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ORIGINAL ARTICLE

Inflammaging phenotype in rhesus macaques is associated with a decline in epithelial barrier-protective functions and increased pro-inflammatory function in CD161-expressing cells

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Abstract The development of chronic inflammation, called inflammaging, contributes to the pathogenesis of age-related diseases. Although it is known that both B and T lymphocyte compartments of the adaptive immune system deteriorate with advancing age, the impact of aging on immune functions of Th17-type CD161-expressing innate immune cells and their role in inflammaging remain incompletely understood. Here, utilizing the nonhuman primate model of rhesus macaques, we report that a dysregulated Th17-type effector function of $CD161^+$ immune cells is associated with leaky gut and inflammatory phenotype of aging. Higher plasma levels of inflammatory cytokines IL-6, TNF-α, IL-1β, GM-CSF, IL-12, and Eotaxin correlated with elevated markers of gut permeability including LPS-binding protein (LBP), intestinal fatty acid binding protein (I-FABP), and sCD14 in aging macaques. Further, older macaques displayed significantly lower frequencies of circulating Th17-type immune cells comprised of $CD161^+$ T cell subsets, NK cells, and innate lymphoid cells. Corresponding with the increased markers of gut permeability, production of the type-17 cytokines IL-17 and IL-22 was impaired in $CD161^+$ T cell subsets and NK cells, along with a skewing towards IFN-γ cytokine production. These findings suggest that reduced frequencies of $CD161^+$ immune cells along with a specific loss in Th17-type effector functions contribute to impaired gut barrier integrity and systemic inflammation in

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aging macaques. Modulating type-17 immune cell functions via cytokine therapy or dietary interventions towards reducing chronic inflammation in inflammaging individuals may have the potential to prevent or delay age-related chronic diseases and improve immune responses in the elderly population.

Keywords Inflammaging · Leaky gut · CD161+ cells · I-FABP · LBP · sCD14

Introduction

Older adults are more susceptible to infections and generate poor vaccine responses due to decline in immune functions along with a chronic inflammatory status characteristic of the aging process. This gradual increase in sub-clinical, low-grade inflammation with aging is referred to as inflammaging (Ciabattini et al. 2018; Franceschi et al. 2000). Inflammaging is characterized by increased plasma levels of multiple pro-inflammatory cytokines (IL-6, IL-8, IL-15, etc.), coagulation factors, and acute phase reactants (Franceschi et al. 2000) and is associated with several age-related pathologies (Calder et al. 2017; Franceschi et al. 2000). Elevated levels of IL-6, in particular, have been linked to cardiovascular disease (Cesari et al. 2003), impaired mobility (Penninx et al. 2004), cancer (Il'yasova et al. 2005), cognitive decline (Yaffe et al. 2003), and all-cause mortality (Reiner et al. 2013; Simanek et al. 2011) in older people.

The association between inflammaging and the loss of intestinal barrier function has been established in a wide range of species. Studies in *Drosophila melanogaster* have shown that systemic inflammation of aging is associated with dysregulated intestinal immune signaling and a breakdown in intestinal barrier functions due to age-related changes in the intestinal microbiome (Clark et al. 2015; Rera et al. 2012). Thevaranjan et al. demonstrated a similar pattern of intestinal barrier dysfunction promoting systemic inflammation in mice (Thevaranjan et al. 2017). Further, a cross-sectional study of young and elderly donors has shown that plasma biomarkers of intestinal epithelial barrier damage and microbial translocation correlated with systemic inflammation in aging individuals (Steele et al. 2014). However, the precise cellular and molecular mechanisms responsible for age-related functional decline in the mucosal immune system and their contribution to gut epithelial barrier damage and microbial translocation are poorly understood.

T helper 17 (Th17) cells are key players in the maintenance of mucosal immune homeostasis in response to commensal organisms and protection against pathogens via production of cytokines IL-17, Il-21, and IL-22 (Mucida and Salek-Ardakani 2009). Besides classical Th17 cells (IL-17-producing CD4+ T cells), lymphocyte subsets including CD8+ T cells, gammadelta T cells, NKT cells, and ILCs are capable of producing Th17-type cytokines. These cells are characterized by surface expression of CD161, a C-type lectin-like receptor originally associated with NK cell inhibitory functions (Giorda et al. 1990; Lanier et al. 1994). Even though CD161-expressing cells are heterogeneous in their phenotype, functions, and recognition of antigens, these cell subsets share a common transcriptional signature and display innate-like function independent of antigen-specific stimulation (Fergusson et al. 2014). Furthermore, we and others have shown that CD161-expressing cells are enriched at barrier sites including the gut mucosa and lungs of humans and nonhuman primates and display enhanced Th17-type functions (Fergusson et al. 2016; Rout 2016). It appears that effector functions develop through stimulation by cognate antigens expressed by commensal microbes/endogenous ligands in mucosal tissues and have a key role in maintenance of epithelial barrier functions.

Nonhuman primates are physiologically and genetically similar to humans and have been used as animal models to better understand the aging process in humans (Didier et al. 2016). The goal of this study was to characterize the inflammaging phenotype and the gut barrier-protective immune cell functions in aging rhesus macaques (*Macaca mulatta*). We assessed pro-inflammatory mediators and markers of gut permeability in plasma obtained from a large group of macaques and investigated the relationship between advancing age and functionality of Th17-type innate immune cells. Our data demonstrate significant correlation between circulating pro-inflammatory cytokines and biomarkers of loss of gut mucosal barrier function (leaky gut) in aging macaques. Furthermore, this inflammaging phenotype was associated with lower type-17 cytokine production by Th17-type innate immune cells of older macaques. Overall, our study demonstrates that the inflammaging phenotype in aging macaques is similar to that reported in humans and provides insights into the role of Th17-type immune cell functions in inflammaging.

