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T Regulatory Cell Subpopulations Associated with Recent Ultraviolet Radiation Exposure in a Skin Cancer Screening Cohort

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Abstract

UV radiation (UVR) causing DNA damage is a well-documented risk factor for nonmelanoma skin cancer. Although poorly understood, UVR may also indirectly contribute to carcinogenesis by promoting immune evasion. To our knowledge, we report the first epidemiological study designed to investigate the association between quantitative measures of UVR, obtained using a spectrophotometer, and circulating T regulatory (Treg) cells. In addition to total Treg cells, the proportion of functionally distinct Treg cell subsets defined by CD45RA and CD27 phenotypic markers, graded expression of FOXP3 and CD25, and those expressing cutaneous lymphocyte–associated Ag and the chemokine receptor CCR4 were enumerated in 350 individuals undergoing routine skin cancer screening exams and determined not to have prevalent skin cancer. No associations were identified for UVR exposure or the overall proportion of circulating Treg cells; however, Treg cell subpopulations with an activation-associated phenotype, $CD45RA^{-}/CD27^{-}$, and those expressing cutaneous homing receptors were significantly positively associated with UVR. These subpopulations of Treg cells also differed by age, sex, and race. After stratification by natural skin tone, and adjusting for age and sex, we found that spectrophotometer-based measures of UVR exposure, but not self-reported measures of past sun exposure, were positively correlated with the highest levels of these Treg cell subpopulations, particularly among lighter-skinned individuals. Findings from this large epidemiologic study highlight the diversity of human Treg

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cell subpopulations associated with UVR, thus raising questions about the specific coordinated expression of CD45RA, CD27, CCR4, and cutaneous lymphocyte–associated Ag on Treg cells and the possibility that UVR contributes to nonmelanoma skin cancer carcinogenesis through Treg cell–mediated immune evasion.

Ultraviolet radiation (UVR) is an environmental factor that contributes to the development of nonmelanoma skin cancer (NMSC), one of the most frequently diagnosed cancers in the United States (1, 2). The two most common types of NMSC, squamous cell carcinoma and basal cell carcinoma, occur most often on areas of sun-exposed skin (2, 3). UVR is involved in several stages of carcinogenesis (1), including induction of DNA damage, and possibly through immune suppression, enabling malignant cells to grow unchecked by T cells or other immune population(s). Although the exact mechanism of the latter is not well understood, immune suppression associated with skin cancer is marked by both a reduction in conventional T cell functions (4, 5) independent of, and as a consequence of, T regulatory (Treg) cells (as reviewed in Ref. 6).

Treg cells, characterized by the expression of the transcription factor FOXP3, CD4, and the IL-2 receptor α-chain (CD25), are expanded systemically and within the tumor of various cancers, where they uniformly have negative prognostic significance (7-9). Differentiation markers on Treg cells have been studied in humans with autoimmune disease, viral infection (10-13), and cancer and include the protein8 tyrosine phosphatase (encoded by the *PTPRC* gene) CD45RA, CD62L (L-selectin), and CD27. Although the coordinated differentiation of conventional T cells in humans, and Treg cells in mice, have been well delineated, the differentiation path for Treg cells in humans is less well defined (as reviewed in Ref. 14). Both CD45RA and CD27, a costimulatory molecule involved in activation and memory development, have the potential to distinguish functionally distinct Treg cell subsets (15-17). All of these markers are expressed on naive, resting T cells and medullary thymocytes but are downregulated after TCR activation (18). Patterns of chemokine receptors are also useful in distinguishing functional Treg cell populations that exhibit directional localization within inflammatory environments, including the skin (19).

In mice, the frequency of neuropilin-1^+, thymic-derived, natural Treg cells increased following exposure to low doses of UVB radiation in the absence of tumors (20). UVR-induced expansion of Treg cells is mediated by Ag activation (21), which, under specified conditions, enables their suppressive mechanisms and triggers tissue-homing to the skin (22, 23) (as reviewed in Ref. 20). Ag activation of Treg cells occurs through self-antigens and, in some tissues, the microbiome (24). The coordination of UVR exposure and Treg cell expansion suggests that both may contribute to tumor growth in keratinocyte carcinogenesis.

Functionally distinct Treg cell subpopulations characterized by specific phenotypic surface markers have been studied in various disease settings (11, 13, 25). Thymic-derived Treg cells expressing CD45RA decline with age in mice (26) during chronic viral infections (13) and following organ transplantation rejection (11). We found previously that $CD45RA^-$/$CD27^-$ Treg cells were expanded prior to disease progression and were specifically associated with poor survival in myelodysplastic syndrome (25). Although the $CD45RA^-$/$CD27^-$ Treg cell subset is more suppressive compared with $CD45RA^-/CD27^+$ Treg cell

subtypes on an individual cell basis, Treg cell population dynamics in the context of UVR, age, sex, and race are poorly characterized (14, 25).

Epidemiological studies have reported associations between prevalence of chronic autoimmune diseases such as multiple sclerosis, lupus erythematosus, and rheumatoid arthritis and distance from the equator, thereby indicating a plausible role for UVR exposure in immune function (27-30). Other studies have used UVR as a treatment for multiple sclerosis and psoriasis and have reported decreased immune function as a result of UVR photochemical therapy, primarily by inducing Treg cells within the lymph nodes, followed by altering their skin migratory behavior (31-33). Among patients with psoriasis, dysfunction in circulating Treg cell populations was restored after treatment with photochemical therapy, suggesting an increase in immunosuppressive activity of Treg cells as a result of UVR (34).

To our knowledge, this is the first epidemiological study to investigate the association between UVR and immune response mediated by Treg cells among a cohort of individuals undergoing routine skin cancer screening exams using a quantitative, spectrophotometer-based measure of UVR. Circulating Treg cells were characterized using flow cytometry of cells in the peripheral blood, as defined previously on the basis of FOXP3, CD4, and CD25 (25, 35). The percent of total circulating conventional T cells and Treg cells was quantified (in addition to specific Treg cell subpopulations), defined by the expression of CD45RA and CD27 markers (11, 13, 25) and markers associated with bidirectional trafficking between lymph node and skin (36, 37) [including cutaneous lymphocyte–associated Ag (CLA) and the chemokine receptor CCR4 (11, 13, 22, 25, 33, 36, 38)], and graded expression levels of FOXP3 (35), as described previously. We hypothesized that UVR would be associated with higher levels of a circulating Treg cell population or a higher number of total Treg cells in this cohort.

Materials and Methods

Study design and population

Cross-sectional, baseline data from participants enrolled in the first year of the Viruses and Skin Cancer Study, an ongoing, 4-y prospective cohort study of UVR, cutaneous viral infections, and skin cancer being conducted at the Moffitt Cancer Center, were used for the current analysis. Individuals undergoing routine skin cancer screening exams at the University of South Florida Dermatology Clinic were eligible for the study if they were at least 60 y of age and had not had both squamous cell carcinoma and basal cell carcinoma previously. At the time of study enrollment, participants underwent a total-body skin examination, and suspicious lesions were biopsied as a part of routine clinical care. Study participants with a pathologically confirmed NMSC at baseline were excluded. Questionnaire data, peripheral blood samples, and skin pigmentation measurements were also required at study entry. Individuals that failed to complete these study-related activities were excluded from the analysis. The study was approved by the University of South Florida Institutional Review Board, and all patients provided written informed consent at the time of enrollment.

