Distinctive immunoregulatory effects of adenosine on T cells of older humans

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ABSTRACT **A role for adenosine in immunosenescence was investigated in T cells from older (≥65 yr) and younger (24–45 yr) healthy humans. Adenosine concentrations in cultures of activated T cells were significantly higher ($P<0.0001$) for older (145±47 nM, mean±SD) than younger (58±5.5 nM) subjects. Expression of the activation coreceptor CD28 was suppressed significantly by 0.1 to 1 μM exogenous adenosine, with greater effects of 1 μM ($P<0.01$) on T cells of younger (mean suppression of 67 and 65% for CD4 and CD8 T cells, respectively) than older (means of 42 and 46%) subjects. T-cell chemotaxis to CCL21 was suppressed significantly by 0.3 and 1 μM exogenous adenosine, with mean maximum decreases of 39 and 49%, respectively, for younger subjects and 28 and 31% for older subjects. Generation of IL-2 and IFN-γ by T cells of younger and older subjects was suppressed substantially only at adenosine levels of 3 μM or higher. Lower baseline expression of CD28 and chemotaxis to CCL21 and S1P for T cells from older subjects attributable to endogenous adenosine were reversed completely by two different A_{2A} adenosine receptor antagonists without affecting T cells of younger subjects. Adenosine is an endogenous T-cell immunosuppressor in older humans, and A_{2A} antagonists reverse adenosine-induced T-cell deficiencies of aging.—Hesdorffer, H. S., Malchinkhuu, E., Biragyn, A., Mabrouk, O. S., Kennedy, R. T., Madara, K., Taub, D. D., Longo, D. L., Schwartz, J. B., Ferrucci, L., Goetzl, E. J. Distinctive immunoregulatory effects of adenosine on T cells of older humans. *FASEB J.* 26, 1301–1310 (2012). www.fasebj.org**

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ADENOSINE IS A PROMINENT mediator in many organ systems, in which the specific cellular effects are regulated by control of local tissue concentrations and levels of expression of one or more of its G-protein-coupled receptors. T cells generate adenosine through the sequential conversion of ATP to AMP by the ecto-nucleoside triphosphate diphosphohydrolase-1 activity of CD39 and AMP to adenosine by the ecto-5′-nucleotidase function of CD73 (1, 2). Of the T-cell subsets, only $Foxp3^+$ CD4 T-regulatory (Treg) cells express both CD39 and CD73 (3). CD39 also is expressed by memory CD4 T cells of the Th1, Th2, and Th17 types, which lack Treg functions and do not bear CD73 (4). CD73 is also expressed by most CD8 T cells and activated CD4 effector T cells, lacking Treg functions, and is especially prominent on tissue-infiltrating T cells as a result of its mediation of their binding to vascular and lymphatic endothelium in inflamed tissues (5–7). Thus, it is clear that optimal adenosine production by T cells other than Tregs requires the concerted participation of both effector and memory sets. Levels of adenosine in blood are normally 100 to 300 nM, and in microdialysates of murine extracellular fluid, levels were 30 ± 5 nM (mean±SE) for normal subcutaneous tissues and 0.2 to 2.4 μM for subcutaneous murine and human tumors (8). Concentrations of adenosine in lymphoid tissues normally and during immune responses have not been determined, but most *in vitro* studies of its effects have employed 5 to 25 μM adenosine (9–12). Human and mouse T cells express predominantly the A_{2A} subtype of adenosine receptor, with lower abundance of the A_{2B} and A_3 subtypes, and little of the A_1 subtype (13, 14). The levels of the A_{2A} receptor, and to a lesser extent, the A_{2B} and A_3 subtypes, on T cells are increased nearly 10-fold by immunological stimulation (14). The functional dominance of T-cell A_{2A} receptors is amplified greatly by their ~100-fold greater affinity for adenosine than the A_{2B} and A_3 subtypes (13). Although quite

Abbreviations: APC, allophycocyanin; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; PE, phycoerythrin; S1P, sphingosine 1-phosphate; Treg cell, T-regulatory cell; UPLC, ultra-high pressure liquid chromatography.

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preliminary, existing data for mouse T cells suggest that inhibition by adenosine of cytokine generation and expression of some cell-surface antigens may be preferentially transduced by A_{2A} receptors, whereas adenosine prevention of proliferation may be largely signaled by A_3 receptors (13).

T-cell immune effects of adenosine often are considered in 2 timeframes because regulation of T-cell trafficking was observed predominantly acutely, and both reduction of telomerase activity and decreases in expression of CD28 by human CD8 T cells have required chronic adenosine exposure. Single additions of 5 to 25 μM adenosine to mouse or human T cells suppressed proliferation and the generation of IL-2, IFN-γ, and IL-4 acutely, and multiple additions of the same concentrations of adenosine to T-cell cultures over several weeks diminished IL-2 gene transcription (9–12). Generation of adenosine was necessary for optimal Foxp3Treg-cell suppressive activity in short-term cultures (3). In the initial 24 h of mouse or human T-cell responses, adenosine reduced activation-induced apoptosis acting through the A_{2A} adenosine receptor, but its ability to influence survival for longer periods remains controversial (15). Adenosine at the same 5 to 25 μM levels rapidly decreased the expression of CD28 in murine mixed T cells, but much longer incubations were needed for the same effects in human CD8 T cells (10, 12).

The most complex immune activity of the adenosine mediator system appears to be acute regulation of T-cell trafficking in afferent lymphatics. The T cells in afferent lymph and afferent lymphatic endothelium, but not efferent lymphatic endothelium, both express high levels of CD73, which presumably generates adenosine as well as enhances T-cell adhesiveness (7). In two different experimental systems, deletion of CD73 on lymphocytes gave opposite results of decreased migration to draining lymph nodes by >50% in one and no difference in lymphocyte trafficking in the other (6, 7). Nonetheless, the importance of adenosine as a primary mediator capable of decreasing T-cell movement in afferent lymphatics was demonstrated by the striking suppression of their entry into lymph nodes in CD73-deficient mice when a selective adenosine agonist was administered to restore the usual level of adenosine receptor signaling (6).

