Original Article

Impact of Aging and HIV Infection on the Function of the C-Type Lectin Receptor MINCLE in Monocytes

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Abstract

Both aging and HIV infection are associated with an enhanced pro-inflammatory environment that contributes to impaired immune responses and is mediated in part by innate immune pattern-recognition receptors. MINCLE is a C-type lectin receptor that recognizes trehalose-6,6'-dimycolate or "cord factor," the most abundant glycolipid in *Mycobacterium tuberculosis*. Here, we evaluated MINCLE function in monocytes in a cohort of HIV-infected and uninfected young (21–35 years) and older adults (≥60 years) via stimulation of peripheral blood mononuclear cells with trehalose-6,6'-dibehenate, a synthetic analog of trehalose-6,6'-dimycolate and measurement of cytokine production (interleukin [IL]-10, IL-12, IL-6, tumor necrosis factor- α) by multicolor flow cytometry. Our studies show an age- and HIV-associated increase in cytokine multifunctionality of monocytes both at the population and single cell level that was dominated by IL-12, IL-10, and IL-6. These findings provide insight into the host response to *M. tuberculosis* and possible sources for the pro-inflammatory environment seen in aging and HIV infection.

Keywords: Innate, Immune, TDB, Multifunctional

The C-type lectin receptor family of innate immune pattern recognition receptors (PRRs) is primarily expressed on myeloid cells and recognizes both carbohydrate and lipid moieties (1). The C-type lectin receptor MINCLE (*Clec4e*; macrophage-inducible C-type lectin) (2,3) recognizes trehalose-6,6'-dimycolate (TDM), also known as cord factor—the most abundant glycolipid in the cell wall of *Mycobacterium tuberculosis* (MTB) (4). TDM induces formation of granulomas when injected into mice (5), and deletion of TDM from MTB reduces its ability to survive within macrophages (6,7). MINCLE, through its association with FcR γ , signals inflammatory responses in antigen-presenting cells in both mice and humans, via the nonreceptor tyrosine kinase Syk and the CARD9 pathway (8,9). However, human MINCLE function remains incompletely understood, and the effects of aging and HIV infection have not been studied. The potential role of MINCLE in defense against MTB is particularly relevant to older adults, who are at increased risk for

reactivation of latent tuberculosis (10–12) and have higher rates of disseminated disease and worse treatment outcomes (13–15). Similarly, HIV infection is also associated with increased rates of reactivation disease (16–18), and more rapid clinical progression (10). Consequently, we evaluated MINCLE function in a cohort of HIV-infected and uninfected young and older adults. We focused on monocytes, the most abundant innate cells in the peripheral blood functioning as antigen-presenting cells that may differentiate into macrophages at sites of inflammation. Monocytes can be subdivided into the following subsets as defined by their expression of CD14 and CD16: classical (CD14+CD16lo); inflammatory (CD14+CD16+), known to be expanded in the setting of aging and HIV infection; and the nonclassical (CD14+CD16hi) subset, also associated with HIV disease. Monocytes can be further defined by expression of activation markers such as CD11b of which the expression is increased in the setting of aging and HIV infection (19–23). Our results show

for the first time an age- and HIV-associated increase in MINCLE-dependent cytokine production and cytokine multifunctionality (production of multiple cytokines) in monocytes both at the single cell and monocyte population level, suggesting that MINCLE signaling could contribute to the heightened pro-inflammatory milieu influencing impaired immune responses to pathogens that is associated with age or HIV infection (21,23).

Materials and Methods

Clinical Study Design and Recruitment of Participants

Older (age ≥ 60 years) and younger adults, (age 21–35 years) were recruited from the Yale Health Services, Yale Primary Care Center, and the Nathan Smith HIV Clinic at Yale-New Haven Hospital. Informed consent was obtained according to a protocol approved by the Human Research Protection Program of the Yale School of Medicine. Participants were evaluated for clinical characteristics by chart review and self-report for demographic information, medications, CD4 count, HIV viral load, and comorbid conditions. Participants were excluded for the following reasons: an acute infection or antibiotic use within 2 weeks of recruitment; pregnancy; history of current cancer; history of stem cell, bone marrow, or solid organ transplant; cirrhosis of the liver; kidney disease requiring dialysis; immunodeficiency other than HIV; and active Hepatitis B or C infection.

Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll gradient centrifugation (Histopaque Sigma) as previously described (24,25). Freshly isolated PBMCs were stimulated with 5 $\mu\text{g/mL}$ of TDB (trehalose-6,6-dibehenate)—a synthetic analog of cord factor (InvivoGen)—for 18 hours at 37°C in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and Penicillin/Streptomycin/l-Glutamine. Brefeldin-A (GolgiPlug BD-Biosciences) was added for the last 6 hours of stimulation. Cells were then surface stained with anti-CD14-PE-CF594 (3G8, BD-Biosciences), anti-CD16-PE-Cy7 (3G8, BD-Biosciences), and anti-CD11b-APC-Cy7 (ICRF44, BD-Biosciences). Cells were fixed in Cytofix buffer (BD-Biosciences) and stored at -80°C in freezing medium. On the day of analysis, cells were thawed, washed, and permeabilized with Cytofix/Cytoperm (BD-Biosciences) and Perm/Wash buffer (BD-Biosciences) for intracellular cytokine staining with anti-interleukin-10 (IL-10) Pacific Blue (JES3-9d7, eBioscience), anti-interleukin-12 p70 (IL-12) PE (20C2, BD-Biosciences), anti-interleukin-6 (IL-6) fluorescein isothiocyanate (MQ2-13A5, eBioscience), and anti-tumor necrosis factor- α (TNF- α) Alexa Fluor 700 (MAB11, BD-Biosciences). Samples were run using a Fortessa LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (FlowJo, LLC), including the Boolean gating analysis. Cells were also analyzed for MINCLE surface expression (anti-MINCLE-15H5, InvivoGen) by flow cytometry.

Statistical Analyses

Patient characteristics were summarized as counts and percentages according to HIV status. Because some of the cytokine outcomes departed from the Gaussian model, nonparametric Wilcoxon Rank Sum Tests were conducted to compare cytokine outcomes according to age group and HIV status. Such tests were conducted, as possible, in four strata consisting of the older only, the younger only,

the HIV-positive only, and the HIV-negative only, and for each of four monocyte groups—classical, non-classical, inflammatory, and activated. A similar analytical plan was applied to MINCLE expression outcomes. All p values, with the exception of multivariable model results and meta-analyses of MINCLE expression, were adjusted with a false discovery rate correction (26) to account for the multiplicity of 11 outcome comparisons within the aforementioned groupings.

Where initial screenings identified approximately Gaussian outcomes and evidence of age group and HIV status differences, multivariable linear regression models were fit including indicator variables for older age, HIV-positive status, an interaction term crossing them, and selected covariates. Covariates included gender, race, recreational drug use, history of a positive QuantiFERON or Purified Protein Derivative test response, percentage of life span with HIV (since diagnosis), and number of comorbid conditions. Models were pruned of nonsignificant variables ($p > .20$) by a forward selection process. All regression models were assessed for goodness of fit using residual plots, influence diagnostics, and goodness-of-fit statistics. Reported model results are supplemented by least squares means for the four groups obtained by the cross-tabulation of the age group and HIV status variables.

SAS 9.4 statistical software was used for all analyses. R version 3.3.3 statistical software was used to create heat maps of cytokine production with the pheatmap R package (27) and to perform the meta-analysis of *CLEC4E* expression by combining effect sizes (mean differences) using the *rma()* function from the metafor R package (28). P values less than .05 for two-sided tests were interpreted as statistically significant. Gene data of *CLEC4E*/MINCLE expression from 5 recruitment years are available through the Gene Expression Omnibus Database with accession numbers GSE59635, GSE59654, GSE59743, GSE101709, and GSE101710.

