

T Cell Subsets in Healthy Black Smokers and Nonsmokers

Evidence for Ethnic Group as an Important Response Modifier¹⁻³

DAVID J. TOLLERUD, LINDA MORRIS BROWN, WILLIAM A. BLATTNER, DEAN L. MANN, LUBA PANKIW-TROST, and ROBERT N. HOOVER

Introduction

Since the association between cigarette smoking and lung cancer was described in the 1950s, the catalog of tobacco-related health effects has grown to include a variety of other malignancies as well as diverse respiratory, cardiovascular, and cerebrovascular diseases (1, 2). Variations in prevalence rates of such conditions between ethnic groups has been largely attributed to differences in smoking rates, dietary influences, and socioeconomic factors (3, 4). The degree to which these and other known risk factors can explain ethnic differences in disease prevalence and survival within the United States, however, remains controversial (5).

Cigarette smoking has been shown to be associated with a variety of immunologic alterations among white subjects, including depressed immunoglobulin levels, decreased numbers and function of natural killer cells, and altered T cell subsets (6-11). Comparable data are not available for black populations. Because blacks and whites differ in a number of immunologic parameters (12-15), we investigated the influence of cigarette smoking on mononuclear cell subsets in healthy adult black subjects.

Methods

Study Population

Study subjects were enrolled from a population-based survey of healthy adults aged 20 to 69 in the Greater Washington, D.C. metropolitan area. The stratification scheme and sampling protocol have been described in detail elsewhere (16). Briefly, random digit dialing and a short household screening questionnaire were utilized to select a random sample stratified by age, ethnic group, gender, and smoking status. Demographic, life-style, and medical information was collected through telephone and self-administered questionnaires. This information was used to exclude individuals with life-style characteristics (intravenous drug use or homosexual activity)

SUMMARY The influence of cigarette smoking on T cell subsets has been studied in white subjects, but comparable data are not available for blacks. We analyzed peripheral blood mononuclear cell subsets in a population-based, stratified, random sample of healthy black adults using monoclonal antibodies and flow cytometry. The study population consisted of 94 men and 79 women, including 73 smokers (CS) and 100 nonsmokers (NS). Cigarette smoking was associated with a significant elevation in leukocyte (WBC) count (CS $7,270 \pm 230$ cells/mm³ versus NS $6,260 \pm 160$ cells/mm³; $p = 0.001$), although WBC counts for both groups were substantially lower than those reported for white smokers and nonsmokers. Smokers had a significantly lower proportion of CD4⁺ cells than nonsmokers (CS $55.4 \pm 0.9\%$ versus NS $58.7 \pm 0.9\%$; $p = 0.01$), adjusting for age and gender. No significant smoking-related changes were observed for CD8⁺ cells, the CD4/CD8 ratio, or total T cells (CD3⁺), monocytes (CD14⁺), or natural killer cells (CD16⁺). Among black smokers, a significant dose-related decrease in CD4⁺ cells was observed as the number of cigarettes smoked per day increased. Among black exsmokers, the level of WBC and CD4⁺ cells returned to the level observed in never smokers within 2 to 5 yr after smoking cessation. These results contrast sharply with the previously reported increase in CD4⁺ cells and decrease in natural killer cells associated with cigarette smoking in whites. The data suggest that the immunologic effects of cigarette smoking may be significantly modified by ethnic characteristics. AM REV RESPIR DIS 1991; 144:612-616

or medical conditions (blood product transfusion since 1975, recent hospitalization, severe allergies, use of steroid medications, history of connective tissue disease, or recent pregnancy) that might influence the immunologic parameters under investigation. Eligible subjects were asked to undergo phlebotomy at a mobile field station near the home.

The initial survey included only white and black nonsmokers (16); black smokers were ascertained but not studied because of budgetary and logistical constraints. The following year, black smokers were enrolled, using the same questionnaires and study procedures. Included in this report are results for the 73 black smokers and 100 black nonsmokers (including 91 previously described nonsmokers [14] and 9 newly enrolled nonsmokers). Participation rates were 78% for the telephone interview and 57% for phlebotomy.

Sample Preparation and Flow Cytometry Analysis

Phlebotomy was performed by a nurse-phlebotomist at a specially equipped mobile van. Blood samples were submitted to a commercial laboratory for routine hematology and chemistry analyses. Peripheral blood mononuclear cells were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation, washed, counted, and resuspended in modified RPMI 1640 medium as previously described (10). Aliquots

of 10 million mononuclear cells were then cryopreserved in dimethylsulfoxide (DMSO) using a controlled rate freezer and stored in liquid nitrogen until needed for flow cytometry analysis.