Chronic effects of repeated additions of adenosine on human CD8 T cells together resembled replicative senescence, including decreased proliferative potential, reduced IL-2 gene expression, diminished surface levels of CD28, and lower telomerase activity (12). Development of these T-cell characteristics of an aging immune system required exposure of cultured human T cells to 10 μM adenosine for 70 d or longer. The purpose of the present study of immunosenescence was to establish the levels of endogenous adenosine usually present in the extracellular fluid of lymphoid tissue T cells in older and younger subjects and to identify differences between human T cells from older and younger subjects in their functional responses to these physiological concentrations of adenosine.

MATERIALS AND METHODS

Subject selection and study design

Each of 18 healthy subjects aged 65 yr or older was studied concurrently with a healthy younger control of age 24 to 45 yr, with older to younger matching based on sex and race. There were 13 healthy younger controls, of whom 5 served as controls for 2 older subjects. Subjects were recruited from the Clinical Research Unit and Baltimore Longitudinal Study of Aging populations of the U.S. National Institute on Aging and from long-term care and ambulatory populations of the Jewish Home of San Francisco. Every participant was informed of the procedures and potential risks of donating 60 ml of venous blood before signing a consent form, and the study protocol was approved by the committees for human research at the two institutions. None of the subjects was receiving any immunoactive drug or hormone. The mean ± SD age of the older subjects was 77 ± 6.6 yr with a range of 65–88 yr; 11 were Caucasian, 5 were African-American, and 2 were Asian. The mean ± SD age of the older women (n=9) was 75 ± 7.5 yr with a range of 65 to 85 yr. The mean ± SD age of the older men (n=9) was 78 ± 5.6 yr with a range of 72 to 88 yr. The mean ± SD age of the younger subjects was 37 ± 7.8 yr with a range of 24 to 45 yr; 9 were Caucasian, 2 were African-American, and 2 were Asian. The mean ± SD age of the younger women (n=5) was 35 ± 9.3 yr with a range of 26 to 45 yr. The mean ± SD age of the younger men (n=8) was 37 ± 7.1 yr with a range of 24 to 45 yr. There were no significant group age differences between men and women in either age group.

Chemicals

The A_{2A} receptor antagonists ANR94 and SCH58261 and the A_{2A} receptor agonist CGS21680 hydrochloride (Tocris Biosciences, Ellisville, MO, USA), the A_3 receptor antagonist 2-phenylamino-N6-endo-norbornyladenine (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (EHNA), adenosine, sphingosine 1-phosphate (S1P), and recrystallized bovine serum albumin (Sigma Chemicals, St. Louis, MO, USA) were obtained from the designated suppliers.

Isolation of T cells

Heparinized blood was diluted 1:1 (v:v) with calcium- and magnesium-free PBS, and 30-ml portions were centrifuged on 10-ml cushions of Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 30 min at 400 *g* to resolve mixed mononuclear leukocytes at the interface from other blood cells, as described previously (16). The total population of T cells was obtained at >96% purity by 2 cycles of immunomagnetic depletion of all non-T-cell mononuclear leukocytes with an antibody cocktail negative selection kit (Miltenyi Biotec, Auburn, CA, USA).

Determination of the concentrations of adenosine and inosine in T-cell culture supernates

Three sets of duplicate suspensions of 1×10^6 T cells in 1 ml of RPMI 1640 with 10% fetal bovine plasma, 100 U/ml of penicillin G, and 50 μg/ml of streptomycin (complete RPMI)

were incubated in 24-well plates without (1 set) and after (2 sets) precoating with 1 μg each of anti-human CD3 plus anti-human CD28 antibodies, as described previously (17). At 24 h, the T cells were washed twice and resuspended in plasma-free RPMI for an additional 24 h of incubation in original wells. The ADA inhibitor EHNA was added to one duplicate set of antibody-precoated duplicate wells at 10 μM. Plates then were centrifuged at 3000 *g*, and the supernates were harvested, passed through 0.22-μm-pore filters (Millex; Sigma-Aldrich), and stored at −80°C. To quantify adenosine and inosine, 9-μl aliquots of each T-cell supernatant were injected in triplicate into an ultra-high-pressure liquid chromatography (UPLC) mass spectrometer. The UPLC apparatus with a nanoAcquity autosampler (Waters, Waltham, MA, USA) was fitted with a Waters HSS T3 C18 column (1×100 mm, 1.8 μm) coupled to a Waters/Micromass Quattro Ultima II triple-quadrupole mass spectrometer in positive mode, operating with multiple reaction monitoring. Quadrupole settings were capillary voltage equal to 3 kV, source temperature equal to 140°C, cone voltage equal to 35 kV, and collision energy equal to 20 eV. The elution program was equilibration prior to injection in 100% mobile phase A (10 mM ammonium formate in 0.15% formic acid), a rapid step from 100% A to 77% A:23% B (acetonitrile) that was continued for 2 min, and a rapid step back to 100% A. Calibration was performed with a 1 nM to 1 μM 5-point standard curve.

Flow-cytometric analyses of T-cell expression of surface protein antigens

T cells were harvested, washed twice in flow-cytometry buffer (PBS with 1 g/100 ml of BSA and 0.1 g/100 ml of sodium azide), incubated 10 min at room temperature in human Fc receptor blocking solution (Biolegend, San Diego, CA, USA) diluted 1:20 with flow-cytometry buffer, and incubated for 30 min at 4°C in the dark with multiple fluorescently labeled antibodies at recommended dilutions in flow-cytometry buffer. After fixation in 1% paraformaldehyde and washing in flow-cytometry buffer, T-cell labeling was examined in a FACScalibur system equipped with CellQuest software (BD-Pharmingen, San Diego, CA, USA), as described previously (18). Antibodies and their sources were mouse anti-human CD8-allophycocyanin (APC) and mouse anti-human CD39-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), mouse anti-human CD4-APC and mouse anti-human CD73-phycoerythrin (PE; clones OKT4 and AD2, respectively; Biolegend), and anti-human CD28-PE (clone CD28.2; R&D Systems, Minneapolis, MN, USA). A mouse monoclonal IgG2a anti-human adenosine A_{2A} receptor antibody (Life Span Biosciences, Seattle, WA, USA) was labeled with PE using a standard kit (Lightning-Link PE-Cy5.5 Tandem conjugation kit; Innova Biosciences, Cambridge, UK).