Results

We recruited a total of 81 HIV-negative and HIV-positive younger (21–35 years) and older (≥ 60 years) adults; the characteristics of enrolled individuals are shown in Table 1. The HIV-positive and HIV-negative groups were comparable in age and gender distribution, incidence of diabetes, metabolic syndrome, cardiovascular disease, and pulmonary disease. HIV-positive adults, compared to the HIV-negative cohort, had higher rates of recreational drug use and number of comorbidities and differed in distribution of self-reported race and Hispanic ethnicity. Most of the HIV-infected cohort were on antiretroviral therapy and had CD4 counts $>200/\text{mm}^3$. The percent life span with HIV-infection (since diagnosis) was calculated for each HIV-infected participant, and is represented as a median with an interquartile range (25 percentile, 75 percentile). Of note, there were four congenitally HIV-infected participants, all in the young group.

Freshly isolated PBMCs were stimulated with TDB, a synthetic analog of cord factor that is a MINCLE agonist (8) and analyzed via intracellular cytokine staining and multicolor flow cytometry. We determined intracellular IL-10, IL-12, IL-6, and TNF- α production in classical (CD14+CD16lo), inflammatory (CD14+CD16+), activated (CD11b+CD14+), and nonclassical monocytes (CD14+CD16hi) monocytes (20). In classical monocytes (Figure 1a), we found a substantial, age-associated increase in IL-10 production ($p = .006$), with the least square mean (LSM) in HIV-negative individuals noted to be 5.66 in young adults versus 14.68 in older adults; larger magnitude, age-associated increases were also noted for IL-12 ($p = .001$,

Table 1. Descriptive Statistics Stratified by HIV Status (*N* = 81)

	HIV positive (n) (%)	HIV negative (n) (%)	<i>p</i> value
<i>Age (in years)</i>			
21–35	36 (44.4)	45 (55.6)	.48 [†]
≥60	14 (38.9)	21 (46.7)	
<i>Gender</i>			
Male	22 (61.1)	24 (53.3)	.21 [†]
Female			
<i>Race</i>			
Black	21 (58.3)	20 (44.4)	<.001 [†]
White	15 (41.7)	25 (55.6)	
Asian			
Other	15 (41.7)	6 (13.3)	
<i>Ethnicity</i>			
Hispanic	13 (36.1)	33 (73.3)	
<i>Number of Co-morbidities</i>			
Co-morbidities (0)	0 (0.0)	4 (8.9)	.01 [†]
Co-morbidities (1–3)	18 (50.0)	12 (26.7)	.04 [‡]
Co-morbidities (4–7)	7 (19.4)	10 (22.2)	
<i>Specific Co-morbidities</i>			
Diabetes	8 (22.2)	11 (24.4)	.81 [†]
Metabolic syndrome (DM + HTN+ HLD)	5 (13.9)	11 (24.4)	.24 [†]
Cardiovascular disease	10 (27.8)	12 (26.7)	.91 [†]
Pulmonary disease	11 (30.6)	12 (26.7)	.70 [†]
<i>Substance use</i>			
Smoking	9 (25.0)	5 (11.1)	.10 [†]
Recreational drugs	9 (25.0)	2 (4.4)	.01 [†]
<i>Tuberculosis risk factor</i>			
History of positive PPD/QuantiFERON	6 (16.7)	2 (4.6)	.13 [‡]
<i>HIV disease factors</i>			
CD4 count >200/mm ³	35 (97.2)	Not measured	
On antiretroviral therapy	34 (94.4)	N/A	
HIV VL > 100	4 (11.1)	N/A	
% life span HIV	*29.7 (19.0, 43.7)	0.0 (0.0, 0.0)	

Note: Demographic information for HIV-positive and HIV-negative cohorts. DM = diabetes mellitus; HLD = hyperlipidemia; HTN = hypertension; PPD = purified protein derivative.

*The value of % life span HIV (since diagnosis) represented in the table represents an interquartile range, with the median (25 percentile, 75 percentile). *p* values less than or equal to .05 were considered significant.

[†]*p* values calculated using the Pearson chi-square test

[‡]*p* values calculated using the Fisher exact test.

LSM-young [0.04], LSM-older [12.5]) and IL-6 ($p = .001$, LSM-young [0.12], LSM-older [15.9]). Cells from HIV-positive young adults produced significantly more IL-10 ($p = .002$, LSM [29.67]), IL-12 ($p < .001$, LSM [24.8]), and IL-6 ($p = .008$, LSM [14.2]) compared to HIV-negative younger adults following MINCLE stimulation. However, cytokine production in monocytes from HIV-positive older adults (IL-10-LSM [23.6]; IL-12-LSM [19.3]; IL-6-LSM [12.58]) was comparable to that in monocytes from HIV-negative older and HIV-positive young adults. MINCLE-induced TNF- α production was comparable among all age/HIV status groups.

