotype that is observed with T cells isolated from aged donors.

We have recently reported that the dysregulated production of IL-2 and IFNγ by PPARα−/− T cells from adult donors was due, in part, to acceleration in the expression of the transcription factor T-bet (49). Stemming from the similarities between young PPARα−/− T cells and aged WT T cells, we questioned whether timing of the activation-induced expression of T-bet is altered in aged T cells as well. To address this question, T-bet mRNA and protein levels were analyzed in $CD4^+$ T cells isolated from 2-month- and 20-month-old DO11.10 mice prior to T-cell activation and at 8 h and 24 h post activation with immobilized anti-CD3 and soluble anti-CD28. As shown in Fig. 2d, western blot analysis determined that T-bet protein synthesis post activation in aged DO11.10 T cells was accelerated when compared with T cells isolated from young DO11.10 animals. A parallel analysis of mRNA for T-bet by qRT-PCR established that activated $CD4^+$ T cells from aged murine donors expressed much higher levels of T-bet mRNA, and expressed it earlier (maximal at 3 h) than was found in activated $CD4^+$ T cells from young adult mice (data not shown). Thus, differences in the kinetics of T-bet expression post activation might represent a contributing factor in the differences observed in cytokine production in young and aged T cells.

Therapeutic effects of vitamin E on the dysregulated cytokine production by aged T cells

We have previously reported that the age-associated decline in basal levels of PPARα expression within splenocytes could be normalized to levels comparable to young following the supplementation of aged splenocyte donors with vitamin E (70). This led us to question whether supplementation of aged animals with vitamin E would increase basal PPARα levels in T lymphocytes to those observed in young mice. We additionally questioned whether supplementation of aged mice with vitamin E might also lengthen the time post activation before T-bet protein can be detected in T cells from the supplemented aged donors. T cells were isolated from 2-month- and 24-month-old mice as well as from 24-month-old mice whose diets had been supplemented with vitamin E. mRNA levels for PPARα were then analyzed in these three different populations of T cells. As previously demonstrated with whole splenocyte preparations, basal levels of PPARα transcripts within T cells from aged mice were much lower than the levels of PPARα transcripts within T cells from young donors. However, the levels of mRNA for PPARα within the T cells from the vitamin E-treated aged animals were quite similar to mRNA levels seen in T cells from young mice (Fig. 3a). This