The following directly fluorescein-conjugated monoclonal antibodies, purchased from Ortho Diagnostics, Raritan, NJ (ORTHO) or Becton Dickinson Monoclonal Center, Mountain View, CA (BD) were utilized (10): OKT3 (CD3⁺ T cells; ORTHO); OKT4A (CD4⁺ helper-inducer T cell subset; ORTHO); OKT8 (CD8⁺ suppressor-cytotoxic T cell subset; ORTHO); anti-Leu 12 CD19⁺ B cells; BD); anti-Leu M3 (CD14⁺ monocytes; BD); anti-

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³ Correspondence and requests for reprints should be addressed to David J. Tollerud, M.D., M.P.H., Department of Environmental and Occupational Health, A-718 Graduate School of Public Health, University of Pittsburgh, 130 DeSoto Street, Pittsburgh, PA 15261.

Leu 11A (CD16⁺ natural killer cells; BD); anti-HLA-DR (nonpolymorphic HLA-DR antigen; BD); and mouse IgG₁ (clone 11-63; BD) and IgG₂ (a + b) (clones 11-4.1 and MPC-11; BD) as negative control reagents.

A direct immunofluorescence staining procedure was employed, after which the stained samples were fixed with paraformaldehyde (17). Prepared samples were analyzed on a FACS[®] II cell sorter (Becton Dickinson, Mountain View, CA) interfaced to a PDP 11/24 DEC computer (Digital Equipment Corporation, Landover, MD). The forward-angle light scatter window was set to exclude electronic noise, debris, and damaged or dying cells; the right-angle light scatter window was set to exclude monocytes (18). Standard window settings were determined for each monoclonal antibody. After gating, less than 2% of cells were Leu M3⁺, with no significant differences between men and women, smokers and nonsmokers, or older and younger subjects. For analysis of CD 14⁺ cells (monocytes), the right-angle light scatter window was opened to allow viewing of all mononuclear cells. For each monoclonal antibody tested, 10,000 gated events were collected. The percentage of immunofluorescence-positive cells was determined by subtracting the negative control fluorescence, determined by analysis of cells stained with mouse IgG of the appropriate isotype for each monoclonal antibody. Intraanalysis reproducibility was excellent, with a coefficient of variation less than 5% for the major T cell subsets. Samples were analyzed in random order, without knowledge of the demographic characteristics or smoking history of the study subjects.

Statistical Analysis

The study population was grouped into two smoking categories, nonsmokers and current smokers, to assess the influence of cigarette smoking on leukocyte count and mononuclear cell subset proportions. Persons who smoked cigarettes at the time of the interview were considered current smokers (CS); all others were considered nonsmokers (NS). Nonsmokers who had smoked at some time in the past were also designated exsmokers (ES), and subjects who had smoked less than one pack of cigarettes in their lifetimes were considered never smokers (NvS). The usual number of cigarettes smoked per day (intensity) and the total number of years smoked (duration) were ascertained from the questionnaires.

Mononuclear cell subset absolute cell counts (cells/mm³) were calculated as the product of the cell subset proportion and the absolute lymphocyte count. Statistical analyses were performed using the SAS statistical analysis package (SAS Institute, Cary, NC). Student's *t* tests were used to compare mean values for selected variables by sex and smoking category. Analyses were also performed using logarithmically transformed values for leukocyte count and arcsin-transformed values for mononuclear cell subset proportions to achieve a more normal distribution of

values. The resulting significance estimates were similar to the analyses presented for the non-transformed values. Linear regression analysis and analysis of variance were used to test whether there were significant differences for mean WBC count and mononuclear cell subset proportions by smoking status, usual number of cigarettes smoked per day, and total number of years smoked, independent of age, sex, or correlates of smoking (education and coffee and alcoholic beverage consumption). Similar analyses were performed for exsmokers alone to assess the influence of time since smoking cessation.