Materials and methods

Rhesus macaques and blood sampling

Indian-ancestry rhesus macaques used in this study were housed in outdoor field cages at the Tulane National Primate Research Center (TNPRC) in Covington, LA, USA. Animals were evaluated by experienced Animal Care and Veterinary Technicians and were considered healthy if no clinical abnormalities that required veterinary intervention were noted at the time of sample collection. Procedures for venipuncture and physical examination were performed during biannual preventive medicine evaluations under anesthesia to minimize stress and were approved by the Institutional Animal Care and Use Committee of Tulane University in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The rhesus macaques were part of the specific pathogen-free (SPF) colony and as part of the biannual preventive medicine surveillance, have been tested twice per year to assure that they remained seronegative for SIV, SRV, herpes B virus and STLV, and PCR negative for SRV (with the exception of > 20-year-old animals that were part of the conventional colony). Exclusion criteria included antibiotic treatment in the past 2 months and current pregnancy. Blood collected in EDTA tubes (Sarstedt S-monovette Blood Collection Tube EDTA, Sarstedt Inc. Newton, NC) was processed immediately. Blood was centrifuged at 14000*g* for 5 min at 4 °C and plasma aliquots were cryopreserved at − 80 °C until used. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation (Lymphocyte Separation Medium; MP Biomedicals Inc., Solon, OH) at 1825*g* for 20 min at 20^0 C using deceleration without braking and used for phenotyping and in vitro functional assays.

Flow cytometry

Multi-color flowcytometric analysis was performed on cells according to standard procedures using anti-human mAbs that cross-react with rhesus macaques. For phenotype analysis, PBMC were surface stained with CD3 APC-Cy7 (BD clone SP34-2), CD4 BV605 (BD clone L200), CD8 BV650 (BD clone SK1), CD14 (BD clone M5E2), CD20 (Biolegend clone 2H7), HLA-DR (Biolegend clone L243), CD127 PE (Beckman Coulter clone R34-34), CD161 PE-Cy7 (Biolegend clone HP-3G10), and TCR Vα7.2 BV421 (Biolegend clone 3C10). Surface staining was carried out by standard procedures as earlier described, (Rout et al. 2012). Briefly, 1 to 2 million PBMC resuspended in 100 μl wash buffer (PBS with 2% FBS) and incubated with surface antibodies for 30 min at 4 °C. After washing, the cells were fixed in 2% paraformaldehyde. All intracellular cytokine staining (ICS) assays were carried out on mitogen-stimulated cells (as described in the functional analysis section). Following 16 h incubation, cells were washed in PBS containing 2% FCS and 0.5 mM EDTA and stained for surface markers in wash buffer for 30 min at 4 °C. The cells were then washed and permeabilized using the BD Cytofix/Cytoperm reagent for 20 min at 4 °C and washed with BD Perm/Wash Buffer. Permeabilized cells were stained intracellularly with antibodies for CD69 PE-CF594 (Dazzle) (Biolegend clone FN50), IFN-γ BV510 (Biolegend clone 4S.B3), IL-17 PerCP-Cy5.5 (eBioscience clone eBio64DEC17), and IL-22 APC (Invitrogen clone IL22JOP). Cells were finally washed in wash buffer and fixed in 1% paraformaldehyde in PBS. Flow cytometric acquisition was performed on the BD Fortessa instrument with FACSDiva software.

Quantification of circulating markers of inflammation, microbial translocation, and intestinal damage

EDTA-preserved plasma samples were centrifuged (14,000*g* for 5 min at 4 °C) and aliquots were frozen at − 80 °C until used. Prior to assay, once-thawed plasma samples were pre-cleared using Ultrafree Centrifugal Filters (Millipore, Billerica, MA). The filtered plasma samples were used for simultaneous quantification of cytokines, chemokines, and growth factors using the multiplexed-bead assay Non-Human Primate Cytokine & Chemokine & Growth Factor 37-plex ProcartaPlex (Invitrogen, Life Technologies), following manufacturers' instructions. Data were acquired with a Bio-Plex 200 analyzer (Bio-Rad, Hercules, CA) and analyzed using Bio-Plex Manager software v6.1 (BioRad). Analysis of markers of leaky gut and microbial translocation, LBP, I-FABP, and sCD14, in stored plasma was carried out by using commercially available ELISA kits. All tests were performed according to the manufacturer's guidelines. The assays were performed in duplicate, and data were analyzed using Gen 5 software (BioTek). Plasma LBP and I-FABP were quantified with 1:2 sample dilution using the Monkey LBP ELISA Kit

and Monkey PP/FABP2 ELISA kit (MyBioSource, San Diego, CA), respectively. The ELISA kit for Human sCD14 (R&D Systems, Minneapolis, MN) applied plasma samples diluted 1:200.

In vitro stimulation for functional analysis

For detecting intracellular cytokine production, 1 million PBMC were stimulated with Phorbol-12-Myristate-13-Acetate (PMA, 10 ng/ml, Sigma-Aldrich) and Ionomycin (1 μg/ml, Sigma-Aldrich) in the presence of brefeldin A (5 μg/ml, Sigma-Aldrich) for 12–16 h at 37 °C in 5% CO2. After activation, the cells were washed in PBS containing 2% FBS and 0.5 mM EDTA and stained appropriately for detection of cytokine production.

Statistical analysis and software

All statistical analysis was performed using Prism Software (Version 8, GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was assessed using the Mann-Whitney *U* test for unpaired data. For all statistical analyses, two-tailed *p* values below 0.05 were regarded as significant. Comparisons across the 3 age-groups were made using a nonparametric test (Kruskal-Wallis) followed by post-hoc comparison by Dunn's test with Bonferroni adjustment. The Mann-Whitney test was used for comparisons between each group. Associations between age and each biomarker measured were examined using the Spearman rank correlation that is robust to transformations and outliers. Analysis of flow cytometric data was performed using FlowJo software (version 9.9.5; TreeStar, Ashland, OR). For experiments measuring polyfunctional responses, Boolean gating was used to partition cells into specific response categories followed by data analysis using PESTLE v2 and SPICE 6 software (https://niaid.github.io/spice).