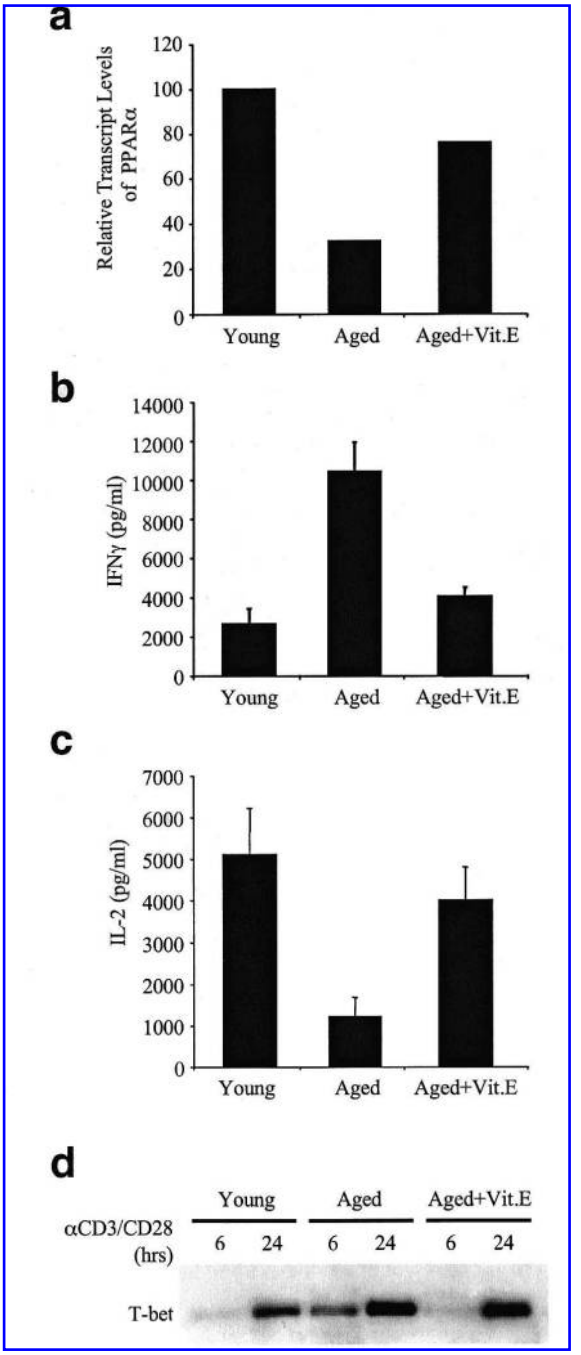

FIG. 3. Dietary supplementation of aged mice with vitamin E increases PPARα levels in T cells and restores control over T-bet expression. Twenty-four-month-old C57BL/6 mice were maintained on a control diet or a vitamin E-supplemented diet for a period of 2 weeks. After this, basal PPARα transcript levels (**a**) were analyzed in splenic T cells isolated from 2-month-old mice, 24-month-old mice, and vitamin E-supplemented 24-month-old mice. T cells isolated from these same groups were then activated for 24 h with immobilized anti-CD3 and soluble anti-CD28. The levels of IFNγ (**b**) and IL-2 (**c**) in the culture supernatants were then analyzed by ELISA. (**d**) The levels of T-bet protein were analyzed by western blot in T cells isolated from these three groups at 6 h and 24 h post activation.

supplementation-induced increase in PPARα mRNA levels within T cells correlated with corrective changes in activation-induced IFNγ and IL-2 protein levels that now resembled those observed in T cells from young mice (Fig. 3b and c). When T-bet protein was analyzed kinetically from T cells isolated from the three donor groups, T-bet protein levels from the untreated aged mice showed a rapid up-regulation post activation when compared with T cells from young donors. T cells from the vitamin E-treated mice, which expressed normal PPARα levels, now initiated T-bet expression post activation with kinetics similar to that seen in T cells obtained from young mice (Fig. 3d). These data demonstrate a correlation in the therapeutic ability of vitamin E to correct the age-associated decline in PPARα expression within T cells, the kinetics of T-bet expression post activation, and the ability of vitamin E dietary supplementation to restore control over the dysregulated cytokine production by these same cells.

Dietary supplementation with vitamin E does not restore control over the dysregulated cytokine production by T cells from PPARα−/− donors

To determine if the ability of vitamin E to bring about the correction in cytokine production in aged mice is dependent on its ability to restore the basal levels of PPARα that are observed in young T cells, we questioned whether supplementation of young PPARα−/− animals with vitamin E could normalize the levels of IL-2 and IFNγ produced by T cells isolated from these animals. To address this question, T cells were purified from the spleens of WT, PPARα−/−, and vitamin E-treated PPARα−/− mice. These T cells were then evaluated for activation-induced production of IL-2 and IFNγ. As shown in Fig. 4, the levels of IL-2 and IFNγ produced from the T cells isolated from the vitamin E-treated PPARα−/− mice remained dysregulated and were similar to the levels produced by T cells harvested from the untreated PPARα−/− animals. These data suggest that the ability of vitamin E supplementation of aged mice to correct the dysregulated T-cell production of IL-2 and IFNγ appears to be due, in part, to its ability to restore normal basal levels of PPARα and its functional properties within these cells.