Results

Study Population

The study population consisted of 173 healthy black adults ages 20 to 69 yr, including 84 men and 79 women. A total of 73 subjects currently smoked cigarettes, 27 had stopped smoking at least 1 month before the study, and 73 subjects were never smokers. Never smokers and current smokers were similar in age (NvS, 39.8 yr versus CS, 37.0 yr; $p > 0.1$), but exsmokers were significantly older (ES, 47.0 yr; $p < 0.05$ compared to NvS or CS). Current smokers reported smoking an average of 14.7 cigarettes per day (range, 1 to 60) and had smoked for an average of 18 yr (range 3, to 58). Fewer than 10% smoked more than 1 pack of cigarettes per day. Exsmokers had quit smoking an average of 12.6 yr before the study. There were no significant differences between exsmokers and never smokers for the leukocyte count, differential cell count, T cell subsets, or CD16⁺ (natural killer) cells. For example, the proportion of CD4⁺ cells among exsmokers was 58.7% compared to 58.6% among never smokers ($p > 0.9$). Therefore, never smokers and exsmokers were considered together as "nonsmokers" for subsequent analyses of these subsets. Exsmokers had a lower proportion of CD19⁺ B cells than either never or current smokers (ES, 11.7% versus NvS, 14.6% and CS, 15.6%; $p < 0.05$); therefore, smoking groups were not combined for analyses of this subset.

Leukocyte Count and Differential

Cigarette smokers had a significantly higher total white blood cell (WBC) count than nonsmokers (figure 1). The cell counts for all cell types except monocytes were increased among smokers, although only the difference in lymphocyte count reached statistical significance. The differential was also significantly different in smokers than in nonsmokers, with smokers having a higher proportion of lymphocytes and a lower propor-

tion of neutrophils and monocytes compared to nonsmokers. There was no significant difference in the number or proportion of eosinophils in smokers compared to nonsmokers. These smoking-associated differences in cell number and proportion were apparent across the entire age range and for men and women separately. Among smokers, there was no detectable significant effect ($p > 0.05$) of smoking intensity (cigarettes smoked per day) or smoking duration (years of smoking) on the WBC count or differential. Repeating the analysis with adjustment for correlates of smoking (age, educational level, and alcohol and coffee consumption) did not alter the observed association between cigarette smoking and leukocyte levels.

Mononuclear Cell Subsets

The distribution of mononuclear cell subsets among black smokers and nonsmokers is shown in table 1. Smokers had a significantly lower proportion of CD4⁺ cells than nonsmokers ($p < 0.02$), adjusting for age and gender. No significant smoking-related differences were observed for CD8⁺ cells, the CD4:CD8 ratio, or total T cells (CD3⁺), monocytes (CD14⁺), or natural killer cells (CD16⁺). As noted previously, no consistent smoking-related differences in B cells (CD19⁺) were detected, with similar levels found in current smokers and never smokers ($p > 0.1$) and lower levels observed among exsmokers. Because of the smoking-related increase in lymphocytes, the calculated cell counts (cells/mm³) for all mononuclear cell subsets were higher in smokers than in nonsmokers (CD3⁺ cells, CS 1915 versus NS 1527; CD4⁺ cells, CS 1530 versus NS 1281; CD8⁺ cells, CS 538 versus NS 443; CD16⁺ cells, CS 152 versus NS 127; CD19⁺ cells, CS 430 versus NS 304; HLA-DR⁺ cells, CS 431 versus NS 362; p value [CS versus NS in an analysis of variance, ANOVA, model adjusting for age and gender] < 0.05 for all except CD16⁺ and HLA-DR⁺ cells). The sum of CD4⁺ and CD8⁺ cells exceeded the CD3⁺ cell count in both smokers and nonsmokers, suggesting that a small proportion of cells may have been labeled with both OKT4A and OKT8. The single-parameter flow cytometry system utilized in this study did not permit further analysis of this phenomenon. The analyses were repeated with adjustment for correlates of smoking, with no significant change in the results.