Results

Elevated levels of circulating pro-inflammatory cytokines in aging rhesus macaques

Since increased levels of circulating inflammatory cytokines in chronologically aged humans have been linked to the development of aging-associated chronic disorders, we aimed to determine whether macaques display a similar increase in circulating pro-inflammatory mediators with age. To this end, multiple cytokines and chemokines were evaluated in plasma samples obtained from adults of different ages. The macaques were stratified as young (5–10 years old), aging (15–20 years old), and old adults (≥ 20 years old). The average lifespan of rhesus macaques in captivity is ~ 27 years and maximal lifespan is ~ 40 years (Mattison et al. 2012). Rhesus macaques age approximately 3–3.5 times that of humans so the young, aging, and old group of macaques are nearly equivalent to humans aged 15–45 years, 45–70 years, and over 70 years of age, respectively (Lane 2000; Roth et al. 2004). A comparison of data obtained from the simultaneous detection of plasma levels of 22 cytokines/chemokines that were within the limits of detection by multiplex assay is shown in Table 1. Multiplex analyses revealed that the concentrations of a panel of six circulating cytokines/chemokines, TNF-α, IL-6, GM-CSF, Eotaxin, IL-1β, and IL-12p70 were significantly higher in the plasma of older macaques (Fig. 1a). In particular, the average levels of Eotaxin, TNF-α, GM-CSF, and IL-6 were greater in older animals in comparison to both the young and aging group of animals (Fig. 1b; heat map showing mean values in each age-group,), indicating a significant increase in an inflammatory phenotype in the oldest group of macaques. In contrast, the circulating levels of the anti-inflammatory cytokines IL-4, IL-5, IL-10, and IL-13 did not differ significantly between the young and aging groups (Fig. 1c). IL-1R antagonist (IL-1Ra) was the only anti-inflammatory protein that was found at significantly elevated levels in the old group of macaques (Fig. 1c, d). Furthermore, a significant positive correlation was observed between chronological age and plasma levels of TNF-α, MCP-1, and GM-CSF, suggesting a progressive increase of pro-inflammatory mediators in aging macaques in the absence of overt infection (Fig. 2). No significant differences between the young and aging groups were observed in circulating levels of the γ-chain cytokines IL-2, IL-7, and IL-15, as well as growth factors including BDNF, bNGF, FGF-2, PDGF-BB, VEGF-A, and VEGF-D (ESM_1). Although the aging group of macaques in our study was predominantly female owing to the demographics of the colony at TNPRC, no significant differences were found for any of the cytokines and analytes in the multiplex assay between the males and females in the young adult group (data not shown).

Table 1 Plasma cytokines and chemokines in young, aging, and old macaques

Analyte	Young		Aging		Old		*p* value	
	Mean (pg/mL)	SE	Mean (pg/mL)	SE	Mean (pg/mL)	SE	Young vs aging	Young vs old
*GM-CSF	10.40	5.25	12.99	2.73	45.07	19.73	0.080	*0.030*
IFN-γ	5.46	1.90	6.31	2.46	12.22	5.77	0.450	0.530
*IL-1β	4.81	1.53	5.72	1.06	17.10	7.34	0.220	*0.040*
*IL-12p70	0.53	0.52	0.79	0.54	4.29	1.92	0.580	*0.016*
IL-17A	11.37	4.36	9.60	1.39	22.72	11.44	0.960	0.06
IL-18	0.54	0.53	1.41	1.40	5.68	5.67	0.740	0.750
IL-23	8.02	4.02	11.10	2.41	25.23	12.48	0.090	0.080
*IL-6	11.11	3.15	9.70	1.87	38.62	18.66	0.860	*0.040*
IL-8	238.16	26.63	167.64	18.39	187.45	38.81	*0.026*	0.056
*TNF-α	24.40	5.75	27.67	3.72	67.52	21.14	0.230	*0.020*
*IP-10	2.82	0.72	4.60	0.78	6.72	2.51	0.054	*0.040*
*MCP-1	76.01	6.60	56.82	4.88	99.81	17.04	0.260	*0.040*
MIP-1α	1.13	0.78	2.76	1.52	3.54	2.08	0.400	0.200
*MIP-1β	32.25	4.99	26.58	12.75	20.24	11.88	0.062	*0.030*
IFN-α	0.01	0.00	0.01	0.00	4.71	4.70	> 0.9999	0.750
I-TAC	4.48	3.07	39.65	39.38	22.96	13.77	0.855	0.390
*Eotaxin	142.53	9.76	148.28	10.53	206.82	12.70	0.845	*0.002*
IL-10	4.26	0.95	3.75	0.00	6.31	1.86	> 0.9999	> 0.9999
IL-13	10.83	3.13	9.99	3.16	12.64	4.71	> 0.9999	0.730
IL-4	11.21	2.24	11.62	2.52	23.60	6.80	0.935	0.056
IL-5	11.38	3.70	6.77	0.68	19.96	5.03	0.260	0.171
*IL-1RA	173.32	49.05	178.09	26.35	503.17	183.02	0.643	*0.006*

Mean and standard error (SE) values are shown for each analyte in young (5–8 years old, $n = 18$), aging (15–20 years old, $n = 17$), and old (≥ 20 years old, $n = 15$) rhesus macaques. Statistically significant differences between young and old by the Mann-Whitney *U* test are italicized and the analytes are marked with an asterisk

Increased markers of microbial translocation in aging macaques

Loss of gut mucosal barrier function, referred to as "leaky gut," has been reported with advanced age in a wide range of species including humans (Ghosh et al. 2015; Rera et al. 2012; Thevaranjan et al. 2017). This allows greater translocation of microbial antigens (microbial translocation) via the intestinal wall into the circulation, thereby contributing to systemic inflammation. Since elevated levels of multiple pro-inflammatory mediators were observed in the older macaques in our study, we next examined markers of microbial translocation in the different groups of macaques using two endotoxin biomarkers, LPS-binding protein (LBP) and soluble CD14 (sCD14). Additionally, intestinal fatty acid binding protein (I-FABP) was also examined as a non-invasive marker of enterocyte damage and loss of mucosal barrier function. Leaky gut markers in old macaques were significantly elevated as mean I-FABP levels were 3-fold greater than young and aging macaque groups ($p = 0.0002$; Fig. 3a). Circulating levels of LBP, which serves as a direct measure of translocated Gram-negative bacterial products, were also significantly greater in old macaques in comparison to young and aging macaques ($p = 0.0018$; Fig. 3a). Accordingly, plasma sCD14 that can be secreted by monocytes and other immune cells following exposure to LPS was significantly elevated in old macaques compared to young ($p < 0.0001$; Fig. 3b) as well as aging macaque groups ($p = 0.002$; Fig. 3b). In contrast to the similar levels of I-FABP and LBP between the young and aging macaques, levels of sCD14 were significantly higher in the aging group in comparison to the young adults ($p < 0.0001$; Fig. 3b), demonstrating an increase from young to middle-aged and then again to the oldest