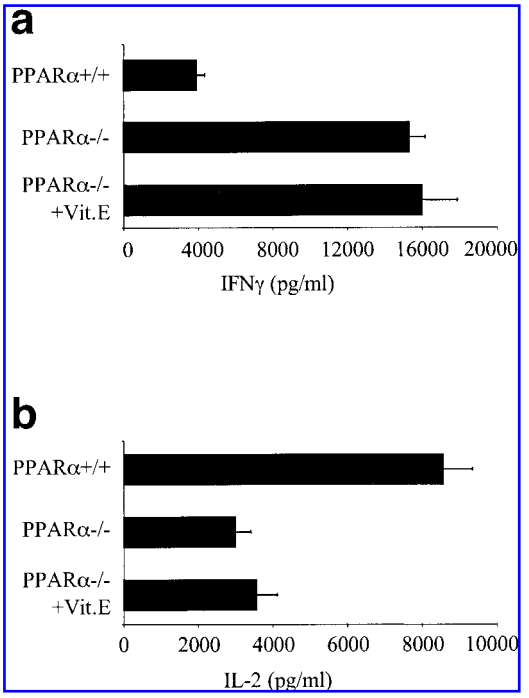

FIG. 4. T cells isolated from vitamin E-supplemented PPARα−/− animals exhibit dysregulated production of activation-induced IL-2 and IFNγ. Freshly isolated splenic T cells from WT, PPARα−/− and vitamin E-supplemented PPARα−/− mice were activated on immobilized anti-CD3 plus soluble anti-CD28. Following a 24-h activation period, the levels of IFNγ (**a**) and IL-2 (**b**) in the culture supernatants were measured by ELISA.

DISCUSSION

The studies from our laboratory support the concept that the oxidative stress excesses that occur as a consequence of aging are involved in the mechanism leading to the immunosenescence that is observed in aged individuals. We have demonstrated that elevated ROS within aged animals contributes to the constitutive activation of NFκB in a number of secondary lymphoid organs. This constitutively active NFκB contributes to the dysregulated production of numerous proinflammatory cytokines, whose activities contribute to the decreased competence of the immune system that occurs with advancing age (70, 73, 74). We have additionally demonstrated that the dysregulated activities of NFκB are paralleled by depressions in the cellular expression and activities of the nuclear hormone receptor PPARα (70). Furthermore, an implementation of therapeutic strategies using known PPARα ligands that resulted in an increase in cellular PPARα expression in the tissues of aged mice correlated with the elimination in constitutively active NFκB as well as the dysregulated production of proinflammatory cytokines and ROS-generating enzymes under the transcriptional control of NFκB (70). However, similar therapeutic strategies failed to correct the aberrant levels of nuclear-localized NFκB or the aberrant proinflammatory cytokine production in aged PPARα−/− mice (70). These data suggest that certain aspects of the aged immunological phenotype are a result of depressions in the level of functional PPARα within lymphoid tissues.

In the present report, we provide suggestive evidence that a decreased expression of PPARα within T cells from aged animals is linked with their depressed capacity to produce IL-2 following activation as well as with the dysregulated overproduction of IFNγ. As has been reported previously (70), we confirmed that providing vitamin E supplementation to aged mice increases the levels of PPARα mRNA in their T cells to levels similar to those observed in T cells from normal young donors. Dietary supplementation of old mice with vitamin E additionally restored normal temporal control over T-bet expression, and largely eliminated the age-related dysregulations in IL-2 and IFNγ production. Vitamin E dietary supplementation did not restore the dysregulations in IL-2 and IFNγ

production in PPARα−/− mice, further supporting the concept that a reduced expression of PPARα may be a major contributing factor of the altered T-cell functions that occur as a consequence of advanced age.

The dysregulation in T-cell function observed in aged individuals contributes to the increased incidence of infections, autoimmune disease, and failure of the elderly to respond successfully to vaccination (36–38, 64). Reduced IL-2 production by activated T cells from aged individuals and aged experimental animals has long been proposed to be responsible for the inadequate lymphocyte expansion and depressions in effector T-cell development that occur in the elderly (40, 41, 57). In addition, it has been demonstrated that the supplementation of cell cultures containing aged T cells with recombinant IL-2 reverses the defects in clonal expansion following their activation, and facilitates their differentiation into efficient effector T cells (40). As activities by IL-2 are critical in the initial stages following T-cell activation, PPARα's ability to sustain IL-2 production post activation would be very advantageous to T-cell physiology. Furthermore, it is now appreciated that IL-2 is critical for the induction and maintenance of regulatory T cells (35). Consequently, the age-associated reductions in IL-2 may also be involved in promoting the development of autoimmunity in the elderly by reducing the efficiency of regulatory T-cell development.