Spectrophotometer data collection

Skin pigmentation readings were obtained using a spectrophotometer (CM-600D; Konica Minolta Sensing Americas) with SpectraMagic NX Lite USB Ver. 2.5 software, using the specular component included mode of the instrument, which minimizes the influence of the gloss and texture of the skin-on-skin pigmentation readings, as described previously (39, 40). The instrument was calibrated against a white tile each morning, per manufacturer guidelines. The spectrophotometer readings were obtained by study personnel at the initial study visit when blood samples were collected. This instrument measures color on three different axes: lightness on a scale of 0 (black) to 100 (white), axis *a*, indicating color within the red through green range, and axis *b*, measuring color within the yellow through blue range, with increasing values on *a*- and *b*-axes, indicating saturation of color (41). Natural skin tone was assessed using spectrophotometer readings of the sun-unexposed underside of the upper arm (i.e., the axilla). The degree of recent tanning in response to recent UVR exposure was measured by calculating the difference between the color readings ($\Delta E \times ab$) on an area of sun-exposed skin (top of the forearm or forehead) and the axilla. Three readings were obtained at each anatomic site, and the average values were recorded. A strong, intraindividual correlation was observed between spectrophotometer readings obtained at the forearm and forehead ($r = 0.6079$; $p < 2.2 \times 10^{-16}$) (Supplemental Fig. 1). Therefore, the average of these two values was used as the single measurement of recent UVR exposure.

Blood collection and flow cytometry data acquisition and analysis

Blood samples were collected in three 10-ml heparin sodium–containing blood collection tubes. Samples were processed for isolation of PBMCs by Ficoll–Hypaque centrifugation using 10–20 ml of Lymphocyte Separation Media (Ficoll), according to manufacturer guidelines (Amersham Pharma Biotech, Piscataway, NJ). Freezing medium (10% DMSO and 90% FBS) was used to viably freeze PBMCs in preparation of the future cytometry analysis. Frozen PBMCs stored in liquid nitrogen were thawed on ice and placed into 1 ml PBS. Approximately 10^6 PBMCs were first labeled with the viability dye Ghost Dye Red 780 (1 μl; Tonbo Bioscience) for 20–30 min at room temperature to exclude dead cells from the analysis. PBMCs were then treated with 1 μl FcR Blocking Reagent (Miltenyi Biotec) and labeled with Abs to detect the following cell surface Ags: anti-CD3/allophycocyanin, anti-CD45RA/PerCPCy5.5 (Tonbo Bioscience), anti-CD4/BV785, anti-CCR4/BV421, anti-CLA/FITC, PD-1/PE (BioLegend), anti-CD25/BUV395 (BD Horizon), and anti-CD27/BUV737 (BD Horizon), using 1 μl each and for 30 min at 4°C. Cells were fixed with a Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's protocol and labeled with 5 μl PE/Dazzle 594 anti-human FOXP3 Antibody (BioLegend) for 30 min.

After staining and fixation, cell populations were analyzed within 24 h on a BD LSR II flow cytometry instrument (BD Biosciences) using the gating strategy defined in Fig. 1 and Supplemental Fig. 2 and quantified using FlowJo v9 (FlowJo). The PBMC samples from all patients were thawed and evaluated for viability. Those with $<1.0 \times 10^5$ viable lymphocytes were excluded from the final data analysis ($n = 31$). In samples included in the analysis, $3.1 \times 10^5 \pm 2.2 \times 10^5$ viable cells were present. Samples were evaluated for $CD8^+$ T cells and

$CD4^+$ T cells of $CD3^+$ lymphocytes. $CD4^+$ T cells were further defined by $CD25^-FOXP3^-$ conventional Th cells (Th CD4) and total $CD25^+FOXP3^+$ Treg cell subpopulations: $CD45RA^+/CD27^+$ (naive) Treg cells, $CD45RA^-/CD27^-$ activated, effector Treg cells (i.e., equivalent to conventional T effector cells), $CD45RA^+/CD27^-$ (terminal effector memory) Treg cells (i.e., equivalent to conventional T cells with an exhausted phenotype), $CD45RA^-/CD27^+$ (central) Treg cells, CLA^+ (skin migratory) Treg cells, and chemokine receptor $CCR4^{hi}$ (skin migratory) Treg cells, as previously described (11, 13, 22, 25, 33, 36, 38). All population data are described as a frequency of the parent population. In addition to the described gating strategy, $CD4^+$ T cells samples were also divided into six populations, according to Miyara et al. (35) (Fig. 1D), to define $CD45RA^-CD25^{++}FOXP3^{lo}$ as resting Treg cells (Group I), $CD45RA^-CD25^{+++}FOXP3^{hi}$ (Group II) as activated Treg cells, and defined $CD45RA^-CD25^{++}$ $FOXP3^{lo}$ (Group III) as nonsuppressive Treg cells.

All gating strategies were confirmed by use of fluorescence-minus-one control samples in which cells are stained with all colors except the Ab noted (Supplemental Fig. 2A, 2B, 2E, 2F).

Statistical methods

Correlations between Treg cells and UVR were calculated using Spearman rank, and a trend line was created to visualize the associations. The correlations between T cell populations were described using Spearman rank correlation coefficient. Additionally, given that previous studies have reported effect modification of UVR-associated physiologic effects by one's natural skin tone (42, 43), and analysis by lighter versus darker skin tone was performed, based on the median value of the spectrophotometer readings of the sun-unexposed axilla, to investigate whether the associations between UVR and Treg cell phenotypes differed by natural skin tone. Linear regression was used to examine the associations between baseline characteristics and Treg cell phenotypes, adjusted for age and sex. Differences in UVR by sex and age were examined using the Wilcoxon rank sum test and Spearman correlation, respectively. Logistic regression was used to calculate odds ratios and 95% confidence intervals to estimate the associations between recent UVR exposure and quartiles of the Treg cell phenotypes, adjusted for age and sex. Quartiles were used to account for the possible nonlinear relationship between Treg cells and recent UVR and to avoid use of data transformation within the multivariate models. Statistical analysis was conducted using R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Characterization of Treg cells associated with UV exposure

During the first year of recruitment (July 2014 through July 2015), 917 individuals were approached, of whom, 448 (49%) consented to participate. No significant differences were observed between study participants and individuals who declined participation, with respect to sex ($p = 0.17$) or age ($p = 0.16$). Participants were asked to complete a website-based questionnaire, including demographic and skin cancer risk factor information. In addition, blood samples and skin pigmentation measurements were obtained from each participant. Blood samples were collected from 409 patients, of which, 378 samples were found to have

sufficient numbers of viable cells available for flow cytometry. Individuals with a pathologically confirmed NMSC at baseline ($n = 23$) or missing skin pigmentation readings ($n = 5$) were excluded. Therefore, the current analysis includes 350 individuals with complete information on both Treg cells and UVR exposure.

Skin pigmentation readings were used as a marker of recent UVR exposure and for natural skin tone, consistent with previous epidemiologic studies (30, 31). Males had significantly higher spectrophotometer readings (14.61 ± 3.12) than females (9.37 ± 2.62; $p < 0.0001$), whereas there was no association with age ($p = 0.81$).