Cytokine ELISAs

The concentrations of IL-2 and IFN-γ in duplicate aliquots of T-cell supernates were quantified at respective dilutions of 1:5 and 1:300 with colorimetric ELISAs (MiniKits from Thermo Scientific-Pierce Biotechnology, Rockford, IL, USA). Color intensity was determined in a VersaMax Microplate Reader (Sunnyvale, CA, USA).

Quantification of T-cell chemotaxis

Transwell plate permeable upper inserts with a 5-μm-pore filter (Corning Life Sciences, Lowell, MA, USA) were preincubated overnight at 4°C in human type IV collagen, washed, and dried, as described previously (19). Each of duplicate upper inserts received 1×10^6 T cells in 0.1 ml of RPMI 1640 with 10% dextran- and charcoal-extracted FBS (lipid-depleted FBS; Univeristy of California–San Francisco Cell Culture facility) and was placed in the plate over 0.6 ml of RPMI-lipid-depleted FBS without (control) or with 50 nM CCL21 (Peprotech, Rocky Hill, NJ, USA) or 100 nM S1P in its lower compartment. Some suspensions of T cells were preincubated for 10 min at room temperature with adenosine, the ADA inhibitor EHNA, the A_{2A} receptor agonist CGS21680, or the A_{2A} receptor antagonists SCH58261 or ANR94 prior to loading in the chemotactic chamber. After incubation at 37°C for 4 h, the number of T cells in each lower compartment was determined by microscopic counting and expressed as a percentage of the initial number added to the upper insert.

Statistical evaluations

The significance of differences between mean values in any series of studies was calculated by a 2-sample *t* test or with a nonparametric method if data distribution was not normal (GraphPad Software, La Jolla, CA, USA).

RESULTS

Human T-cell generation of adenosine

The capacity of human blood T cells from healthy older and younger donors to produce adenosine was examined using a mixed T-cell population, which expresses the enzymes CD39 and CD73 required to convert ATP to adenosine. Adenosine concentrations in the culture medium of T cells activated by an antigen receptor-

TABLE 1. *Greater generation of adenosine by T cells of older healthy than younger healthy subjects*

Age group	Subjects (*n*)	T-cell activation	ADA inhibitor	Adenosine concentration (nM)	Inosine concentration (nM)
Old	9	+	−	145 ± 47**	100 ± 41*
Young	8	+	−	58 ± 5.5	52 ± 24
Old	6	+	+	333 ± 196	224 ± 193
Young	5	+	+	344 ± 494	187 ± 286
Old	3	−	−	18 ± 4.4	21 ± 3.6
Young	3	−	−	13 ± 1.7	20 ± 5.5

Adenosine and inosine concentrations are standardized for 10^6 mixed T cells/ml; values are means ± SD. Studies were conducted in duplicate, and supernates were assayed in triplicate. Subjects in the bottom 4 rows are subsets of those studied for the top 2 rows. The ADA inhibitor was 10 μM EHNA. A 2-sample *t* test was used to compare results for T cells of older subjects with those of younger subjects, except for values with EHNA, where distribution was non-normal and comparison was with a Mann-Whitney nonparametric test. *$P = 0.0108$; **$P = 0.0001$.

dependent mechanism were substantially higher than those of unactivated T cells and were very significantly higher for older than younger subjects (**Table 1**). Concentrations of inosine, the product of adenosine deaminase biodegradation of adenosine, also were significantly higher in culture medium of activated T cells from older than younger subjects. When EHNA was introduced to inhibit adenosine deaminase, adenosine concentrations were higher, but results were non-normally distributed with greater scatter, and there was no difference between mean values for activated T cells of old and young subjects. These findings indicate that the range of adenosine concentrations most relevant to investigations of effects on T-cell immune functions is 0.1 to 1 μM rather than the previously studied range of 5 to 25 μM (9–12).

Effects of adenosine on human T-cell production of cytokines

A single addition of 0.3 μM adenosine at the beginning of antigen receptor stimulation of T cells from older and younger subjects failed to suppress generation of IL-2 significantly (**Fig. 1**). In contrast, one addition of an optimally effective concentration of the adenosine deaminase inhibitor EHNA at the beginning of the same activating incubation very significantly suppressed generation of IL-2 by T cells from older and younger subjects. As adenosine deaminase inhibition is expected to establish a medium concentration of endogenous adenosine near 0.3 μM (Table 1), this result suggests that a sustained level of adenosine has far greater effects on T-cell cytokine generation than those attained by a transient pulse of the same or higher concentrations. This possibility is supported by a finding that single additions of 1 and 3 μM adenosine did not materially reduce the levels of IL-2 or IFN-γ achieved by activated T cells from older or younger subjects (Fig. 1). Adenosine (10 μM) in a single addition reduced IL-2 and IFN-γ production by activated T cells from older and younger subjects, and the decrease in IL-2 level was similar to that attained by the approximate sustained concentration of 0.3 μM adenosine from adenosine deaminase inhibition (Fig. 1).

To further elucidate the possible importance of persistent adenosine levels in regulation of human T-cell cytokine production, the effects of multiple additions of exogenous adenosine were compared to those of the elevated adenosine level attained by inhibition of adenosine deaminase. Multiple additions of 3 μM adenosine significantly reduced the levels of IL-2 and IFN-γ achieved by activated T cells from older and younger subjects by a mean of 43 to 65% (**Fig. 2**), whereas it had suppressed by only 24% or less after a single addition (Fig. 1). Multiple additions of 10 μM adenosine or EHNA suppressed the production of IL-2 and IFN-γ to the same very significant extent as had a single addition. In the presence of normal levels of adenosine deaminase activity, thus, it appears that the usually expected concentrations of endogenous adenosine would have only modest regulatory effects on cytokine production by T cells of younger or older humans.