In contrast to the influence of cigarette smoking on the WBC count, differences

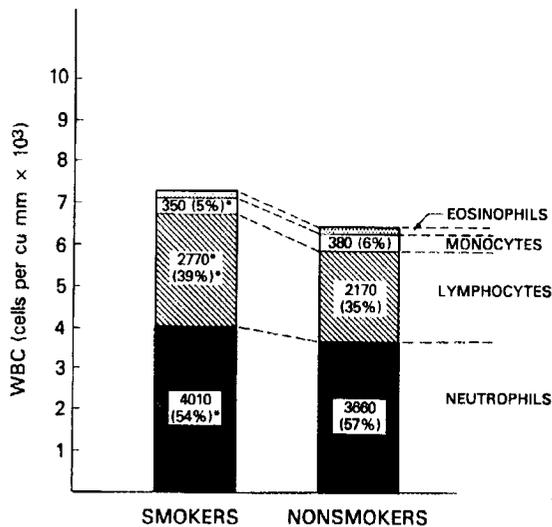


Fig. 1. White blood cell (WBC) count and differential in black smokers and black nonsmokers. Total WBC count was $7,270 \pm 230$ cells/mm³ for smokers versus $6,380 \pm 160$ cells/mm³ for nonsmokers ($p < 0.0001$). Values within bars indicate cell count (cells per mm³) for each type, with the differential proportion for that cell type given in parentheses. Asterisks indicate $p < 0.05$ compared with nonsmokers. Corresponding values for eosinophils: 130 cells/mm³ (1.8% of WBC) (smokers) versus 120 cells/mm³ (1.9% of WBC) (nonsmokers) ($p > 0.1$). Error bars represent 1 SEM.

TABLE 1
PERIPHERAL BLOOD MONONUCLEAR CELL SUBSETS IN
BLACK SMOKERS AND NONSMOKERS*

Cell Surface Antigen	Smokers	Nonsmokers	p Value†
CD3 (pan-T cell)	68.7 ± 1.2	70.1 ± 1.1	0.4
CD4 (helper T cell)	55.5 ± 0.9	58.7 ± 0.9	0.01
CD8 (suppressor/cytotoxic T cell)	19.3 ± 0.8	20.7 ± 0.7	0.2
CD4:CD8 ratio	3.47 ± 0.24	3.14 ± 0.11	0.2
CD18 (NK cell)	5.3 ± 0.4	5.9 ± 0.4	0.3
CD14 (monocyte)‡	12.7 ± 0.5	11.7 ± 0.5	0.2
HLA-DR	15.8 ± 0.7	16.4 ± 0.8	0.6

* Values are expressed as mean \pm SEM percentage of lymphoid cells.

† p Value derived from ANOVA models, adjusting for age and gender.

‡ Right-angle light scatter gate removed for determination of Leu M3⁺ cells.

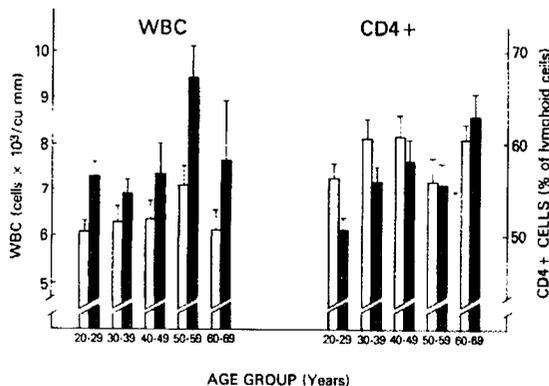


Fig. 2. White blood cells (WBC) and CD4⁺ cells (expressed as percentage of lymphoid cells) by age group for black nonsmokers (open bars) compared with black smokers (solid bars). Note that the vertical scales are discontinuous. Error bars represent 1 SEM.

in the level of CD4⁺ cells between smokers and nonsmokers was most evident among younger subjects and appeared to disappear with age (figure 2). Among subjects under age 45, the proportion of CD4⁺ cells was significantly lower in smokers compared to nonsmokers (CS $54.4 \pm 1.0\%$ versus NS $58.7 \pm 1.2\%$; $p < 0.01$ by two-tailed *t* test), but no such difference was evident in subjects age 45 or older (CS $59.5 \pm 1.6\%$ versus NS $58.6 \pm 1.4\%$; $p = 0.7$). This age-related pat-

tern was evident in both men and women and was not significantly affected by exclusion of exsmokers. Current smokers under age 45 smoked an average of 14.1 cigarettes per day compared to 16.9 cigarettes per day for older smokers ($p = 0.3$).