PBMC samples were analyzed by flow cytometry, as shown in Fig. 1 (25). First, $CD8^+$ T cells and $CD4^+$ T cells of $CD3^+$ lymphocytes were identified, followed by $CD4^+$ T cells that were further defined by $CD25^-FOXP3^-$ conventional Th cells (Th CD4) and total $CD25^+FOXP3^+$ Treg cells. Low expression of CD127 on $CD25^+FOXP3^+$ Treg cells was confirmed, as described previously, but was not included in this analysis (data not shown) (25). Recent UVR exposure was plotted against each population of T cells (Fig. 2). The proportion of CD8 (33 ± 14.4), Th CD4 (66.1 ± 14.6), and total Treg cells (4.29 ± 2.15) were within normal range for peripheral blood of healthy individuals (25). $CD8^+$ T cells ($r = 0.03$, $p = 0.56$), Th $CD4^+$ T cells ($r = 0.09$, $p = 0.09$), and total percent of Treg cells among all $CD4^+$ T cells ($r = -0.03$, $p = 0.54$) were not correlated with UV exposure (Fig. 2A-C). In addition to these populations, functionally distinct Treg cell subpopulations were examined, as defined in Fig.1B-F. Four phenotypically distinct Treg cell populations were evident by flow cytometry, with the vast majority (75.0 ± 10.5%) displaying a $CD45RA^-/CD27^+$ central memory phenotype, as described previously (25). CLA^+ and $CCR4^{hi}$ Treg cells did not express CD45RA, consistent with prior Ag engagement, but consisted of both $CD27^+$ and $CD27^-$ subpopulations (Fig. 1E, 1F), similar to conventional Th $CD4^+$ T cells (Supplemental Fig. 2C, 2D). The $CD45RA^-/CD27^-$ activated, effector Treg cells (Fig. 2G), CLA^+ Treg cells (Fig. 2F), and $CCR4^{hi}$ Treg cells (Fig. 2I) were positively correlated, with recent UVR exposure among all individuals ($r = 0.19$, $p < 0.01$; $r = 0.13$, $p = 0.01$; $r = 0.17$, $p < 0.01$; respectively), whereas $CD45RA^+/CD27^+$ Treg cells (i.e., naive) showed a less-pronounced, negative correlation ($r = -0.11$, $p = 0.03$) (Fig. 2D), indicating that these specific Treg cell subsets, not total Treg cells or conventional T cells, correlated with recent UVR exposure as measured by spectrophotometer. Of note, no self-reported measures of past UVR exposures or sun-susceptibility factors were associated with these four Treg cell populations (Supplemental Table I).

To further explore the Treg cell populations and their association to UVR, $CD4^+$ T cells were also segregated into six populations defined previously (Miyara et al.) using CD45RA, CD25, and levels of FOXP3 (Fig. 1G, 1H), with Groups I–III all expressing FoxP3, consistent with having a Treg cell phenotype (35). Group II ($CD45RA^-CD25^{+++}FOXP3^{hi}$) and Group I ($CD45RA^+CD25^{++}$ $FOXP3^{lo}$) are functionally suppressive, whereas Group III with a $CD45RA^-CD25^{++}FOXP3^{lo}$ phenotype was shown previously to be nonsuppressive, but capable of actively secreting cytokines. Although the $CD45RA^-/CD27^-FOXP3$ population showed an association to UVR exposure, no association was observed with the $CD45RA^-$ Treg cells defined as Group II or Group III (Fig. 2K, 2L, respectively).

Interestingly, Group I Treg cells were negatively associated with recent UVR exposure ($r = -0.16$, $p < 0.01$) (Fig. 2J).

To further define the characteristics of the Treg cells and conventional T cells through association studies, a correlation matrix was prepared for all T cell populations identified. Group I and Group II Treg cell populations were highly correlated with total Treg cells, but were not associated with Treg cells defined by other surface markers, including CD27, CLA, and CCR4 (Fig. 3). Indeed, we found there to be a high positive correlation between $CD45RA^+/CD27^+$ Treg cells, naive Treg cells, and the Group I population, resting Treg cells (Fig. 3). These results are consistent with the negative association between both Group I and $CD45RA^+/CD27^+$ Treg cell populations. Moreover, overlays of the flow cytometry expression patterns reveal CD27, CLA, and CCR4 to be represented throughout Group II–IV populations (Fig. 1I), indicating that these surface markers further differentiate Treg cells. Gating on such populations within Group II–III may be necessary to observe correlations between Treg cells defined by this strategy and recent UVR exposure.

Natural skin tone represents an effect modifier of the UVR and Treg cell association

Previous studies of nevus development in children have reported effect modification of UVR-associated physiologic effects by one's natural skin tone (42, 43). Therefore, we stratified the current analysis by lighter versus darker skin tone, based on the median value of the spectrophotometer readings of the sun-unexposed axilla, to investigate whether the associations between UVR and Treg cell phenotypes differed by natural skin tone. Interestingly, a significant positive correlation between recent UVR and the proportion of activated, effector $CD45RA^-/CD27^-$ Treg cells ($r = 0.30$, $p < 0.01$) was observed only in individuals of lighter skin (Fig. 4). The $CD45RA^+/CD27^+$ naive Treg cell population was negatively correlated with recent UVR exposure in lighter-skinned individuals, although the trend was not as striking as the association with increased $CD45RA^-/CD27^-$ Treg cells (Fig. 4). The positive correlation between UVR and CLA^+ Treg cells was also restricted to the lighter-skinned individuals ($r = 0.15$, $p = 0.04$). However, UVR and $CCR4^{hi}$ Treg cells were positively correlated among both darker-skinned ($r = 0.18$, $p = 0.02$) and lighter-skinned individuals ($r = 0.14$, $p = 0.06$), with the former being statistically significant. These results suggest that individuals with a lighter natural skin tone may be at higher risk for UVR-induced changes in Treg cells.

Demographics are associated with Treg cells

Associations between demographic factors and Treg cell subpopulations, including naive $CD45RA^+/CD27^+$, activated effector $CD45RA^-/CD27^-$, CLA^+, and $CCR4^{hi}$ Treg cells (based on results shown in Fig. 1), are presented in Table I. A statistically significant inverse trend was observed between age and naive $CD45RA^+/CD27^+$ Treg cells (p-trend < 0.01). Conversely, $CD45RA^-/CD27^-$ Treg cells were positively associated with age (p-trend = 0.04). This is similar to the expected loss of conventional naive T cells because of thymic involution and shown previously for Treg cells in mice (26, 44-46). Significant positive trends were also observed between age and the two skin migratory Treg cells, CLA^+ (p-trend = 0.02), and $CCR4^{hi}$ (p-trend = 0.01) Treg cell populations (Table I).