We used Boolean gating (29) to determine whether multiple cytokines were being produced at the single cell level within the classical monocyte subset after stimulation with TDB (Figure 1b and c), by examining all possible combinations of cytokines produced within a single cell. Notably, CD14+CD16lo classical monocytes from HIV-negative older adults showed significantly increased production of multiple cytokine combinations compared to HIV-negative younger adults, including IL-10/IL-12 ($p = .002$, LSM-young [3.18], LSM-older [14.39]), IL-10/IL-6 ($p = .002$, LSM-young [4.69], LSM-older

[16.89]), IL-12/TNF- α ($p = .017$, LSM-young [0.8], LSM-older [6.6]), IL-6/IL-12 ($p = .001$, LSM-young [0.77], LSM-older [14.15]), IL-10/IL-12/IL-6 ($p = .001$, LSM-young [0.62], LSM-older [4.8]), and IL-10/IL-12/IL-6/TNF- α ($p = .002$, LSM-young [1.21], LSM-older [8.55]). HIV-positive young adults showed markedly increased multifunctionality or production of multiple cytokines compared to HIV-negative young adults, with increased levels of monocytes producing the following cytokine combinations: IL-10/IL-12 ($p < .001$, LSM [30.46], IL-10/IL-6 ($p = .001$, LSM [26.67]), IL-12/TNF- α ($p = .001$, LSM [21.37]), IL-6/IL-12 ($p = .001$, LSM [25.6]), IL-10/IL-12/IL-6 ($p = .001$, LSM [4.19]), and IL-10/IL-12/IL-6/TNF- α ($p = .001$, LSM [18.28]). Classical monocytes from HIV-positive older adults showed multifunctionality that was not significantly different as observed in this study when compared to HIV-negative older adults or HIV-positive younger adults. Taken together, these data indicate that increased age (particularly in HIV-negative adults) and HIV infection contribute to increased MINCLE-dependent multifunctionality at both the population and single-cell level within classical monocytes.

Figure 1. Effects of age and HIV infection on Mincle-induced cytokine production in CD14+CD16_{lo} classical monocytes. The cohort consists of HIV-negative young adults (age 21–35) ($n = 21$), HIV-negative older adults (age ≥ 60) ($n = 24$), HIV-positive young adults ($n = 14$), and HIV-positive older adults ($n = 22$). (a) Dot plots showing percent change in production of interleukin IL-10, IL-12, IL-6, and tumor necrosis factor- α (TNF- α) compared to baseline after trehalose-6,6-dibehenate (TDB) stimulation in classical monocytes in the indicated populations of HIV-negative and HIV-positive, young, and older adults as measured by intracellular cytokine staining and flow cytometry. The following comparisons, indicated by asterisks, were statistically significant using a Wilcoxon two-sample test with t approximation, which were then adjusted with a false discovery rate (FDR) calculation for multiple comparisons: HIV-negative older adults versus young adults, IL-10 ($p = .002$), IL-12 ($p = .001$), and IL-6 ($p = .001$); HIV-positive versus HIV-negative younger adults, IL-10 ($p = .002$), IL-12 ($p < .001$), IL-6 ($p = .001$). All other comparisons were not significant as observed in this study. (b) Boolean