The ability of PPARα to delay the expression of IFNγ in activated T cells may be as important to T-cell function as sustaining the production of IL-2. IFNγ has been reported to play a central role in the maintenance of peripheral tolerance (33). Signaling through the IFNγ receptor induces the expression of certain genes, like indoleamine 2,3-dioxygenase (IDO) and iNOS within antigen presenting cells (46, 58). The expression of IDO and iNOS provides antigen presenting cells with mechanisms to inhibit T-cell proliferation and induce T-cell apoptosis (46, 58). Therefore, the rapidly induced production of IFNγ by aged T cells following their stimulation could promote an accelerated expression of IDO or iNOS within the peptide-antigen presenting DCs and inhibit the responding T cells from undergoing effective clonal expansion. We have recently found that the increased IFNγ production by T cells isolated from aged donors results in an increase in the level of iNOS and nitric oxide produced by neighboring DCs that are presenting antigen to the T cells (unpublished results). The increases in nitric oxide levels within these cultures contribute to the significant decrease in aged T-cell proliferation. Therefore, an increase in the expression of iNOS and/or IDO by peptide presenting DCs, coupled with the decreased IL-2 production by aged T cells subsequent to activation, would severely compromise the ability of antigen-specific T cells to undergo adequate clonal expansion.

Due to the numerous immune responses that become altered as a consequence of the T-cell dysfunctions observed in aging, a number of studies have proposed therapies designed to restore normal T-cell function in the elderly. Numerous reports exist demonstrating the beneficial effects of vitamin E supplementation on aged T-cell function, including a restoration of IL-2 and IFNγ production to levels that are observed in young T cells. Here, we demonstrate that the ability of vitamin E to reestablish control over IL-2 and IFNγ production is due in part to its ability to increase the levels of PPARα expression in aged T cells.

A number of molecular mechanisms may be responsible for depressing basal levels and activities of PPARα within aged T cells. It is known that the activities of various transcription factors that control the expression of PPARα, including the glucocorticoid receptor and Sp1, are susceptible to alterations in cellular redox state (1, 55). Increases in oxidative stress that are observed in tissues from aged animals may contribute a reduced ability of these transcription factors to effectively transcribe the genes, including PPARα, that are under their control (70). Additionally, the aberrant overproduction of the proinflammatory cytokines IL-1β, IL-6, and TNFα by aged individuals may also contribute to the age-associated decrease in the cellular levels of PPARα expression (5, 68). The molecular mechanism(s) through which these cytokines mediate their suppressive effects have yet to be uncovered, although it has been reported that both IL-1β and TNFα can inhibit Sp1 activity, a transcription factor for which seven putative binding sites have been identified within the promoter region of the PPARα gene (26, 54). Interestingly, it is an excess in ROS that can induce each of the mechanisms that potentially down-regulate PPAR expression. The ability of vitamin E supplementation to reduce the age-associated redox imbalance may explain how this therapeutic intervention increases PPARα expression within lymphoid cells isolated from aged donors and exerts its corrective influences on T-cell cytokine production.

Fluctuations of PPARα levels within T cells can influence the timing and quantity of IL-2 and IFNγ produced following activation. As these two cytokines are important in the initiation, regulation, and maintenance of immune responses, any influences over the transcriptional expression of PPARα and its activities might prove important in designing interventions to enhance immune competence and reduce immunopathologies in the elderly.

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ABBREVIATIONS

DC, dendritic cell; DHEA, dehydroepiandrosterone; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of κB; IDO, indoleamine 2,3-dioxygenase; IFNγ, γ-interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor κB; PMSF, phenylmethylsulfonyl fluoride; PPARα, peroxisome proliferator-activated receptor-α; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; T-bet, T-box expressed in T cells; TCR, T-cell receptor; TNFα, tumor necrosis factor-α; WT, wild-type.

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