Interestingly, Treg cell populations differed significantly by sex after adjustment for age, including higher numbers of $CD45RA^{-}/CD27^{-}$ Treg cells in males (14.5 ± 6.8 in males versus mean 13.0 ± 7.0 in females; $p = 0.02$), higher CLA^{+} Treg cells in males (59.3 ± 10.7 in males versus mean 56.0 ± 11.3 in females; $p = 0.01$), and higher $CCR4^{hi}$ Treg cells in males (84.5 ± 8.4 in males versus 82.8 ± 8.4 in females; $p < 0.01$). Race was also associated with $CD45RA^{-}/CD27^{-}$ Treg cells (mean 13.5 ± 6.8 in white versus mean 19.9 ± 9.6 in others; $p < 0.01$). Ethnicity was not associated with Treg cell populations (Table I). Treg cells defined as Group I–III (Fig. 1D) were also analyzed by demographic information. No association was found among Group I–III populations with age, sex, or ethnicity. Group II populations were significantly associated with race, with white participants having higher numbers (Table II). This finding is consistent with the significant racial difference observed for the distinct Treg cell subpopulation defined by $CD45RA^{-}/CD27^{-}$ expression, which was strongly negatively correlated with age, independent of Group I–III distribution (Fig. 3).

Recent UVR and Treg cells are independently associated

Associations between recent UVR and quartiles of circulating Treg cells are presented in Table III. We observed a positive trend between recent UVR and quartiles of $CD45RA^{-}/CD27^{-}$ Treg cells in all individuals (p-trend = 0.02) (Table III). After stratification by natural skin tone, the UVR association with $CD45RA^{-}/CD27^{-}$ Treg cells was particularly pronounced among lighter-skinned individuals, with each unit increase in spectrophotometer readings associated with a 32% increase in odds of being in the uppermost versus lowermost quartile of $CD45RA^{-}/CD27^{-}$ Treg cells (odds ratio = 1.32, 95% confidence interval = 1.12–1.61), after adjustment for age and sex. The strength of this association supports its biological relevance. Interestingly, we observed a significant inverse trend with recent UVR exposure and quartiles of $CD45RA^{-}/CD27^{+}$ Treg cells among all individuals (p-trend = 0.04) and lighter-skinned individuals (p-trend = 0.01), but not among darker-skinned individuals (p-trend = 0.22). Among darker-skinned individuals, UVR exposure was significantly lower among those in the second and third quartiles of $CD45RA^{+}/CD27^{-}$ Treg cells compared with the first, although no clear trend was observed, taking into account the highest quartile (p-trend = 0.11). No associations were observed between UVR exposure and quartiles of total Treg cells, CLA^{+} Treg cells, and $CCR4^{hi}$ Treg cells, overall or after stratification, by natural skin tone (Supplemental Table II).

Discussion

NMSC is the most common malignancy in the United States with older age (>60 y), representing an important risk factor (47). To our knowledge, this is the first epidemiologic study of recent UVR exposure and Treg cells in a population of individuals age 60 y and older at high risk for NMSC. In 350 individuals undergoing skin cancer screening, recent UVR exposure was positively associated with activated, effector $CD45RA^{-}/CD27^{-}$ Treg cells, whereas an inverse association was observed between recent UVR and $CD45RA^{-}/CD27^{+}$ Treg cells. Furthermore, these associations were specific to lighter-skinned individuals. No associations were observed between recent UVR exposure and the total percentage of Treg cells relative to conventional $CD4^{+}$ or $CD8^{+}$ T cells.

To the best of our knowledge, previous findings of UVR-related changes in Treg cells have been exclusively reported in in vitro or in mouse models in vivo, in which subsets of T cells were not specifically studied (18, 36, 48). Treg cells are largely thought of as migratory populations both in circulation and in lymph nodes, but many are retained in cutaneous tissue, where they curb autoimmune diseases of the skin and contribute to tumor progression, allergic responses, and microbial immunity (as reviewed in Refs. 49 and 50). In cutaneous tissues, overrepresentation or increased suppressive functions of Treg cells may contribute to immune evasion and development of skin cancer.

We have reported previously that $CD45RA^{-}/CD27^{-}$ Treg cells represent a more suppressive subset on a per-cell basis in T cell suppression assays (25). Because of the similarity between $CD45RA^{-}/CD27^{-}$ Treg cells and conventional T effector cells, these cells were termed effector Treg cells. Downregulation of CD45RA and CD27 is aligned with acute Ag activation in the context of MHC class II, which, in the case of Treg cells, is largely induced by stimulatory autoantigens (51-53). This precise Treg cell subset was also expanded in a higher risk cohort of patients with a premalignant human disease myelodysplastic syndrome characterized by a high rate of leukemia transformation (25). In this disease, the total number of Treg cells increases during later stages of leukemia progression, suggesting that an activation-associated phenotypic change and expansion occur at different points of the disease, suggesting that Ag activation may precede the accumulation of Treg cells as a whole (54, 55). Collectively, our results show an increase in the $CD45RA^{-}/CD27^{-}$ Treg cells in association with UVR exposure, which may occur through local tissue damage and Ag release. This increase is in tandem to low UV exposure in individuals with higher levels of naive $CD45RA^{+}/CD27^{+}$ Treg cells.

Alternative gating strategies on CD4 T cells by CD25 and CD45RA to define Treg cells, rather than by FOXP3 and CD25, resulted in no significant findings among this cohort (Figs. 1G, 1H, 2K, 2L, Table II). These populations have been previously shown to be phenotypically distinct, in which $CD45RA^{-}CD25^{+++}$ $CD4^{+}$ cells, defined as Group II, exhibit more repressive activity on conventional T cells than other Treg cell populations (35). Indeed, these populations expressed the highest intracellular FOXP3 expression in the cohort presented in this study, but no association was found with recent UVR exposure. This population correlated with populations of total Treg cells defined by FOXP3 and CD25, which also showed no correlation with recent UVR exposure. Given that $CD45RA^{-}/CD27^{-}$ Treg cells were found to be present within Groups II, III, and IV, loss of CD27 could contribute to a distinct functional attribute with clinical relevance to UVR exposure. Analysis of this process could yield a better understanding of Treg cell activity in response to UVR exposure.

Two skin-homing receptors were also measured on Treg cells in the context of UVR, chemokine receptor CCR4, and CLA (23, 56). CLA is a carbohydrate epitope induced by the α-(1,3)-fucosyltransferase VII gene and by cytokines and Ag stimulation (57-59), which is consistent with higher $CD45RA^{-}/CD27^{-}$ Treg cells. In our data set, all CLA^{+} cells lacked CD45RA expression, confirming that these are activated Treg cells (Fig. 1C). Recently, CLA expression on the Treg cell surface was shown to occur after exposure to NO, a chemical that is released into the skin upon UVR exposure (56). CCR4 is another skin-associated T

cell marker that has been shown to increase following UVB exposure in mice, suggesting that not only are Treg cells activated, but that they are stimulated to express both homing receptors significant for infiltration into cutaneous tissues (23, 60). Most studies suggest that Treg cells can migrate from lymph nodes to skin and then re-enter the circulation (60). Transcriptional signatures and phenotypes suggest that the migratory versus resident populations can be distinguished (49, 50). However, conjoined parabiotic surgery of two mice that share the same vasculature compartment showed that there are T cells in the skin epidermis that do not equilibrate through blood and are permanently resident in skin (61-64). Because many resident Treg cells remain in the dermis and do not recirculate after activation and migration to skin, Treg cells may accumulate through repeated sun exposure; a well-defined risk factor for skin cancer (65, 66). Although positive correlations were observed between UV exposure and CLA^+ and $CCR4^{hi}$ Treg cells, after adjusting for age and sex, these associations were no longer statistically significant, indicating that the strongest independent marker is the accumulation of $CD45RA^-/CD27^-$ Treg cells. Although it is difficult to delineate the origins of tissue resident cells, future studies are needed to define whether UVR contributes to the population of tissue resident memory Treg cells in human skin.