Among current smokers, the decrease in CD4⁺ cells was significantly related to the self-reported number of cigarettes smoked per day. For each additional 10 cigarettes smoked per day, the propor-

tion of CD4⁺ cells decreased by 1.9 percentage points in a linear regression model for current smokers, adjusting for age and gender ($p < 0.05$). Black smokers reported smoking fewer cigarettes per day (mean 14.7) than did white smokers in the earlier study (mean 22) (10). To examine whether this difference might account for the observed differences in smoking-related T cell subset alterations between blacks and whites, we reanalyzed the earlier study data, including only subjects who smoked 20 cigarettes or less per day. This sub-group of 73 white smokers (mean reported cigarette consumption 15.5 cigarettes per day) was compared with the 174 white nonsmokers in that study. Although excluding the heaviest smokers tended to diminish the differences between white smokers and nonsmokers, cigarette smoking was still associated with an increase in the proportion of CD4⁺ cells (CS $54.3 \pm 1.0\%$ versus NS $52.2 \pm 0.6\%$; $p = 0.06$) and a decrease in the proportion of CD16⁺ natural killer cells (CS $5.5 \pm 0.4\%$ versus NS $6.7 \pm 0.3\%$; $p < 0.05$), adjusting for age and gender.

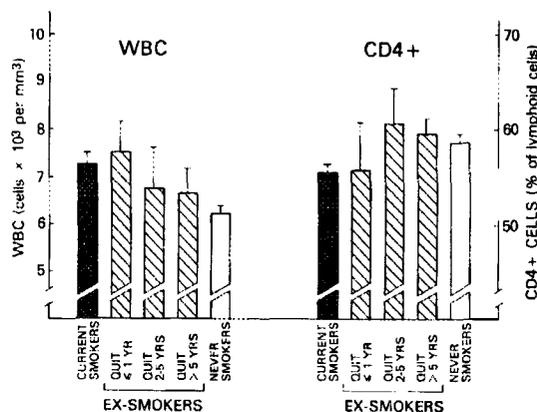
Analysis of Exsmokers

Among the 27 exsmokers in the study population, eight had quit smoking within the past year, six had quit smoking 2 to 5 yr before the study, and 13 had not smoked for over 5 yr. Subjects who had quit smoking within the past year had WBC counts and CD4⁺ cell levels similar to those of current smokers (figure 3). Subjects who had not smoked for 2 yr or more had WBC and CD4⁺ cell levels similar to those of never smokers.

Discussion

Cigarette smoking has been associated with a variety of alterations in both cellular and humoral components of the immune system. Smokers have an increased WBC count, a decrease in circulating IgG and an increase in circulating IgE, a decrease in the number and function of natural killer cells, and alterations in T cell subsets (6-11). However, as in many areas of clinical research and epidemiology, black subjects are conspicuously absent from these studies. A few studies of ethnicity-related immunologic differences have been reported, but we were unable to find any that addressed the potential influence of ethnic group on smoking-related immunologic alterations. Blacks in the United States are more frequently affected than whites by a number of conditions linked to ciga-

Fig. 3. White blood cells (WBC) and CD4⁺ cells among black exsmokers (hatched bars) compared with black current smokers (solid bars) and black never smokers (open bars). Exsmoker categories are QUIT \leq 1 YR (stopped smoking within the past year); QUIT 2-5 YRS (stopped smoking between 1 and 5 yr before this study); and QUIT $>$ 5 YRS (stopped smoking more than 5 yr ago). Note that the vertical scales are discontinuous. Error bars represent 1 SEM.



rette smoking, a disparity largely attributed to higher levels of cigarette and alcohol use and poor access to medical care among American blacks (3, 4). However, recent investigations suggest that other factors may be involved. A report by the Centers for Disease Control estimated that 31% of the excess mortality observed among blacks in the United States was unexplained by six well-established risk factors (smoking, systolic blood pressure, cholesterol, body mass index, alcohol, and diabetes) and family income (5). A recent population-based investigation of a large United States metropolitan area suggests that differences in alcohol and tobacco consumption between blacks and whites cannot account for the excess in esophageal cancer observed in blacks from the same area (L. M. Brown and D. J. Tollerud, unpublished observations). We have previously reported the results of a population-based study of the relationship of cigarette smoking to T cell subsets in healthy whites (10). That study, together with the knowledge that blacks differ from whites in a number of immunologic parameters, led us to investigate whether cigarette smoking would have similar effects in black subjects from the same geographic area.