As the T-cell activation required to increase medium levels of adenosine also may evoke generation of other immunoregulatory factors, it is only possible to distinguish specific effects of adenosine by the application of selective pharmacological probes of the T-cell adenosine system. To examine the role of endogenous adenosine as a suppressor of IL-2 generation, the SCH58261 antagonist of A_{2A} adenosine receptors that predominate in T cells (13–15) was added to cultures of mixed T cells from 3 older and 3 younger healthy subjects twice at 0 and 24 h. In the absence of the antagonist, generation of IL-2 by

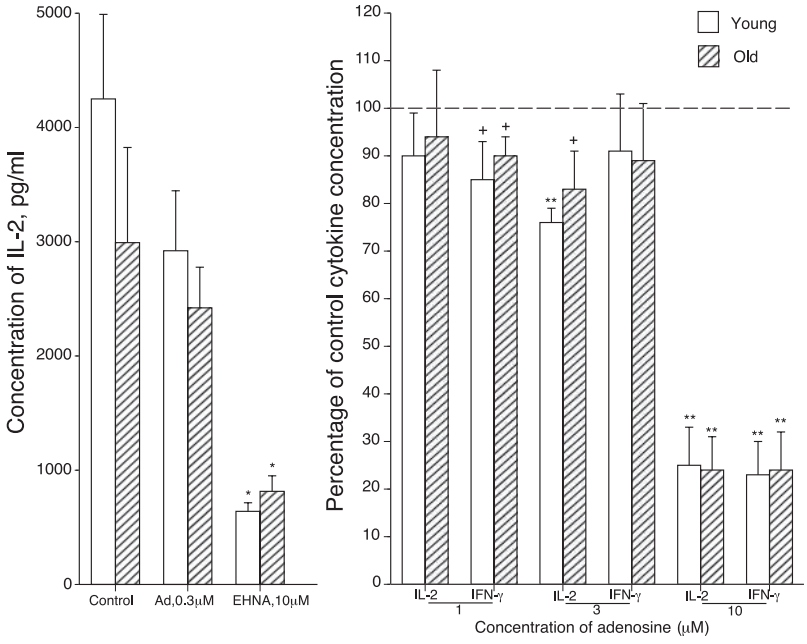

Figure 1. Effects of one addition of adenosine or the adenosine deaminase inhibitor EHNA on T-cell generation of cytokines. T cells were added to wells with adherent anti-CD3 + anti-CD28 antibodies on d 0 after a 10-min preincubation and continued incubation with a single addition of 0.3 to 10 μM adenosine or 10 μM EHNA. Aliquots of supernates were removed on d 1 (24 h) for IL-2 ELISAs and d 2 (48 h) for IFN-γ ELISAs. Bars represent means ± SD of results of studies of T cells from 3 older men and women and 3 younger matched control men and women. In the right panel, control cytokine levels (100%) were 5518 ± 804 pg/ml IL-2 and 45,326 ± 3519 pg/ml IFN-γ for older subjects; 7420 ± 1168 pg/ml IL-2 and 56,775 ± 3915 IFN-γ for younger subjects. Ad, adenosine. $^{+}P < 0.05$, $^{*}P < 0.01$, $^{**}P < 0.001$ *vs.* control.

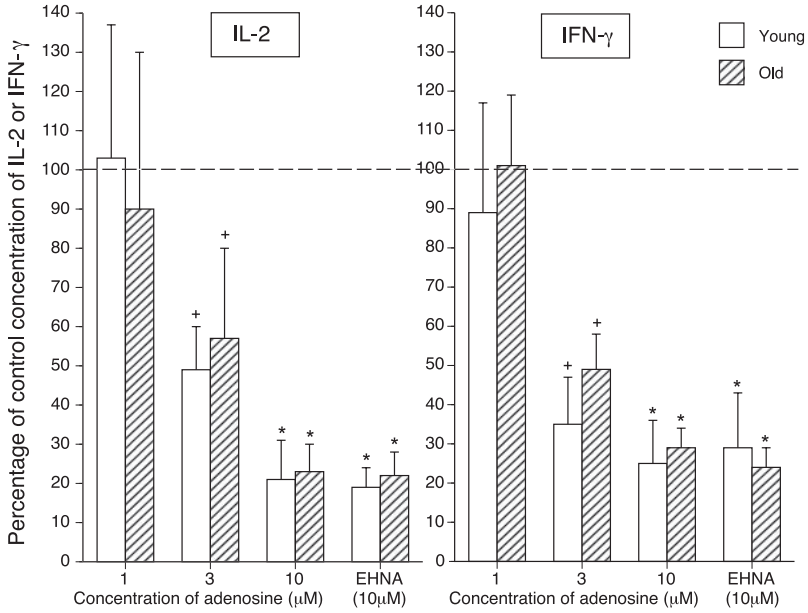

Figure 2. Effects of multiple additions of adenosine or the adenosine deaminase inhibitor EHNA on T-cell generation of cytokines. Procedural protocol was the same as in Fig. 1, except that adenosine and EHNA adenosine deaminase inhibitor were added to T cells on d 0, 1, and 2, with removal of aliquots of supernates at 36 h for IL-2 ELISAs and at 56 h for IFN-γ ELISAs. Bars represent means ± SD of results of studies of T cells from 3 older men and women and 3 younger matched control men and women. Control IL-2 levels (100%) were 1153 ± 485 pg/ml for T cells of older subjects and 1773 ± 609 pg/ml for younger subjects; control IFN-γ levels (100%) were 35,104 ± 6149 pg/ml for T cells of older subjects and 36,426 ± 12,585 pg/ml for younger subjects. $^{+}P < 0.05$, $^{*}P < 0.01$ *vs.* control.

activated T cells at 36 h was a mean ± SD of 1467 ± 153 pg/ml for older subjects and 3346 ± 611 pg/ml for younger subjects. With the introduction of 200 nM SCH58261 at 0 and 24 h, generation of IL-2 by activated T cells at 36 h was a mean ± SD of 1517 ± 282 pg/ml for older subjects and 2981 ± 201 pg/ml for younger subjects. As there were no significant changes after blocking the A_{2A} adenosine receptor, endogenous adenosine appears to have little regulatory effect in either age group. It also is not possible to attribute the known lower levels of IL-2 generation by T cells of older than younger healthy subjects (20–22) to a suppressive effect of endogenous adenosine.