Although several mechanisms control the suppressive functions of Treg cells, UV promotes IL-10 secretion and reportedly exert suppressive activity largely through IL-10 production and express the glucocorticoid-induced TNF family–related receptor [(67, 68), reviewed in Ref. 69]. In cancer studies, engagement of glucocorticoid-induced TNF family–related receptor with agonistic Abs potentiates antitumor immunity by destabilizing FOXP3 expression and reducing Treg cells (70). Given that studies have shown that adoptively transferred UV-activated Treg cells into host mice suppress tumor-immune responses, $CD4^+$ T cell proliferation, and contact hypersensitivity (68, 71-73), it is likely that UV exposure increases the suppressive activity of Treg cells through Ag activation. This is congruent with the data reported in this study in that distribution of total Treg cells is not associated with recent UV exposure (Fig. 2C, Supplemental Table I). The mechanism for both Treg cell expansion and increased suppressive activity has often pointed to the UV/vitamin D production axis (74). Vitamin D is both acquired through the diet and through activation of the first synthesis step by radiation in the UVB range (reviewed in Ref. 74). Topical application of vitamin D's active form increases the suppressive activity of Treg cells (75) and causes comparable effects to UVB radiation in mice. This method for UV-induced immune suppression is controversial given that vitamin D receptor (VDR) knockout mice are still susceptible to UV-activated Treg cells (76). Furthermore, there are reports that VDR is not highly expressed in mouse $FoxP3^+CD4^+$ Treg cells, but there is an inverse relationship between VDR-dependent signaling and FoxP3 regulation (77-80) (as reviewed in Ref. 81). Vitamin D production by UVB exposure is particularly high in lighter-skinned individuals, potentially because of the protectiveness of melanin in individuals with natural dark skin (80, 82-84), which may explain the more pronounced associations between recent UVR exposure and Treg cells among lighter-skinned individuals in our cohort. Given the controversial evidence for vitamin D and its role in UV-induced immune suppression, investigation of vitamin D or VDRs in the context of UVR exposure and Treg cells is an important future step.

The cross-sectional nature of this analysis cannot establish the presence of UVR exposure prior to Treg cell measurement, although the UVR-associated changes in skin pigmentation had to have occurred as a result of exposure prior to the time of blood draw. The exact timing of UVR exposure is unknown, and therefore, the temporal relationship between UVR exposure and associated changes in circulating Treg cells requires further investigation. In mouse studies, UVB-induced Treg cell activation in skin lasted for 2 wk after exposure and then later contributed to expansion of these cells in the peripheral blood (23). Some previous studies of UVR exposure and immune-related conditions have relied on self-report of sun exposures and ecologic measures, such as latitude, to estimate UVR exposure (27, 29, 30, 85). Other studies have demonstrated that Treg cell populations are altered with UVR exposure in a small cohort of patients presenting with autoimmune disorders (31, 34), including one such study that demonstrated that activated $CD45RA^-$ Treg cells are increased in psoriasis patients with recent UV exposure (32). Our study builds upon these findings, suggesting that Treg cell activation occurs with recent UVR in this cohort.

The spectrophotometer readings used in the current study provide an objective and quantitative measure of recent UVR exposure. Future analysis of this prospective cohort will focus on the associations between baseline UVR exposure, Treg cells, and subsequent development of NMSC. In this review, to our knowledge, we report the first quantitative UVR exposure study associated with subpopulations of Treg cells in peripheral blood. Future studies are necessary to understand if UVR leads to the accumulation of tissue-resident Treg cell populations and whether that, in turn, contributes to carcinogenesis. Additionally, our study reports differences in Treg cell subpopulations by age, sex, and race, with potential importance in other immune-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article:

CLA	cutaneous lymphocyte–associated Ag
NMSC	nonmelanoma skin cancer
Treg	T regulatory
UVR	UV radiation

VDR vitamin D receptor

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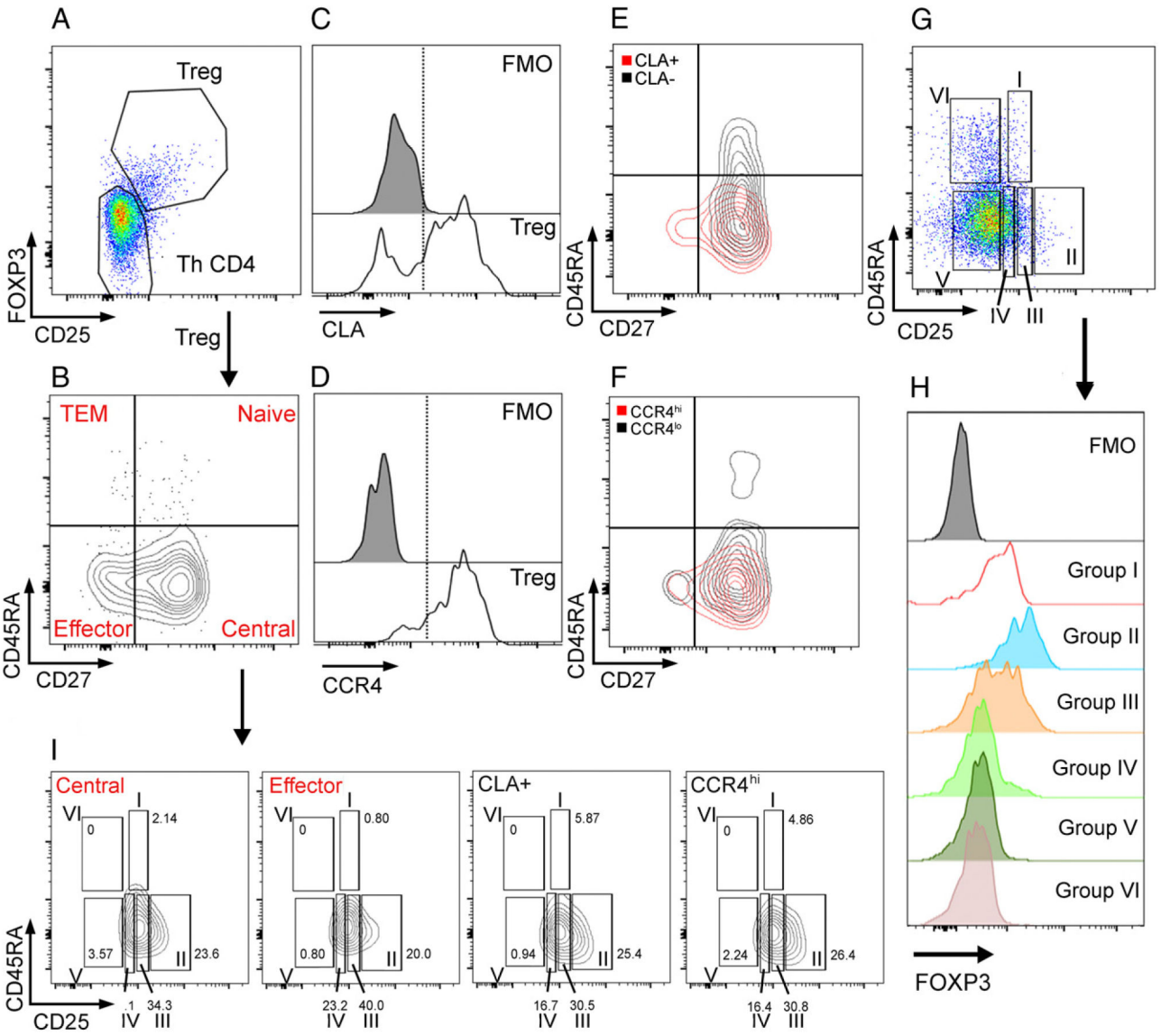

FIGURE 1.