In CD14+CD16₊ inflammatory monocytes (Figure 2a), both IL-6 ($p = .005$) and IL-12 ($p = .007$) production were significantly increased following Mincle activation in HIV-negative older, compared to HIV-negative young adults. IL-6 ($p = .012$), IL-10 ($p = .028$), IL-12 ($p = .001$), and (in contrast to classical monocytes) TNF- α ($p = .001$) production was higher in cells from HIV-positive, compared to HIV-negative young adults. HIV-positive older adults produced significantly more IL-12 ($p = .011$) compared to HIV-negative older adults, but showed comparable production of IL-6 and IL-10. Inflammatory monocytes from HIV-positive young and older adults showed comparable cytokine responses to TDB stimulation. Boolean gating of the inflammatory monocyte subset in the HIV-negative group (Figure 2b) showed increased age-associated multifunctionality at the single-cell level for cells producing IL-6/IL-12 ($p = .005$). HIV infection in young adults was associated with increased multifunctionality for all cytokine combinations compared to HIV-negative young adults: IL-10/IL-6 ($p = .028$), IL-10/IL-12 ($p = .001$), IL-12/TNF- α ($p = .001$; data not shown), IL-6/IL-12 ($p < .001$), IL-10/IL-12/IL-6 ($p = .018$; data not shown), and IL-10/IL-12/IL-6/TNF- α ($p = .013$). Notably, we also found increased multifunctionality in HIV-positive versus HIV-negative older adults for IL-10/IL-12 ($p = .021$) and IL-12/TNF- α ($p = .018$; data not shown). Similar to classical monocytes, no differences in multifunctionality were noted comparing HIV-infected young versus older adults. When the nonclassical monocyte subset was examined, there were no age-associated differences in total cytokine production (Supplementary Figure 1a and b). However, significantly increased production of IL-10 and IL-12 was found upon Mincle stimulation in HIV-positive versus HIV-negative young adults, and increased production of IL-6, IL-12, and IL-10 in HIV-positive versus HIV-negative older adults (Supplementary Figure 1a). Increased multifunctionality at the single cell level was also associated with HIV infection, but not with increased age (Supplementary Figure 1b). Taken together, these findings suggest that HIV status strongly affects Mincle-dependent cytokine production in both inflammatory and nonclassical monocytes.

Finally, in the CD11b+CD14₊ activated monocyte subset (Supplementary Figure 2a), HIV-negative older adults produced significantly more IL-6 and IL-12 than uninfected young adults. HIV status was also associated with increased production of IL-12 and IL-10 for both older and young adults. Boolean gating showed increased multifunctionality with HIV infection, with an age-associated increase in cells producing IL-6/IL-12 found in HIV-negative older, versus young adults (Supplementary Figure 2b).

To evaluate the basis underlying age- and HIV-associated increases in Mincle-dependent cytokine production, we assessed

gating demonstrates increased cytokine multifunctionality at the single cell level in older and HIV-infected adults. Shown is a color heat map of Boolean gating (percent change from baseline) of all cytokine combinations produced at the single cell level for each cohort following TDB stimulation. Number of co-morbidities are indicated in the green heat map symbols. (c) Dot plots showing Boolean gating in the indicated cohorts. HIV-negative older adults versus young adults showed increased proportions of monocytes producing IL-10/IL-12 ($p = .002$), IL-10/IL-6 ($p = .002$), IL-12/TNF- α ($p = .017$, data shown in panel b), IL-6/IL-12 ($p = .001$), IL-10/IL-12/IL-6 ($p = .001$, data shown in panel b), IL-10/IL-12/IL-6/TNF- α ($p = .002$); HIV-positive versus HIV-negative young adults, IL-10/IL-12 ($p < .001$), IL-10/IL-6 ($p = .001$), IL-12/TNF- α ($p = .001$, data shown in panel b), IL-6/IL-12 ($p = .001$), IL-10/IL-12/IL-6 ($p = .001$, data shown in panel b), IL-10/IL-12/IL-6/TNF- α ($p = .001$). All other comparisons were not significant as observed in this study.