Effects of adenosine on human T-cell expression of surface protein antigens

Multiple additions of 1 μM adenosine or 10 μM EHNA adenosine deaminase inhibitor resulted in significant suppression of T-cell surface levels of CD4, CD8, and CD28 on activated T cells of both younger and older subjects after 5 d of incubation (**Fig. 3**). In every instance, the extent of suppression of these surface protein antigens was significantly greater for T cells of younger than older subjects. A greater sensitivity to adenosine of the higher levels of CD28 expression by T cells of younger than older subjects was supported by

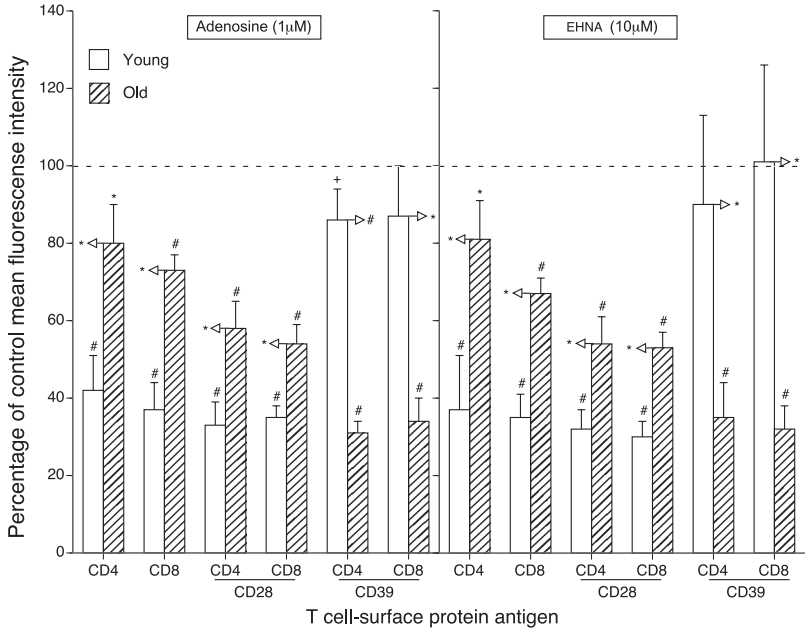

Figure 3. Effects of multiple additions of adenosine or the adenosine deaminase inhibitor EHNA on T-cell expression of surface protein antigens. Adenosine (1 μM) and the adenosine deaminase inhibitor EHNA were added to T cells on d 0 (with a 10-min preincubation before addition to adherent anti-CD3 antibodies), 2 and 4. On d 5, T cells were harvested, washed, and labeled for flow cytometric assessment of expression of CD28 and CD39 separately on the CD4 and CD8 subsets. Bars represent means ± SD of results of studies of T cells from 2 older men and women (n=4) and 2 younger matched control men and women (n=4). Mean ± SD fluorescence intensity of expression of CD28 by control (100%) CD4 T cells and CD8 T cells, respectively, was 673 ± 59 and 756 ± 32 for younger subjects and 409 ± 18 and 475 ± 34 for older subjects (P=0.0001 for young *vs.* old groups in both subsets). Mean ± SD fluorescence intensity of expression of CD39 by control (100%) CD4 T cells and CD8 T cells, respectively, was 265 ± 46 and 264 ± 17 for younger subjects and 163 ± 18 and 150 ± 12 for older subjects (P<0.05 for young *vs.* old groups in both subsets). Symbols at tops of bars indicate statistically significant difference *vs.* corresponding control; symbols next to horizontal arrowheads indicate difference between bars of that set. $^{+}P < 0.05$, $^{*}P < 0.01$, $^{\#}P < 0.001$.

findings of the effects of similar multiple additions of 0.1 μM adenosine over 5 d to stimulated cultures of CD8 T cells from a subgroup of these subjects. CD28 expression was suppressed by a mean ± SD of 28 ± 5% for CD8 T cells of younger subjects ($P<0.01$), but only by an insignificant 7 ± 4% for those of older subjects ($n=3$ young-old pairs). This differential sensitivity may reflect the chronic accommodation of T cells from older subjects to higher levels of adenosine than those usually experienced by the T cells from younger subjects and the higher baseline levels of CD28 expression by T cells of younger than older subjects (Fig. 3 legend).

In contrast, highly significant adenosine suppression of expression of CD39, the enzyme that converts ATP to AMP, was only seen in CD4 and CD8 T cells from older subjects. Activated T-cell expression of CD73, the enzyme that converts AMP to adenosine, was the same in young and old subjects at respective mean ± SD fluorescence intensity levels of 53 ± 11 and 51 ± 12 ($n=5$/group). Expression of CD73 by activated T cells also was not altered by 48 h of incubation with 10 μM adenosine plus 10 μM EHNA, with mean fluorescence intensity levels of 48 ± 10 for younger and 50 ± 11 for older subjects. Thus, it seems unlikely that the higher levels of adenosine generated by T cells of older than younger subjects are attributable to lower feedback suppression of CD39 activity by endogenous adenosine. The greater sensitivity to suppression by adenosine of expression of some immunologically functional surface protein antigens than generation of major immune cytokines suggests a potential rank order of importance of endogenous adenosine in regulation of diverse T-cell functions.

Three concentrations of the A_{2A} adenosine receptor agonist CGS21680 were employed in similar studies designed to confirm the specificity of suppression of T-cell surface protein antigens by the adenosine system. By d 5, multiple additions of 100 nM CGS21680 significantly suppressed expression of CD4 and CD8, as well as CD28, on CD4 and CD8 T cells of both younger and older subjects (**Fig. 4**). At 20 and 40 nM, CGS21680 significantly suppressed expression of CD8 by T cells of younger, but not older, subjects and had no effect on CD4 expression by either group of subjects. At 40 nM, CGS21680 significantly suppressed expression of CD28 by CD4 and CD8 T cells of younger subjects, with only marginal reductions in CD28 expression by these sets of T cells of older subjects. At 20 nM, CGS21680 only substantially suppressed expression of CD28 by CD8 T cells of younger subjects (Fig. 1, right panel). As for adenosine and EHNA, major suppression of CD39 by all concentrations of CGS21680 was evident only for CD4 and CD8 T cells of older subjects and was significantly greater in extent for the T cells of older than younger subjects, even in the 3 instances in which it was significant for the latter group.