Flow cytometry gating strategy for Treg cell analysis. PBMCs were gated first on viability and on CD3 and CD4 positivity. (**A**–**D**) (A) $CD4^+$ Treg cells were defined by high expression of CD25, and intracellular FOXP3 and conventional $CD4^+$ T cells (Th CD4) were defined by low expression of CD25 and FOXP3. (B) Treg cells were then analyzed for CD45RA and CD27 expression, and populations were defined by dual or single expression. (C–**F**) CLA and CCR4 expression was also analyzed among Treg cells. CLA- and CCR4-positive Treg cells were also confirmed to be CD45RA negative with mixed expression of CD27. (**G**–**I**) (E) Six groups of $CD4^+$ T cells were defined by CD45RA positivity and various CD25 expression. (H) Each population was analyzed for FOXP3 expression. (I) Distribution of these populations was analyzed in $CD45RA^-/CD27^+$ (Central) Treg cells, $CD45RA^-/CD27^-$ (Effector) Treg cells, CLA^+ Treg cells, and $CCR4^{hi}$ Treg cells as defined in (B)–(D).

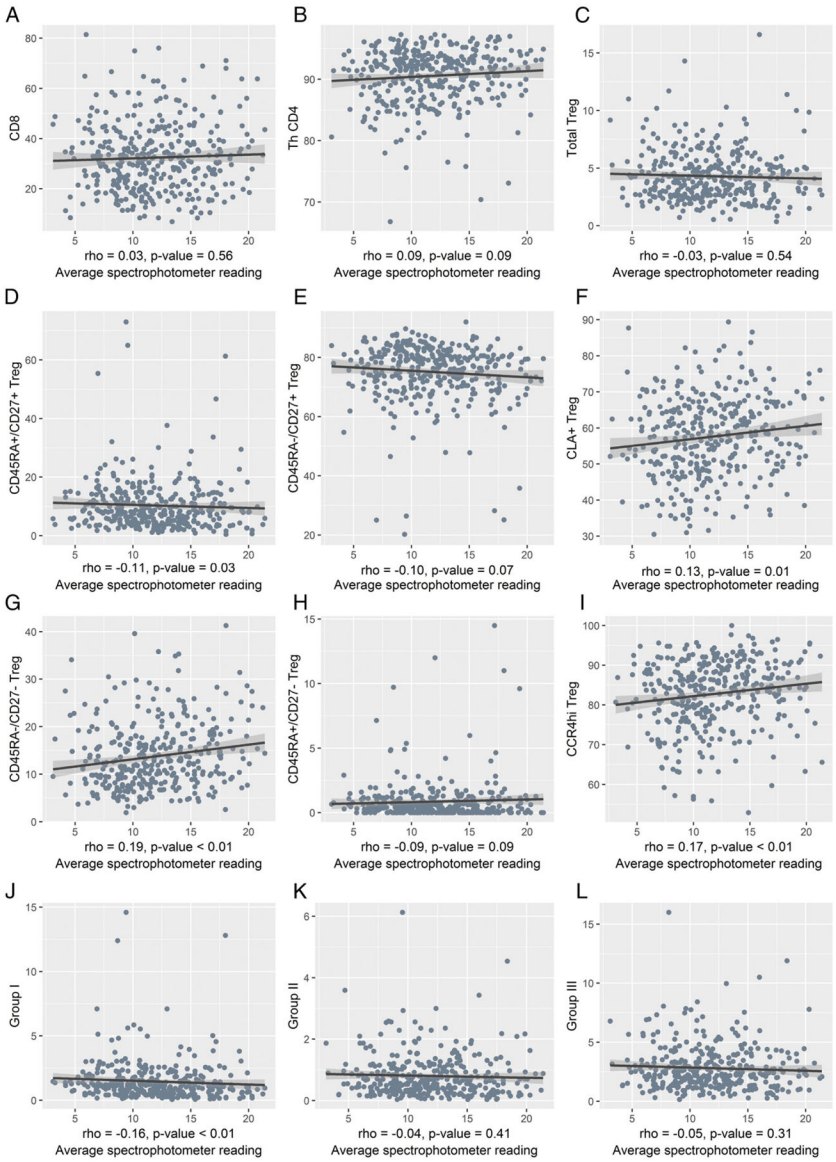

FIGURE 2.

T cell populations in correlation with recent UV exposure among 350 individuals undergoing skin cancer screening. Correlations between Treg cells and UVR were calculated using Spearman rank, and a trend line was created to visualize the associations. (**A**–**C**) Scatterplot of spectrophotometer reading and CD8, Th CD4, and total Treg cells. (**D**–**I**) Scatterplot of spectrophotometer reading and phenotypes of Treg cells defined in Fig. 1A. (**J**–**L**) Scatterplot of spectrophotometer reading and Group I, II, and III Treg cell phenotypes defined in Fig. 1G.

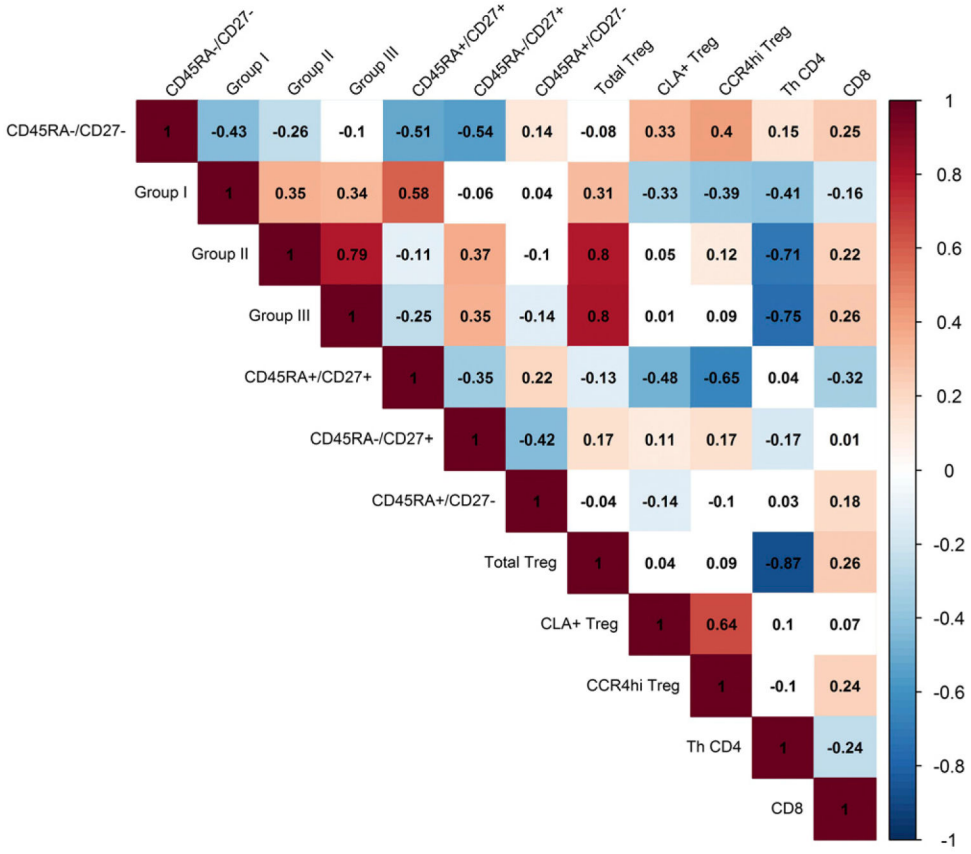

FIGURE 3.