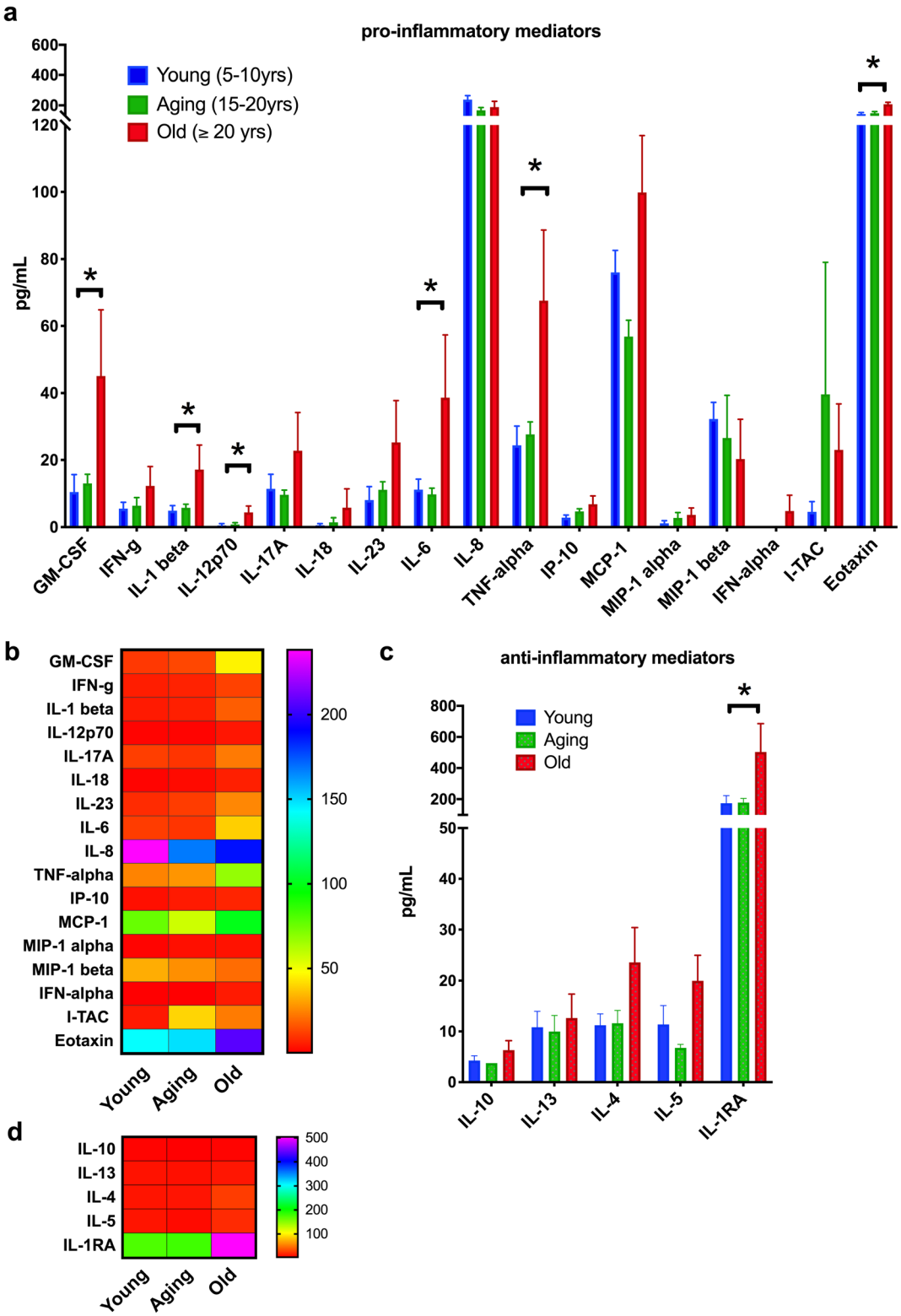
a
pro-inflammatory mediators
Young (5-10yrs)
Aging (15-20yrs)
Old (≥ 20 yrs)
pg/mL
600
400
200
120
100
80
60
40
20
0
*
GM-CSF
IFN-g
IL-1 beta
IL-12p70
IL-17A
IL-18
IL-23
IL-6
IL-8
TNF-alpha
IP-10
MCP-1
MIP-1 alpha
MIP-1 beta
IFN-alpha
I-TAC
Eotaxin
b
GM-CSF
IFN-g
IL-1 beta
IL-12p70
IL-17A
IL-18
IL-23
IL-6
IL-8
TNF-alpha
IP-10
MCP-1
MIP-1 alpha
MIP-1 beta
IFN-alpha
I-TAC
Eotaxin
200
150
100
50
Young
Aging
Old
c
anti-inflammatory mediators
Young
Aging
Old
pg/mL
800
600
400
200
50
40
30
20
10
0
*
IL-10
IL-13
IL-4
IL-5
IL-1RA
d
IL-10
IL-13
IL-4
IL-5
IL-1RA
500
400
300
200
100
Young
Aging
Old

Fig. 1 Circulating levels of pro-inflammatory cytokines and chemokines in healthy young and aging macaques. Data were obtained from Luminex multiplex cytokine assays, showing the levels of 22 cytokines and chemokines in plasma. **a** Pro-inflammatory cytokine and chemokine levels shown in plasma from rhesus macaques stratified into 3 age-groups comprising young adults 5–10 years (blue bars; $n = 18$), aging adults 15–20 years (green bars; $n = 15$), and old adults ≥ 20 years old (red bars; $n = 11$). **b** Pro-inflammatory cytokine and chemokine levels represented as heat map of mean values for each analyte expressing significant differences in the old group. **c** Plasma anti-inflammatory cytokine levels are presented for the 3 age-groups of macaques. **d** Anti-inflammatory cytokine data represented as heat map of mean values showing a significant difference in IL1Ra in the old group. Data are mean ± SEM. Significant differences between each group were determined by the Mann-Whitney test (* $p < 0.05$ was considered statistically significant)

animals. Moreover, all of the three markers of microbial translocation displayed a significant positive correlation with progression of age in these macaques (Fig. 3c–e).