Figure 2. Effects of age and HIV infection on MINCLE-induced cytokine production in CD14+CD16+ Inflammatory monocytes. The cohort consists of HIV-negative young adults ($n = 21$), HIV-negative older adults ($n = 24$), HIV-positive young adults ($n = 14$), HIV-positive older adults ($n = 22$). (a) Dot plots of percent change in production of interleukin IL-10, IL-12, IL-6, and tumor necrosis factor- α (TNF- α) compared to baseline in the indicated groups, as assessed by intracellular cytokine staining, after stimulation with trehalose-6,6-dibehenate. The following comparisons, indicated by asterisks, were statistically significant using a Wilcoxon two-sample test with t approximation, with an additional false discovery rate adjustment: HIV-negative older adults versus young adults, IL-6 ($p = .005$), IL-12 ($p = .007$); HIV-positive versus HIV-negative young adults, IL-6 ($p = .012$), IL-10 ($p = .028$), IL-12 ($p = .001$), and TNF- α ($p = .001$); HIV-positive versus HIV-negative older adults: IL-12 ($p = .011$). All other comparisons were not significant as observed in this study. (b) Dot plots showing Boolean gating of specific cytokine combinations in the indicated cohorts. The following statistically significant comparisons are shown (indicated by asterisks): HIV-negative older versus young adults, IL-6/IL-12 ($p = 0.005$); HIV-positive versus HIV-negative young adults, IL-10/IL-12 ($p = .001$), IL-6/IL-12 ($p < .001$), IL-10/IL-6 ($p = .028$), IL-10/IL-12/IL-6/TNF- α ($p = .013$); HIV-positive versus HIV-negative older adults, IL-10/IL-12 ($p = .021$).

MINCLE protein expression by flow cytometry (Figure 3a–d). In classical monocytes, MINCLE expression was significantly increased in HIV-negative older, compared to HIV-negative young adults

($p = .003$) and in HIV-positive young versus HIV-negative young adults ($p = .005$)—reflecting the patterns noted in analysis of cytokine production. An analogous pattern of MINCLE expression was found in nonclassical monocytes, although MINCLE-dependent cytokine production only differed for HIV-positive, compared to HIV-negative individuals. No significant differences in MINCLE expression were found in the inflammatory and activated monocyte populations, as observed in this study.

To study whether the age-associated increase in MINCLE surface protein in monocyte populations could arise from changes in gene expression, we carried out a meta-analysis of *MINCLE*/*CLEC4E* gene expression from microarray data on PBMCs from cohorts of young and older HIV-negative adults recruited for a study of influenza vaccine response over five consecutive vaccine seasons (Supplementary Figure 3). The effect sizes in each data set were calculated as the mean log-transformed difference in expression between older and young adults. A random-effects model was used to combine effect sizes across data sets. We found expression of *MINCLE*/*CLEC4E* in total PBMCs that was significantly detected above background in at least 90% of participants in every data set (detection $p < .05$), and found that across all 5 years, older adults expressed higher levels of *MINCLE*/*CLEC4E* than young adults ($p = .003$). An analysis comparing the percentage of total, inflammatory, and classical monocytes in young versus older HIV-negative adults did not show statistically significant differences as observed in this study, suggesting that age-related changes in PBMC composition do not account for this change in *MINCLE*/*CLEC4E* expression. Thus, increases in *MINCLE*/*CLEC4E* gene expression and protein levels may contribute to the age-associated increases we find in MINCLE-dependent cytokine production.

We generated a multivariable linear regression model focusing on IL-12 production because it was most strongly associated in unadjusted analyses with increased age and HIV infection in most monocyte subsets (refer to Supplementary Table 2). Classical monocytes were further analyzed in a model that included only explanatory variables for older age, HIV-positive status, and their interaction. This model for IL-12 production yielded a statistically significant interaction term ($p < .0001$) crossing age and HIV infection. Findings from this analysis mirrored those from unadjusted bivariate analyses, with a significant age-associated difference in least squares means for IL-12 production found in HIV-negative adults ($p < .0001$) and a significant difference in least squares means between HIV-positive versus HIV-negative young adults ($p < .0001$). In unadjusted models, the magnitude of IL-12 production was substantially elevated in young adults with HIV infection (LSM-24.81) compared to uninfected young adults (LSM-0.04), and in HIV-positive older adults (LSM-19.33) compared to uninfected older adults (LSM-12.52). The number of comorbid conditions, when added as a covariate to the regression model, contributed significantly to model fit ($p = .023$) and reduced the least squares means in older adults (both HIV-positive and negative; Supplementary Table 2), indicating an important contribution of comorbidity to MINCLE function in older adults (also shown in Figure 1b). We also considered the effect of variability in duration of HIV infection, and found that incorporating a variable for percentage of life span with HIV infection was significant when added to a model also including number of comorbidities as a covariate; however, even with adjustment for these covariates, the differences in MINCLE-dependent IL-12 production remained significant.