CD28 is a T-cell coreceptor for specific surface proteins of antigen-presenting cells and thereby functions with the T-cell antigen receptor to promote T-cell functional activation. The level of expression of CD28 by T cells of older subjects is lower than that by T cells of younger subjects, and this deficiency is considered one basis for relatively lower activation responses to antigens of T cells from older subjects (23). To examine the possibility that higher levels of endogenous adenosine are, in part, responsible for the relatively decreased level of expression of CD28 by T cells of older subjects, T cells from both age groups were incubated without and with adherent anti-CD3 antibodies for 5 d, and some suspensions received the selective A_{2A} adenosine receptor antagonists SCH58261 ($K_i=1.3$ nM) or ANR94 ($K_i=46$ nM) on d 0, 2, and 4. For unstimulated CD4 T cells, CD28 expression was significantly lower for those of older than younger subjects, as expected, and this deficiency was corrected by antagonizing the A_{2A} adenosine receptor with SCH58261 (**Table 2**). Stimulation increased CD28 levels in CD4 T

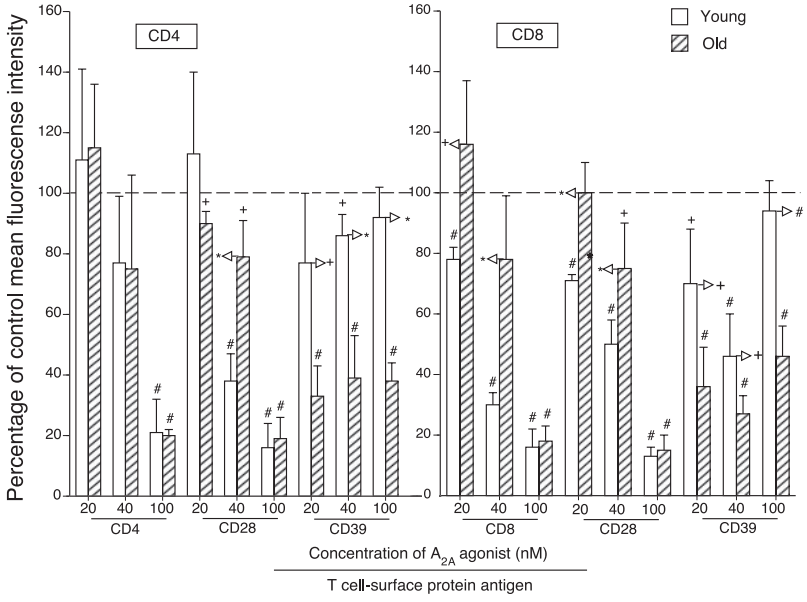

Figure 4. Effects of multiple additions of the A_{2A} adenosine receptor agonist CGS21680 on T-cell expression of surface protein antigens. Protocol is the same as in Fig. 3, including the number of subjects, with the addition of 3 concentrations of CGS21680 on d 0, 2, and 4, and labeling of the harvested T cells on d 5. Symbols at tops of bars indicate statistically significant difference *vs.* corresponding control; symbols next to horizontal arrowheads indicate difference between bars of that set. $^{+}P < 0.05$, $^{*}P < 0.01$, $^{\#}P < 0.001$.

TABLE 2. *Effects of adenosine A_{2A} receptor antagonist SCH58261 on human T-cell expression of CD28 coreceptor*

Age group	TCR stimulation	SCH58261 (20 nM)	Mean fluorescence intensity	
			CD4 T cells	CD8 T cells
Young	−	0	189 ± 44	191 ± 42
Young	−	+	171 ± 42	192 ± 38
Old	−	0	125 ± 45*	141 ± 35
Old	−	+	193 ± 78	220 ± 43#
Young	+	0	821 ± 327	810 ± 255
Young	+	+	735 ± 278	756 ± 246
Old	+	0	483 ± 222	430 ± 171*
Old	+	+	709 ± 247	658 ± 67#

Values are means ± SD of results of flow-cytometric analyses of T cells from 3–5 younger and older healthy subjects. *$P < 0.05$ *vs.* corresponding young age group; #$P < 0.05$ *vs.* corresponding group without SCH58261 incubation; 2-sample *t* test.

cells of both age groups significantly ($P<0.01$) and the lower expression of CD28 by stimulated CD4 T cells of older than younger subjects was corrected by SCH58261. However, this increment was not statistically significant due to high variability of the data. For unstimulated CD8 T cells of older subjects, SCH58261 increased the level of expression of CD28 significantly (Table 2). In stimulated CD8 T cells of older subjects, the baseline expression of CD28 was significantly lower than that for younger subjects, and SCH58261 increased significantly their level of expression of CD28. In contrast, neither 100 nor 300 nM A_3 receptor antagonist 2-phenyl-amino-N6-endo-norbornyladenine (K_i=37 nM) had any restorative effect on depressed levels of CD28 in T cells of older subjects (n=3 pairs, data not shown). In studies of 3 other older subjects, the similarly selective but structurally distinct and less potent A_{2A} adenosine receptor antagonist ANR94 also increased T-cell expression of CD28. For these older subjects, augmentation of CD28 expression (mean± SD) by 460 nM ANR94 was 147 ± 27% (P=0.038) of the untreated control for CD4 T cells and 128 ± 12% (P=0.0154) of control for CD8 T cells. No significant change in expression of CD28 was evoked by 460 nM ANR94 in CD4 T cells or CD8 T cells from the younger subjects (respective means relative to untreated controls of 96 and 107%).

Flow cytometric quantification of T-cell expression of A_{2A} adenosine receptors showed mean ± SD fluorescence intensity levels on unstimulated and activated CD4 T cells of 0.73 ± 0.48 and 24 ± 6.2, respectively, for older subjects (n=6) and 2.4 ± 1.4 and 22 ± 9.0 for younger subjects (n=4). The corresponding values for A_{2A} adenosine receptors on unstimulated and activated CD8 T cells were 1.48 ± 1.01 and 48 ± 11, respectively, for older subjects and 1.0 ± 0.43 and 29 ± 20 for younger subjects. T-cell activation thus very significantly increases expression of A_{2A} receptors on both subsets of T cells from younger and older subjects. Only the difference between A_{2A} receptor levels on activated CD4 and CD8 T cells in older subjects was significant (P=0.0007). Thus, the adenosine-A_{2A} receptor mediator system is a contributor to decreased levels of expression of CD28 by T cells of older subjects, but this age-dependent difference appears to be attributable to higher concentrations of adenosine and/or enhanced postreceptor signaling in the older subjects and not to higher levels of A_{2A} receptor expression.