Higher levels of plasma pro-inflammatory mediators are positively associated with microbial translocation in older macaques

The association of systemic inflammation with microbial translocation was examined by correlation analyses between increased pro-inflammatory mediators and I-FABP, LBP, and sCD14. There was no significant difference in I-FABP, LBP, and sCD14 levels between males and females in the young group (data not shown). Also, in the young group of animals, there was no correlation between plasma I-FABP, LBP, and sCD14 levels and any of the cytokines tested. In the aging and old groups of macaques, in contrast, plasma I-FABP levels exhibited significant positive correlations with several of the pro-inflammatory cytokines (Fig. 4a) including IL-6 ($p = 0.002$), GM-CSF ($p = 0.0003$), TNFα ($p = 0.001$), IL-1β ($p = 0.002$), and IFNγ ($p = 0.03$), suggesting an association between systemic inflammation of aging and compromised gut epithelial barrier. Likewise, increased levels of LBP significantly correlated with elevated levels of IL-6 ($p = 0.02$), GM-CSF ($p = 0.03$), and TNFα ($p = 0.04$) in the aging and oldest macaques (Fig. 4b). IL-6, which is a significant predictor of chronic disease and mortality in the elderly humans, particularly showed direct positive correlations with all three of the microbial translocation markers namely, I-FABP ($p = 0.002$), LBP ($p = 0.02$), and sCD14 ($p = 0.003$; Fig. 4a–c). Overall, these data suggest that increases in circulating inflammatory cytokines and markers of microbial translocation are concurrently associated with aging of rhesus macaques.

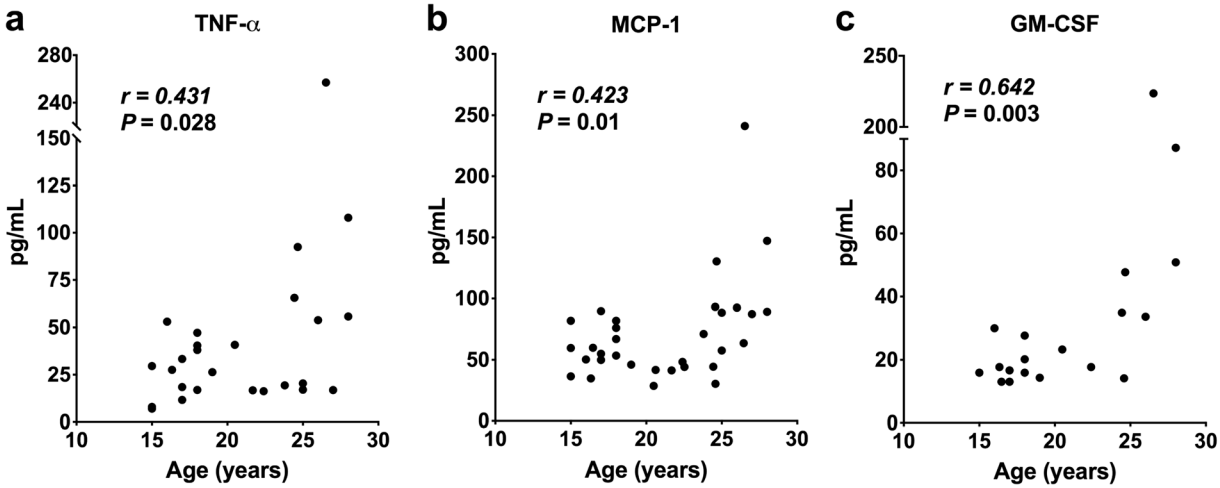

Fig. 2 Associations between age and plasma levels of the pro-inflammatory markers TNF-α, MCP-1, and GM-CSF. Correlation between plasma levels of (**a**) TNF-α, (**b**) MCP-1, and (**c**) GM-CSF and chronological age of macaques at the time of sampling. Data were obtained from 19 to 30 healthy rhesus macaques, and values out of range of detection for individual analytes were excluded from the analysis. The Spearman rank correlation test was applied for comparisons, and $p < 0.05$ was considered statistically significant