Spearman rank correlation matrix for T cell populations among 350 skin cancer screening patients. Spearman correlation coefficients (rho) are presented for all T cell populations. The matrix cells are shaded only if they are significantly correlated at $p < 0.05$ level. Red shading represents a positive correlation, whereas blue shading represents a negative correlation.

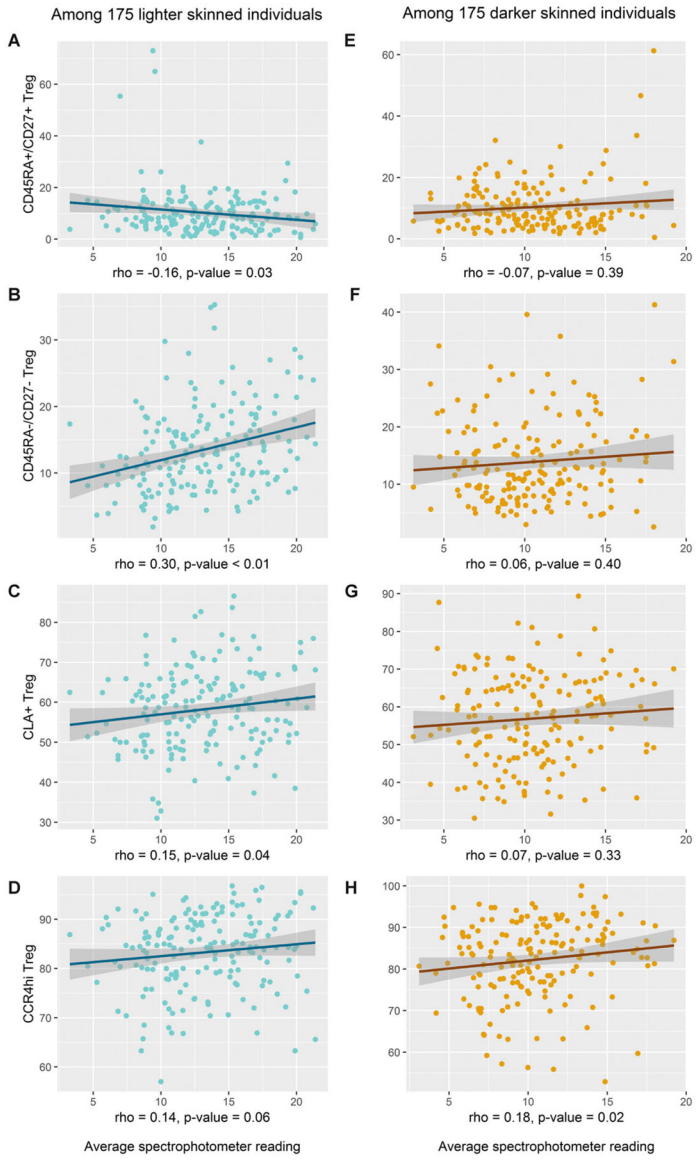

FIGURE 4.

Effect modification by natural skin tone. (**A**–**H**) Scatterplot of spectrophotometer readings and Treg cell subpopulations that were found to have significant associations with UVR after stratification by natural skin tone as defined by median spectrophotometer readings of sun-unexposed underside of the upper arm (i.e., the axilla). Correlations between Treg cells and UVR were calculated using Spearman rank, and a trend line was created to visualize the associations.

Table I.

Circulating Treg cell phenotypes and demographic characteristics

		Treg Cell Phenotypes											
		CD45RA$^+$/CD27$^+$ Treg Cells			CD45RA$^-$/CD27$^-$ Treg Cells			CLA$^+$ Treg Cells			CCR4^{hi} Treg Cells		
	***n* (%)**	**Mean (SD)**	**Coef.[a]**	***p* Value[a]**	**Mean (SD)**	**Coef.**	***p* Value**	**Mean (SD)**	**Coef.**	***p* Value**	**Mean (SD)**	**Coef.**	***p* Value**
Age[b]													
60–64	78 (22.3)	12 (9.3)	−0.0031	< ***0.01***	12.4 (6.9)	0.0042	***0.04***	55.4 (10.8)	0.2228	***0.02***	80.5 (9.1)	383549	***0.01***
65–69	125 (35.7)	10.8 (10.3)			14.2 (7.5)			57.4 (10.6)			82.7 (8.4)		
70–74	78 (22.3)	9.8 (6.9)			13.7 (6.4)			58.1 (11.7)			83.7 (8.1)		
75–79	41 (11.7)	9.5 (6.3)			13.6 (6.6)			58 (11.7)			83.1 (8.5)		
80–89	28 (8.0)	6.4 (4.3)			15.5 (6.3)			62 (11.5)			86 (8.2)		
Gender[c]													
Female	187 (53.4)	10.8 (9.1)			13.0 (7.0)			56.0 (11.3)			81.2 (8.5)		
Male	163 (46.6)	9.8 (8.2)	−0.0187	*0.15*	14.5 (6.8)	0.0588	***0.02***	59.3 (10.7)	3.1290	***0.01***	84.5 (8.4)	7335781	***<0.01***
Race													
White	340 (97.1)	10.3 (8.6)			13.5 (6.8)			57.8 (11.1)			82.8 (8.4)		
Others	10 (2.9)	10.7 (10.8)	−0.0515	*0.18*	19.9 (9.6)	0.2225	***<0.01***	51.0 (11.9)	−6.1680	*0.09*	80.1 (13.4)	−1106525	*0.85*
Ethnicity													
Non-Hispanic	330 (94.8)	10.4 (8.6)			13.7 (6.9)			57.5 (11.2)			82.6 (8.6)		
Hispanic or Latino	18 (5.2)	10.2 (9.8)	−0.0166	*0.57*	14.5 (7.9)	0.0293	*0.61*	59.0 (11.3)	1.7580	*0.51*	84.2 (8.4)	4063970	*0.34*

Bold indicates significance ($p < 0.05$) and italic represents statistical results.

[a]Coefficients and *p* values were calculated using linear regression, adjusted for age, CD45RA$^-$ (categorical), and sex with transformed Treg cell data as follows: CD45RA$^+$/CD27$^+$ Treg cells (eighth root), CD45RA$^-$/CD27$^-$ Treg cells (fourth root), CLA$^+$ Treg cells, and CCR4^{hi} Treg cells (fourth power).

[b]Coefficient and *p*-trend were calculated using linear regression with continuous age, presented in years, and adjusted for sex, with transformed Treg cell data.