Effects of adenosine on human T-cell chemotaxis

Chemotactic responses of T cells from both older and younger subjects to the chemokine CCL21, which is a critical mediator of normal translymphoid tissue trafficking, were suppressed significantly by 0.3 μM and 1 μM adenosine, and by increases in adenosine levels elicited by the adenosine deaminase inhibitor EHNA (**Fig. 5**). For 1 μM adenosine and EHNA, this suppression was significantly greater for T cells of younger than older subjects. As for the greater effects of adenosine on CD28 expression by T cells of younger than older subjects (Figs. 3 and 4), it is presumed that here, again, it is attributable to accommodation of the T cells from older subjects to a chronically higher level of endogenous adenosine. The significant suppression of T-cell chemotaxis by 0.3 μM adenosine, which did not alter either cytokine generation (Figs. 1 and 2) or expression of surface protein antigens (Figs. 3 and 4 and text), further supports the possibility that there is a rank order of susceptibility of diverse T-cell functions to adenosine immunoregulation.

Chemotaxis of T cells from older subjects to most stimuli is lower than that of T cells from younger subjects. In the present studies, the control (100%) responses to CCL21 and S1P of T cells from the older subjects were a mean of 68 to 74% of that of T cells of the younger subjects. To assess a chemotactic suppressive role for higher endogenous concentrations of adenosine in the extracellular fluid of T cells from older subjects, optimally active concentrations of the A_{2A} receptor antagonists SCH58261 and ANR94 were added to the T-cell suspensions before their introduction into chemotactic chambers. Chemotactic re-

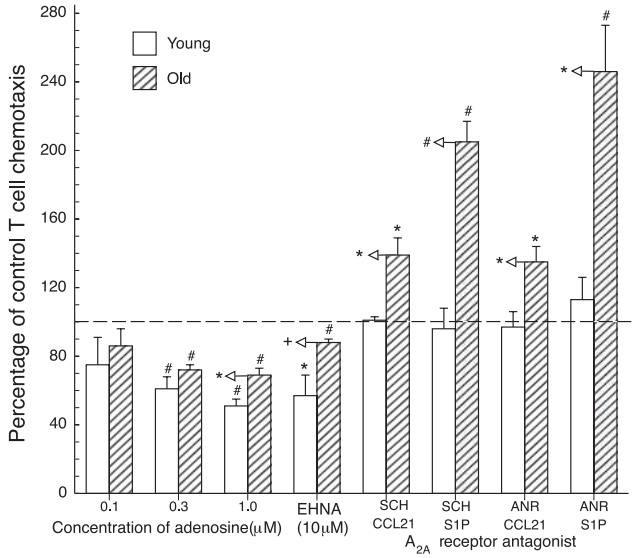

Figure 5. Effects of adenosine and the A2A adenosine receptor antagonists SCH58261 (SCH) and ANR94 (ANR) on T-cell chemotaxis. T cells from 2 older men and women (n=4) and 2 younger matched control men and women were preincubated for 15 min and then continuously with 0.1, 0.3, or 1.0 μM adenosine, 10 μM EHNA, or 20 nM SCH58261 for 4 h of chemotaxis to 50 nM CCL21 or 100 nM S1P. T cells from 2 older men and 1 old woman (n=3) and 2 younger matched control men and 1 woman similarly were preincubated for 15 min and then continuously with 460 nM ANR94 for 4 h of chemotaxis to 50 nM CCL21 or 100 nM S1P. Bars represent means ± SD of each cluster of responses as a percentage of the control responses (100%). Mean CCL21 control chemotactic responses for adenosine, EHNA, and SCH series were 24.6 ± 7.1% of initial T cells for younger donors and 16.7 ± 3.8% of initial T cells for older donors (69% of young control group level). Mean CCL21 control chemotactic responses for ANR series were 31.8 ± 6.5% of initial T cells for younger donors and 24.7 ± 3.5% of initial T cells for older donors (68% of young control group level). Mean S1P control chemotactic responses for SCH and ANR series were 7.2 ± 1.1% of initial T cells for younger donors and 5.3 ± 0.6% of initial T cells for older donors (74% of young control group level). Symbols at tops of bars indicate statistically significant difference *vs.* corresponding control; symbols next to horizontal arrowheads indicate difference between bars of that set. $^{+}P < 0.05$, $^{*}P < 0.01$, $^{\#}P < 0.01$.

sponses of T cells from older subjects, but not those from younger subjects, to CCL21 and S1P were significantly increased by SCH58261 and ANR94 (Fig. 5). To evaluate the extent of these increases, the levels of chemotaxis of the SCH58261-treated and ANR94-treated T cells from older subjects were recalculated as a percentage of that of untreated T cells from younger subjects, yielding a mean of 96% or higher. As these levels are not significantly different from those of untreated T cells of younger subjects, the elimination of adenosine action on the T cells of older subjects by the A_{2A} receptor antagonists effectively restored their chemotaxis to a normal level for younger subjects. In contrast, neither 100 nor 300 nM A_3 receptor antagonist 2-phenyl-amino-N6-endo-norbornyladenine had any restorative effect on depressed levels of chemotaxis of T cells of older subjects (n=2 pairs, data not shown).