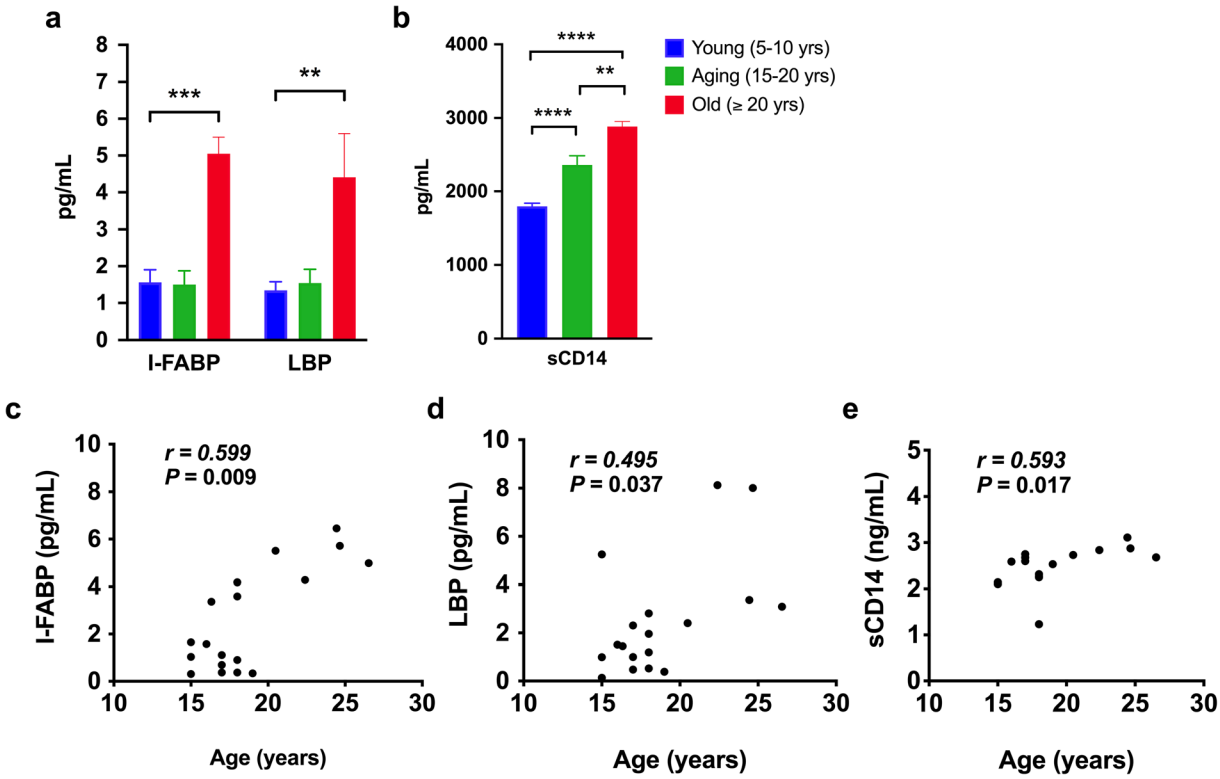

Fig. 3 Elevated levels of biomarkers for leaky gut and microbial translocation in older macaques. Plasma levels of I-FABP, a marker of enterocyte loss and generalized damage to the intestinal epithelium and LPS-binding protein (LBP), and sCD14, as a measure of host response to microbial translocation was measured in young (5–10 years; blue bars, $n = 18$), aging (15–20 years; green bars, $n = 13$), and old (≥ 20 years; red bars, $n = 6$) macaques. **a**, **b** Levels of I-FABP, LBP, and sCD14 in the three groups measured by single ELISAs. Significant differences between each group were determined by the Mann-Whitney test (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). The Spearman rank correlations were measured between age and plasma levels of I-FABP (**c**), LBP (**d**), and sCD14 (**e**) were examined in 14–18 macaques > 15 years old. $p < 0.05$ was considered statistically significant.

Age-associated decline in the frequencies of circulating CD161-expressing immune cell subsets in older macaques

Inflammaging is associated with perturbed immune cell functions in the elderly. CD161-expressing lymphocytes, including the previously described subsets of CD161+ CD4+ T (classical Th17) cells, CD161high CD8+ mucosal associated invariant T (MAIT) cells, and CD161int CD8+ T cells, as well as lineage-negative innate lymphoid cells (ILC), are highly functional immune cells that can regulate the balance between inflammation and epithelial barrier protection in the gut (Fergusson et al. 2014; Povoleri et al. 2018). Since we have previously shown that CD161+ cells are enriched in the gut mucosa of rhesus macaques and have a Th17/Th1 function (Rout 2016), we proceeded to study differences in the frequency of CD161-expressing T cells and ILCs in rhesus macaques across age cohorts. The gating strategy to identify the CD161-expressing immune cell subsets is shown in Fig. 5. In accordance with prior studies (Fergusson et al. 2011; van der Geest et al. 2018), we identified CD161+ cells within the CD4+ T cell compartment, as well as CD8+ T cells with high and intermediate expression levels of CD161, (Fig. 5). The ILCs were identified as CD3/CD14/CD20 lineage-negative cells expressing CD127, and then CD161 expression on these cells as the CD161+ ILC subset. The lineage-negative cells expressing CD8alpha were considered to be surrogates for NK cells and were thus further divided into CD161-expressing NK cell subsets (Fig. 5). Due to low number of animals in the oldest group for the cellular immune assays, the aging and old groups were

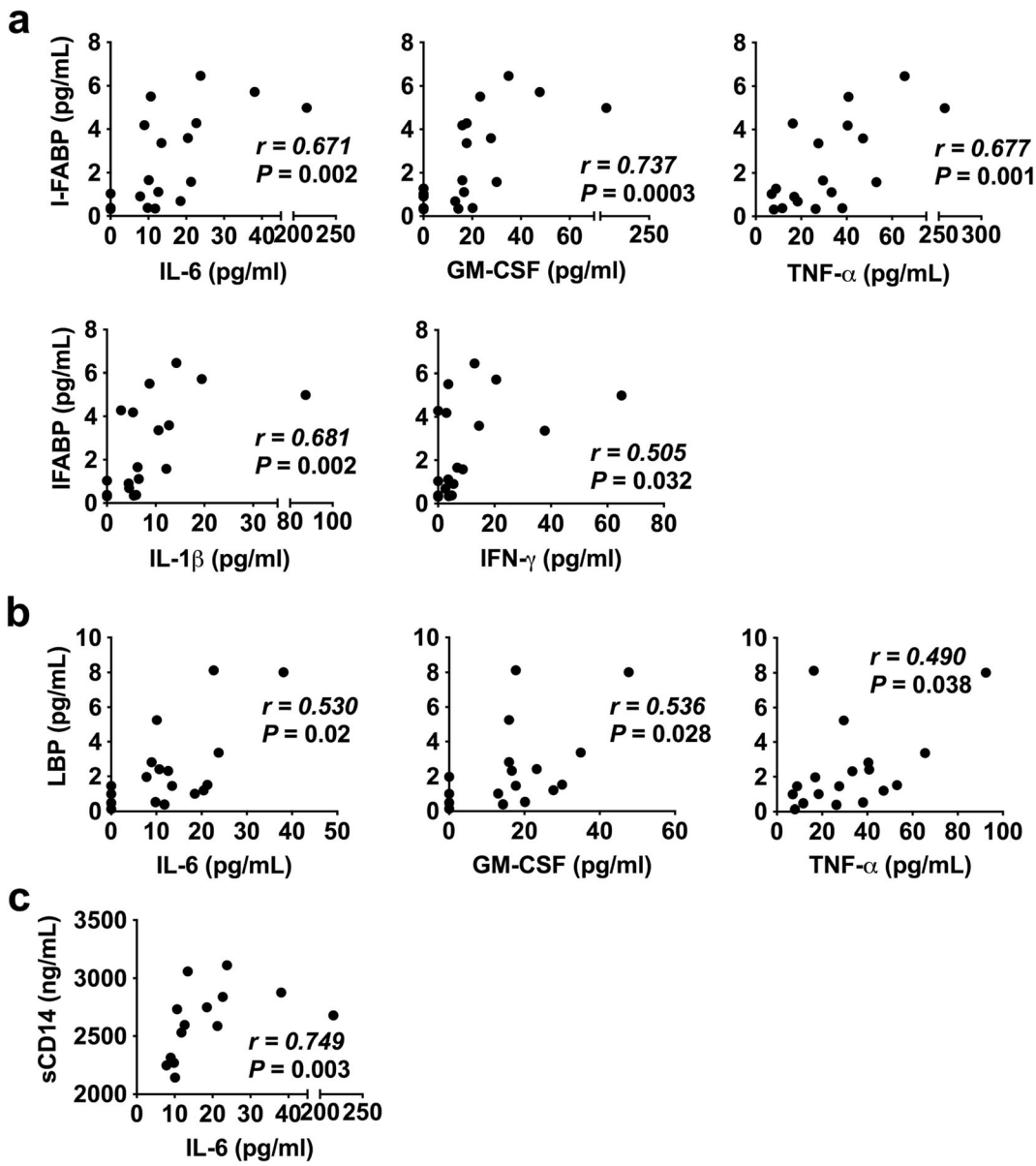

Fig. 4 Significant associations between leaky gut markers and pro-inflammatory cytokines in aging/old macaques (> 15 years old). The Spearman rank correlations are shown between plasma levels of I-FABP (**a**), LBP (**b**), and sCD14 (**c**) against circulating pro-inflammatory cytokines in aging macaques (> 15 years; n = 14–18). Only statistically significant correlations are shown. $p < 0.05$ was considered statistically significant.