Figure 3. Effects of age and HIV infection on MINCLE protein expression in monocyte subsets. HIV-negative young adults ($n = 20$), HIV-negative older adults ($n = 18$), HIV-positive young adults ($n = 10$), HIV-positive older adults ($n = 10$) were assessed. Shown are dot plots depicting MINCLE protein expression (Mean Fluorescence Intensity (MFI)) as assessed by flow cytometry for (a) Inflammatory (CD14+CD16+), (b) Classical (CD14+CD16lo), (c) Activated (CD11b+CD14+), and (d) Nonclassical (CD14+CD16hi) monocytes. Statistically significant comparisons, indicated by asterisks, were noted using a Wilcoxon two-sample test with t approximation, with an additional false discovery rate adjustment: Classical monocytes, HIV-negative older versus young adults, ($p = .003$); HIV-positive versus HIV-negative young adults, ($p = .005$); non-classical monocytes, HIV-negative older versus young adults, ($p = .003$), and HIV-positive versus HIV-negative young adults, ($p = .03$). All other comparisons did not reach statistical significance as observed in this study.

Discussion

We have carried out the first analysis of the effects of age and HIV infection on the function of the C-type lectin receptor family member MINCLE in human monocyte populations. In CD14+CD16lo classical monocytes, MINCLE-induced cytokine production and production of multiple cytokines at the single-cell level were increased in cells from HIV-negative older, compared to young adults, and in HIV-positive, compared to HIV-negative adults. CD14+CD16+ monocytes also showed evidence of an age-associated increase in cytokine production in HIV-negative individuals, but such inflammatory monocytes, as well as CD14+ CD16hi nonclassical monocytes showed substantial increases in cytokine production associated with HIV-infection in both young and older adults. Notably, most previous studies of innate immune PRR activation have shown decreased cytokine production in the setting of aging (21,24,25), for example, in the context of toll-like receptor (TLR) function, with increased basal cytokine production as a potential contributing factor limiting further TLR activation (25). Despite evidence for elevated cytokine levels in peripheral blood from older adults (so-called “Inflammaging” (21,30)), few examples of increased age-associated cytokine production following innate immune PRR activation have been reported.

Our data provide evidence for multifunctionality resulting from MINCLE engagement both at the population and (as assessed by Boolean gating) at the single-cell level. In two previous studies, monocyte stimulation by TLR 2 in HIV-negative and of TLR 2/4/7 in HIV-positive adults was also associated with production of multiple cytokines (31,32). Our findings provide new evidence that dysregulated innate immune receptor

activation may contribute directly to the elevated inflammatory milieu associated with human aging and HIV infection. Interestingly, MINCLE-dependent cytokine production in monocytes from HIV-infected older versus HIV-infected young adults showed no significant age-associated potentiation, suggesting that the effects of HIV infection supercede those of aging.

Increased production of IL-12 was strongly associated with both older age and HIV infection, particularly in the classical, inflammatory, and activated monocyte subsets. When IL-12 production in classical monocytes was examined within the context of a multi-variable linear regression model, both older age and HIV infection remained strongly associated with IL-12 production. We found that number of comorbid medical conditions appeared to correlate with increased multifunctionality (Figure 1b); indeed, there was a significant interaction when number of comorbidities was included as a covariate in the regression model. We also considered variation in duration of HIV disease as another potential source of heterogeneity among HIV-infected individuals. Consequently, we included percent life span with HIV infection (since diagnosis) in the regression model together with comorbidity number; although the percent life span variable following HIV diagnosis underestimates the true duration of infection, incorporation of this covariate also decreased means in both HIV-infected older and younger adults. These findings suggest that number of comorbidities and duration of HIV infection are important sources of heterogeneity that should be considered in studies of immune responses in the context of HIV infection and aging. However, it is worth noting that our cohort included four congenitally HIV infected individuals (100% life span with HIV), which could have contributed to the significance of percent life span. Our cohort of HIV-infected individuals contained four individuals with a positive HIV viral load, usually in the setting of noncompliance; however, a sensitivity analysis that removed these individuals did not show substantial changes in the means (data not shown). Another sensitivity analysis to assess the role of CD4 count, excluding individuals with low CD4 counts $<200/\text{mm}^3$, also did not change the conclusions of the study (data not shown).