DISCUSSION

The adenosine mediator system of T cells from younger and older healthy subjects differed in 3 major respects. First, T-cell antigen receptor-directed activation of T cells from older subjects resulted in extracellular fluid concentrations of adenosine that were 2.5-fold higher (P<0.0001) than those of activated T cells from younger subjects (Table 1). Thus, normally the T cells of older subjects respond to antigenic and other stimuli in an environment of much higher adenosine concentrations than those of younger subjects. Further, physiological levels of adenosine in the extracellular fluids of T-cell regions in the immune system would be in the 50 nM to 1 μM range, as was detected for such fluids of other mammalian tissues (8), and not at the 5 to 25 μM concentrations employed in most other studies of adenosine effects on lymphocytes and other immune cells (9–12). Even the introduction of an adenosine deaminase inhibitor known to prevent degradation of adenosine by lymphocytes raised the extracellular fluid concentrations of adenosine only to ~0.3 μM (Table 1). When adenosine concentrations in cultures of T cells were raised to 0.3, 1, 3, or 10 μM by a single exogenous addition at the onset of incubation, the generation of IL-2 and IFN-γ were significantly suppressed only by the 10 μM level (Fig. 1). However, when the adenosine concentration was raised to ~0.3 μM throughout the period of incubation by the presence of an adenosine deaminase inhibitor, IL-2 generation was suppressed to a similar extent as that attained by one addition of 10 μM exogenous adenosine. Multiple additions of exogenous adenosine to maintain a steady fluid-phase concentration were employed thereafter in all subsequent studies. This protocol resulted in significant suppression of generation of IL-2 and IFN-γ by 3 μM adenosine, but again the highest physiological level of 1 μM adenosine had no effect, and there were no differences between the results for T cells of older and younger subjects in any of these cytokine studies (Fig. 2). The generation of cytokines by T cells of older and younger subjects thus is affected little by extracellular fluid concentrations of adenosine usually achieved in immune responses, and the substantial inhibition reported previously is attainable only at pharmacological levels of 5 to 25 μM.

The second major difference between T cells of older and younger subjects was seen in responsiveness to adenosine of expression of several immunologically relevant surface protein antigens. For the T-cell subset-defining antigens CD4 and CD8, both multiple additions of 1 μM adenosine and the ~0.3 μM level of endogenous adenosine established by an adenosine deaminase inhibitor significantly suppressed the expression of CD4 and CD8 on T cells from all sources, with significantly greater effects throughout on T cells of younger than older donors (Fig. 3). Although the highest employed concentration of the A_{2A} adenosine receptor agonist CGS21680 very significantly suppressed CD4 and CD8 expression by T cells of older

and younger donors similarly, only CD8 expression by T cells of younger subjects was reduced significantly by the two lower concentrations of the A_{2A} adenosine receptor agonist (Fig. 4). The result for CD28 expression was similar, as multiple additions of 1 μM adenosine and the ~0.3 μM level of endogenous adenosine established by an adenosine deaminase inhibitor significantly suppressed the expression of CD28 on CD4 and CD8 T cells from all sources, with significantly greater effects throughout on T cells of younger than older donors (Fig. 3). Again, only the lower concentrations of the A_{2A} adenosine receptor agonist CGS21680 distinguished between CD28 expression by T cells based on subject age, with significantly greater effects for those of younger subjects (Fig. 4). Whether this greater sensitivity to adenosine is attributable to higher baseline expression of CD28, accommodation to a typically lower level of adenosine *in vivo*, or another factor has not yet been examined. It also is possible that adenosine suppresses CD28 in T cells by more than one mechanism, including down-regulation of expressed CD28, as in unactivated T cells (Table 2), and prevention of up-regulation of CD28 that follows T-cell antigen receptor-dependent activation. Application of the A_{2A} adenosine receptor antagonists SCH58261 and ANR94 confirmed both the role of endogenous adenosine and the central involvement of the A_{2A} adenosine receptor. SCH58261 and ANR94, but not fully active concentrations of the A_3 receptor antagonist 2-phenylamino-N6-endo-norbornyladenine, significantly increased the depressed levels of expression of CD28 by unstimulated CD4 T cells, unstimulated CD8 T cells, and stimulated CD8 T cells of older subjects (Table 2).

The third major age-dependent difference in effects of adenosine on T cells was observed in chemotaxis. Physiological concentrations of 0.3 and 1 μM exogenous adenosine, as well as an ~0.3 μM level of endogenous adenosine established by an adenosine deaminase inhibitor, significantly suppressed the chemotactic responses to CCL21 of T cells from younger and older subjects, with greater effects on those of younger subjects (Fig. 5). The mean control chemotactic response of T cells from older subjects was significantly lower than that of T cells from younger subjects, as had been shown in other studies (17). Antagonism of the A_{2A} adenosine receptor by SCH58261 or ANR94 significantly enhanced the chemotactic responses of T cells from older but not younger subjects to two different stimuli (Fig. 5). This level of enhancement by SCH58261 or ANR94 raised the chemotactic response of T cells from older subjects to or above that of T cells from younger subjects and thereby supported the possibility that adenosine plays a major role in reducing chemotactic responses of T cells from healthy older subjects. As cytokine generation by T cells was unaffected by physiological concentrations of adenosine that significantly altered T-cell expression of surface protein antigens and T-cell chemotaxis, the endogenous adenosine mediator system clearly has a greater influence on these latter aspects of T-cell function, as well as being a more important mediator in normal immunity of old subjects.

The present findings have several implications for our understanding of human immunosenescence. T-cell immune functions as diverse as expression of functional cell-surface proteins and chemotactic responses may be significantly reduced by the same mediator system. Functional abnormalities of T cells in healthy older subjects that appear to be due to intrinsic changes from aging may actually result from changes with aging in the concentrations of regulatory factors or in the recognition or signaling mechanisms of such factors. Perhaps most important for our clinical approaches to human immunosenescence is the realization that those abnormalities attributable to alterations in regulatory mediator systems are potentially reversible pharmacologically, with specific beneficial effects for host defenses and control of autoimmune reactions.

For more than a decade, selective A_{2A} receptor antagonists of diverse structures have been investigated for their beneficial effects in Parkinson's disease, including motor stimulation, as well as reversal of emotional components, such as depression and anxiety (24). Limited data also suggested possible improvement in cognition by A_{2A} receptor antagonists. In several animal models simulating aspects of Parkinson's disease, A_{2A} receptor antagonists reduced immobility directly and had antidepressant effects that depended in part on dopaminergic signals (25, 26). Two types of selective A_{2A} receptor antagonists now are shown to restore full expression of surface immune antigens and reverse the chemotactic deficiency in T cells of older subjects at concentrations that would be attained when it is administered for therapy of Parkinson's disease. It will be useful to examine other A_{2A} receptor antagonists now in advanced clinical trials for Parkinson's disease to completely delineate their effects on T-cell and other immune functional activities. Immunosenescence could become a near-term target for selective A_{2A} receptor antagonists, in parallel with Parkinson's disease and late-life depression. FJ

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