combined and referred to as aging macaques > 15 years old. No significant differences between the young and aging groups (> 15 years) were observed in the circulating frequencies of total T cell subsets, NK and ILC (ESM_ 2). However, frequencies of CD161-expressing subsets of CD4 T cells and CD8 T cells, as well as MAIT cells, were significantly lower in the aging macaques (Fig. 6a). Similar to the T cell compartment, the frequencies of CD161-expressing NK cells and ILCs were also significantly lower in the aging macaques than observed in young macaques (Fig. 6b). Further,

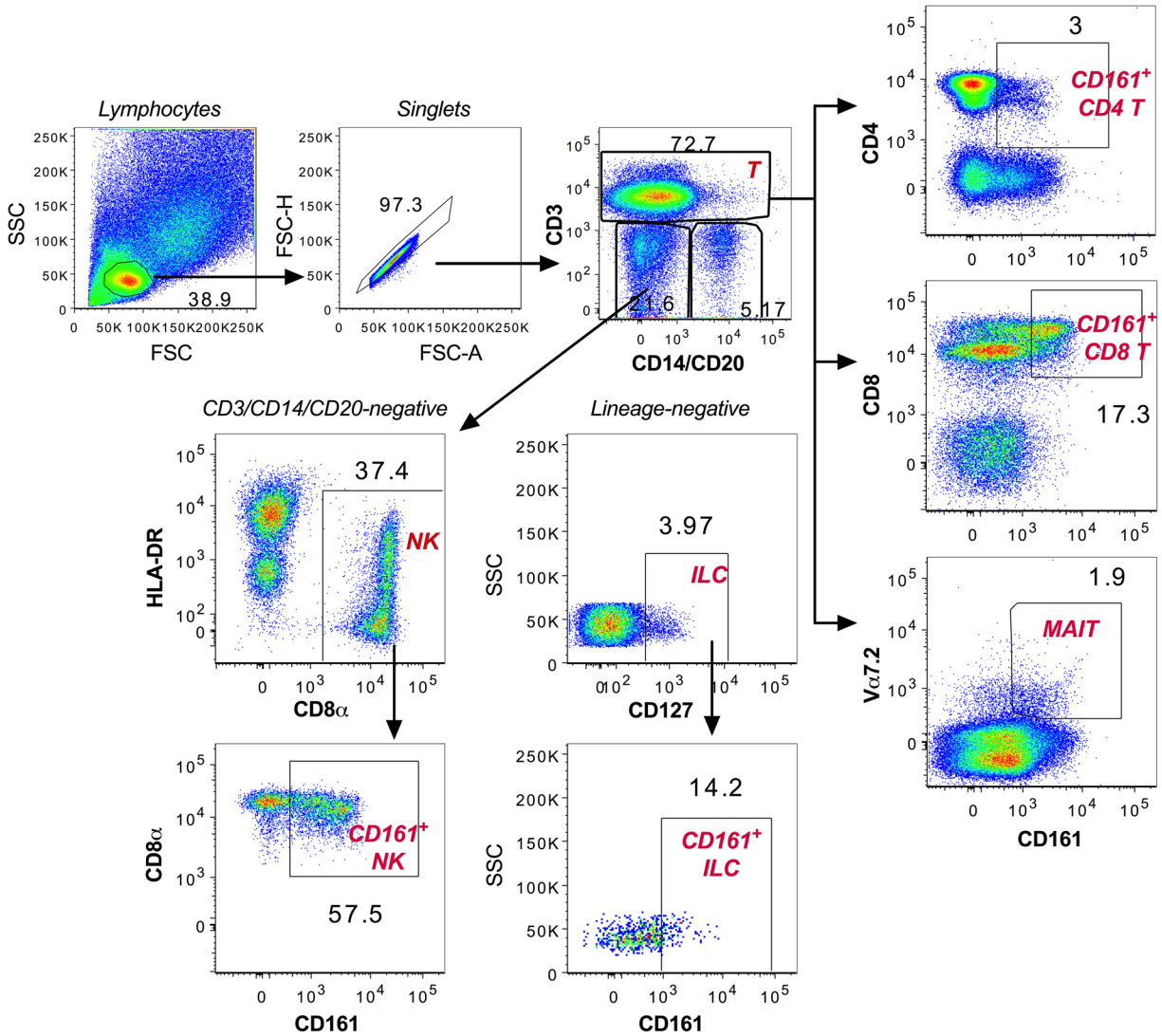

Fig. 5 Gating strategy for flow cytometric analysis of CD161-expressing immune cell subsets. Representative flow plots showing the gating strategy used to describe CD161-expressing subsets of T cells, NK cells, and innate lymphoid cells (ILC) in peripheral blood of rhesus macaques. Lymphocytes were gated based on forward (FSC) and side scatter (SSC) profiles followed by exclusion of doublets. Within this population, CD3 expression was used to define T cells and subsequent CD4+ T, CD8+ T, and Vα7.2 + CD161+ MAIT cell subsets. CD3, CD14, and CD20 was used to exclude T cells, monocytes and B cells, and the lineage-negative cells were further divided into CD8α-expressing NK cells and CD127-expressing innate lymphoid cells (ILC). Each subset was further gated for CD161-expressing cells (labeled in red)

the percent CD161+ immune cells for each subset showed a statistically significant inverse correlation with age in these macaques (Fig. 6c, d), in contrast to the total T cell, NK cell, and ILC frequencies (ESM_ 2). This loss was most significant in the overall CD161-expressing T cells ($p = 0.0005$), followed by the $CD8^+$ T cell subsets ($p = 0.003$). Taken together, these data demonstrated that the numbers of diverse subsets of CD161-expressing T cells and innate immune cells decline with increasing age.