It is notable that our Boolean gating analysis revealed significantly increased levels of monocytes producing both IL-12, a cytokine crucial for Th1 responses, with IL-10, an anti-inflammatory cytokine in monocytes, as a function of age and HIV status. MINCLE has been proposed to have dual roles in inflammation, with both pro- and anti-inflammatory functions (33). Studies in mice have shown production of IL-10 in macrophages following MINCLE activation with concurrent dampening of TLR 2-induced IL-12p40 production (34), along with nitric oxide induction that dampened inflammasome activation (35). IL-10 induction by MTB has been noted in human studies (36–38), and may promote immune evasion by inhibition of phagosome/lysosome fusion in innate cells (36,39). It is notable that TDM/Cord factor—hypothesized to promote evasion of the immune system (33)—suppresses phagosome/lysosome fusion in mice (7). In contrast, TDM also induces pro-inflammatory responses, such as the formation of granulomas when injected into mice (4,5); both TDB and TDM can stimulate human antigen-presenting cells, including monocytes, with strong induction of IL-8, IL-6, and chemokines (8). The mechanisms underlying these opposing functions of MINCLE/TDM remain to be elucidated.

Although IL-12 is an important component of the Th1/IFN- γ (type II interferon) axis, known to be important for control of mycobacterial disease (40), it is unclear whether the enhanced multifunctionality noted in monocyte populations from older and HIV-infected adults represents an improved or detrimental response.

However, it is notable that murine knockouts of components of the Type I IFN pathway are more protected against MTB disease and that an elevated IFN response signature in PBMCs is associated with increased risk for MTB progression in humans (41,42). In addition, MINCLE-induced IL-10 production could influence the development of regulatory T cells (43,44), representing an additional potential mechanism for immune dysfunction. Consequently, our findings of increased multifunctionality may represent immune dysregulation that can interfere with MTB control and contribute to the increased risk for tuberculosis seen in the context of HIV infection or aging. The inflammatory profile seen in these monocytes could also influence future differentiation to macrophages and granuloma formation/resolution in the context of MTB infection (35,42).

Increased multifunctionality in both older adults and HIV-infected adults can in part be explained by increased MINCLE protein expression, particularly in the classical monocyte subset, in both older HIV-negative and HIV-infected adults when compared to younger uninfected adults. Consistent with this, MINCLE RNA expression in PBMCs was also noted to be higher in HIV-negative older, compared to younger adults. However, MINCLE protein expression was unperturbed in inflammatory and activated monocytes, suggesting a role for other mechanisms, such as alterations in intracellular signaling. The basis for increased MINCLE protein expression remains unclear; however, for HIV-infected adults the presence of increased plasma levels of LPS (45,46) suggests a potential means for MINCLE induction, in view of its original characterization as an LPS-inducible receptor (2). Increased serum levels of LPS binding protein have been reported in older, compared to young individuals—a potential surrogate marker for increased LPS and microbial translocation (47).

Conclusion

TDB, the cord factor analog, is now in human trials as an adjuvant (48,49). Whether the age- and HIV-dependent increase in MINCLE-induced cytokine production we observed results in an enhanced or dysregulated immune response to TDB-adjuvanted vaccines in these populations remains to be determined. Finally, it is notable that previous studies have suggested that elevated levels of both pro- and anti-inflammatory cytokines are found in older, compared to young adults (50). The source for the age-related pro-inflammatory environment remains incompletely understood, but innate immune PRR activation by increased levels of endogenous damage-associated molecular patterns has been proposed as a potential mechanism (21,30). Our findings of increased induction of IL-12, IL-6 as well as IL-10 in response to MINCLE stimulation provide evidence for a direct contribution of innate immune PRR signaling to age- or HIV-associated inflammatory dysfunction.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

L.B. has received honoraria from Gilead for being a consultant. S.A. reports honoraria from Janssen R&D. All other authors report no conflicts of interest.

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