Black smokers had a 16% higher WBC count than black nonsmokers, virtually identical to the difference between white smokers and nonsmokers from the same area (10). Unlike whites, in whom cigarette smoking is associated with a symmetric increase in all cell types, neutrophils showed a smaller increase than lymphocytes in black smokers compared to black nonsmokers. Blacks may also have a blunted neutrophil response to other stimuli. In a study of bacterial meningitis in infants, Sadowitz and Oski reported that infected black infants mounted a significantly lower neutrophil response than white infants, although there were

no detectable differences in clinical outcome (19). It is tempting to speculate that the less vigorous neutrophil response to cigarette smoking in black adults and the decreased neutrophilia in black infants with meningitis may relate to a basic difference in hematopoietic homeostasis between blacks and whites.

A direct comparison of T cell subset values in the present study with those in our previous study of healthy whites is not possible because of differences in laboratory methodology and equipment. However, the influence of cigarette smoking on mononuclear cell subsets within the black population can be compared with the smoking-related effects observed among whites in the earlier study. White smokers had a significantly higher proportion of CD4⁺ cells, a slightly higher CD4:CD8 ratio, and a significantly lower proportion of CD16⁺ natural killer cells than white nonsmokers (10). In contrast, black smokers had a significantly lower proportion of CD4⁺ cells than black nonsmokers, with no significant differences in CD4:CD8 ratio or CD16⁺ natural killer cells. Among whites, these alterations appeared relatively uniform across the age range of 20 to 69 yr, and the differences in blacks were observed primarily among younger subjects. In both blacks and whites, a dose-response effect was observed between the number of cigarettes smoked per day and the level of CD4⁺ cells. For whites, among whom smokers had an increase in CD4⁺ cells, smoking 10 additional cigarettes per day increased the proportion of CD4⁺ cells by 1.2 percentage points. For blacks, among whom smokers had a decrease in CD4⁺ cells, the same increment in cigarette consumption was associated with a decrease in the proportion of CD4⁺ cells by nearly 2 percentage points. In both populations, analysis of exsmokers indicated that the smoking-related effects

on WBC and CD4⁺ cells resolved within a few years of smoking cessation.

Black smokers reported smoking fewer cigarettes per day (mean 14.7) than white smokers in the earlier study (mean 22) (10). Novotny and colleagues have reported a similar tendency for blacks to smoke less heavily than whites in an analysis of data from the 1985 National Health Interview Survey, adjusting for socioeconomic status and demographic factors (20). To determine whether differences in smoking intensity could account for the observed black:white differences in T cell subset proportions, we reanalyzed the earlier study data, including only those whites who smoked 20 cigarettes or fewer per day. These analyses confirmed that even light to moderate cigarette smoking among whites was associated with a significant increase in the proportion of CD4⁺ cells and a decrease in the proportion of CD16⁺ natural killer cells. Within the black population, smoking intensity among younger smokers (age $<$ 45 yr) was similar to that in older smokers. Thus, it appears unlikely that differences in smoking habits can account for the differential effect of smoking on the CD4⁺ cell subset in blacks compared with whites or in older blacks compared with younger blacks. We cannot exclude the possibility that analysis of a larger population of older blacks might reveal smoking-related differences that were not detectable in our limited population. In addition, the participation rate for blacks in this study (78% for the telephone interview and 57% for phlebotomy) was lower than that for whites in the earlier study (85% for the interview and 74% for phlebotomy), despite the use of a well-trained panel of black and white interviewers and provision of modest remuneration. The potential bias introduced by this disparity in participation rates is uncertain, although adjustment for potential confounders and surrogate measures of socioeconomic status did not significantly alter the results. It should be noted that not all investigators have found a smoking-related increase in CD4⁺ cells among whites. One group has reported a decrease in CD4⁺ cells in white heavy smokers that resolved after smoking cessation (6, 21).

The health implications of these black:white differences are uncertain. Several other immunologic differences between blacks and whites have been reported. In addition to the previously discussed differences in WBC count and differential, blacks have been reported to

have an increase in serum IgE (22), a decrease in serum IgG (12), an increase in circulating B cells and activated T cells relative to whites (14, 15), and a genetically determined heterogeneity in expression of an epitope of the CD4 cell surface antigen that is not observed in whites (23). None of these black:white differences has been associated with specific diseases or detectable differences in health outcome.

In summary, we have presented data from a population-based sample of healthy black adults that suggest that the immunologic effects of cigarette smoking may be significantly modified by ethnic group. It is possible that other common host-environment interactions may also be modified by ethnic characteristics. Additional efforts to define these differences and explore their underlying mechanisms may provide important clues to guide future treatment and prevention strategies.

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