[c]The *p* values were calculated using linear regression adjusted for age (categorical).

Coef, coefficient.

Table II.

Circulating Treg cell phenotypes and demographic characteristics

		Group I		Group II		Group III	
	n **(%)**	**Mean (SD)**	***p*** **Value**	**Mean (SD)**	***p*** **Value**	**Mean (SD)**	***p*** **Value**
Age[a,b]							
60–64	78 (22.3)	1.6 (2)	*0.10*	0.7 (0.4)	*0.44*	2.6 (1.5)	0.62
65–69	125 (35.7)	1.5 (1.6)		0.9 (0.8)		3.1 (2.4)	
70–74	78 (22.3)	1.5 (1.4)		0.7 (0.6)		2.5 (1.4)	
75–79	41 (11.7)	1.5 (1.1)		0.9 (1)		3 (1.9)	
80–89	28 (8.0)	0.9 (0.6)		0.8 (0.8)		2.6 (1.7)	
Gender[b,c]							
Female	187 (53.4)	1.5 (1.7)		0.7 (0.6)		2.8 (2)	
Male	163 (46.6)	1.4 (1.4)	*0.06*	0.9 (0.8)	*0.15*	2.9 (1.9)	0.46
Race							
White	340 (97.1)	1.5 (1.6)		0.8 (0.7)		2.8 (2)	
Others	10 (2.9)	1.3 (1.1)	*0.22*	0.4 (0.2)	***0.04***	2.4 (0.8)	0.74
Ethnicity							
Non-Hispanic	330 (94.8)	1.5 (1.6)		0.8 (0.7)		2.8 (1.9)	
Hispanic or Latino	18 (5.2)	1.6 (1.7)	*0.76*	0.8 (0.7)	*0.93*	3.2 (2.4)	0.51

[a]The *p*-trend was calculated using linear regression with continuous age, presented in years, and adjusted for sex.

[b]The *p* values were calculated using linear regression, adjusted for age (categorical) and sex, with transformed Treg cell data: Group I (eighth root), Group II (fourth root), and Group III (fourth root).

[c]The *p* values were calculated using linear regression adjusted for age (categorical).

Table III.

Quartiles of circulating Treg cell subpopulations and UV spectrophotometer readings among all 350 patients undergoing skin cancer screening

	Average UV Spectrophotometer Reading								
	All (*n* = 350)			Lighter Skin[c] (n = 175)			Darker Skin (*n* = 175)		
Treg Cell Phenotypes[a]	***n***	**Mean (SD)**	**OR (95% CI)[b]**	***n***	**Mean (SD)**	**OR (95% CI)**	***n***	**Mean (SD)**	**OR (95% CI)**
$CD45RA^+/CD27^+$ Treg cells									
Q1	88	12.63 (3.96)	1.0 (ref.)	46	14.19 (3.83)	1.0 (ref.)	42	10.92 (3.36)	1.0 (ref.)
Q2	87	11.58 (3.74)	0.91 (0.81–1.01)	41	12.67 (3.93)	0.84 (0.70–0.98)	46	10.61 (3.30)	0.99 (0.83–1.17)
Q3	87	11.50 (3.86)	0.91 (0.81–1.01)	43	12.91 (3.91)	0.86 (0.73–1.01)	44	10.12 (3.31)	0.93 (0.77–1.12)
Q4	88	11.53 (3.89)	0.92 (0.83–1.02)	45	12.75 (3.89)	0.88 (0.75–1.02)	43	10.25 (3.49)	0.95 (0.79–1.13)
		p-trend[d]: *0.24*			*p*-trend: *0.18*			*p*-trend: *0.62*	
$CD45RA^-/CD27^-$ Treg cells									
Q1	88	10.88 (3.40)	1.0 (ref.)	44	11.48 (3.78)	1.0 (ref.)	44	10.27 (2.89)	1.0 (ref.)
Q2	87	11.50 (3.64)	1.00 (0.89–1.12)	42	12.96 (3.79)	1.08 (0.92–1.27)	45	10.13 (2.94)	0.92 (0.75–1.11)
Q3	89	12.09 (3.87)	1.05 (0.94–1.17)	47	13.41 (3.81)	1.20 (1.03–1.42)	42	10.61 (3.39)	0.84 (0.67–1.04)
Q4	86	12.80 (4.34)	1.13 (1.01–1.27)	42	14.79 (3.65)	1.32 (1.12–1.61)	44	10.89 (4.11)	0.99 (0.83–1.20)
		p-trend: ***0.02***			*p*-trend: < ***0.001***			*p*-trend: 0.91	
$CD45RA^-/CD27^+$ Treg cells									
Q1	88	12.36 (4.15)	1.0 (ref.)	39	13.78 (3.70)	1.0 (ref.)	49	11.23 (4.18)	1.0 (ref.)
Q2	88	11.72 (3.92)	0.94 (0.84–1.04)	43	13.30 (3.90)	0.94 (0.79–1.10)	45	10.21 (3.33)	0.88 (0.75–1.03)
Q3	87	12.42 (3.96)	0.92 (0.82–1.02)	53	13.79 (4.04)	0.85 (0.70–1.00)	34	10.30 (2.74)	0.83 (0.69–0.99)
Q4	87	10.73 (3.22)	0.88 (0.78–0.98)	40	11.53 (3.59)	0.77 (0.63–0.93)	47	10.05 (2.72)	0.93 (0.78–1.10)
		p-trend: ***0.04***			*p*-trend: ***0.01***			*p*-trend: 0.22	
$CD45RA^+/CD27^-$ Treg cells									
Q1	88	12.84 (3.75)	1.0 (ref.)	45	13.60 (4.38)	1.0 (ref.)	43	12.05 (2.80)	1.0 (ref.)
Q2	89	11.26 (3.56)	0.91 (0.81–1.02)	41	13.33 (3.25)	1.01 (0.86–1.19)	48	9.49 (2.80)	0.72 (0.57–0.89)
Q3	85	11.18 (3.67)	0.86 (0.77–0.96)	43	12.32 (3.76)	0.89 (0.76–1.04)	42	10.01 (3.22)	0.77 (0.63–0.93)
Q4	88	11.95 (4.30)	0.99 (0.88–1.10)	46	13.33 (4.09)	1.05 (0.90–1.23)	42	10.43 (4.04)	0.87 (0.70–1.05)
		p-trend: 0.41			*p*-trend: 0.93			*p*-trend: 0.11	

[a] Treg cells were categorized into quartiles using the following cutoffs: CD45RA^{+}/CD27^{+} Treg cells: 4.89–8.34–13.5; CD45RA^{-}/CD27^{-} Treg cells: 8.44–12.25–17.4; CD45RA^{-}/CD27^{+} Treg cells: 71.43–76.9–80.9; CD45RA^{+}/CD27^{-} Treg cells: 0.12–0.43–1.03.

[b] ORs and 95% CIs for average spectrophotometer readings were calculated using logistic regression, comparing each risk group to the reference group Q1.

[c] Natural skin tone based on median spectrophotometer readings in sun-unexposed underside of the upper arm, as described in *Materials and Methods*.

[d] The *p*-trend was calculated using ordinal logistic regression.

CI, confidence interval; OR, odds ratio; Q, quartile; ref., reference.

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