Aging is associated with a decrease in Th17-type cytokine and increased Th1-type cytokine expression in CD161+ cells

Since CD161-expressing T cells and ILCs are potent producers of Th17 and Th1 type cytokines and are

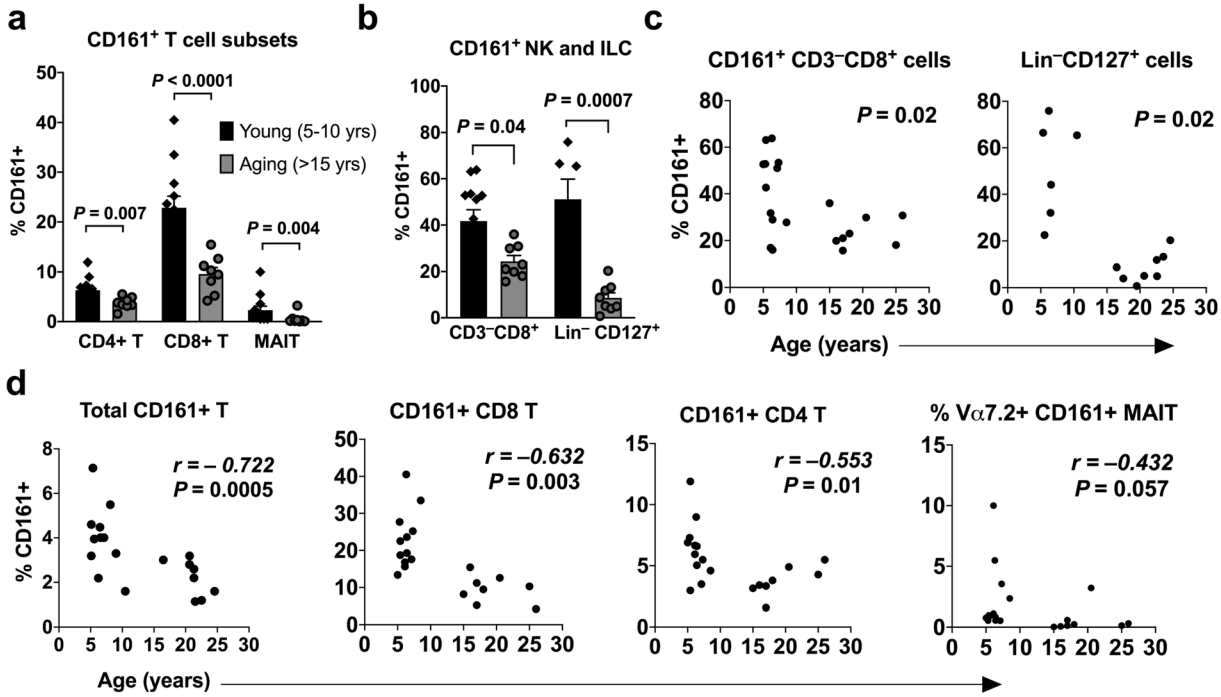

Fig. 6 Effect of age on frequencies of circulating CD161-expressing subsets of T cells and innate immune cells. Frequencies of cells expressing CD161 in (**a**) T cell subsets including CD4+ T, CD8 + T and MAIT cells, and (**b**) innate cells including CD3-CD8α + NK cells and lineage-negative CD127+ ILCs. Data obtained from PBMC of 12 young macaques (black bars) and 8 aging/old macaques > 15 years old (gray bars). Significant differences between each group were determined by the Mann-Whitney test. The Spearman rank correlations were performed between age and frequencies of (**c**) CD161+ NK and ILCs, and (**d**) total T, CD4+ T, CD8+ T, and MAIT cells were examined in the two groups of macaques using the Spearman rank correlations. $p < 0.05$ was considered statistically significant

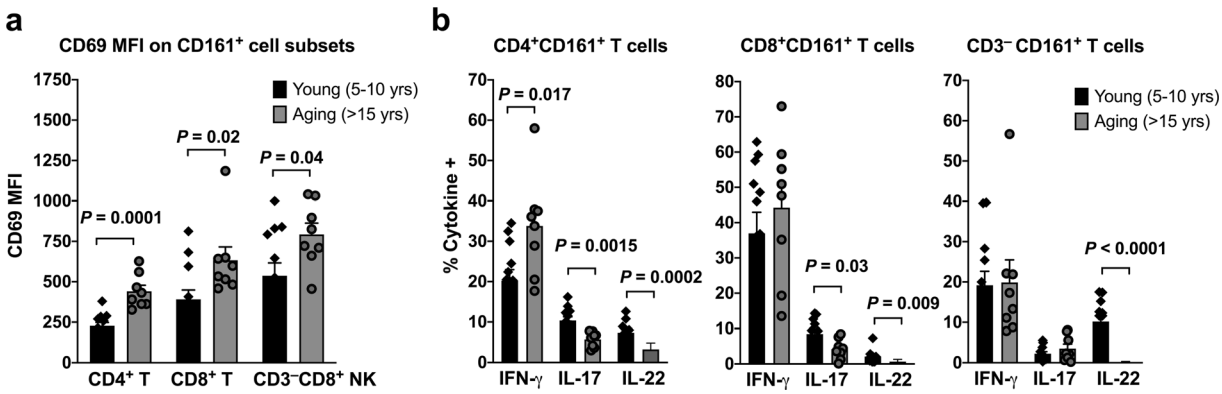

Fig. 7 Dysfunctional Th17-type cytokine production and increased activation of CD161-expressing cells in aging/old macaques (> 15 years old). **a** Mean fluorescence intensity of the activation marker CD69 and **b** intracellular cytokine production of IFN-γ, IL-17, and IL-22 by CD161-expressing T and NK cells were determined by flow cytometry. Intracellular cytokine staining was performed on freshly isolated PBMC stimulated for 16 h with PMA/Ionomycin. Data were represented as mean + SEM for each pair after subtraction of background values from unstimulated cells incubated with medium alone. Significant differences between each group were determined by the Mann-Whitney test and $p < 0.05$ was considered statistically significant.

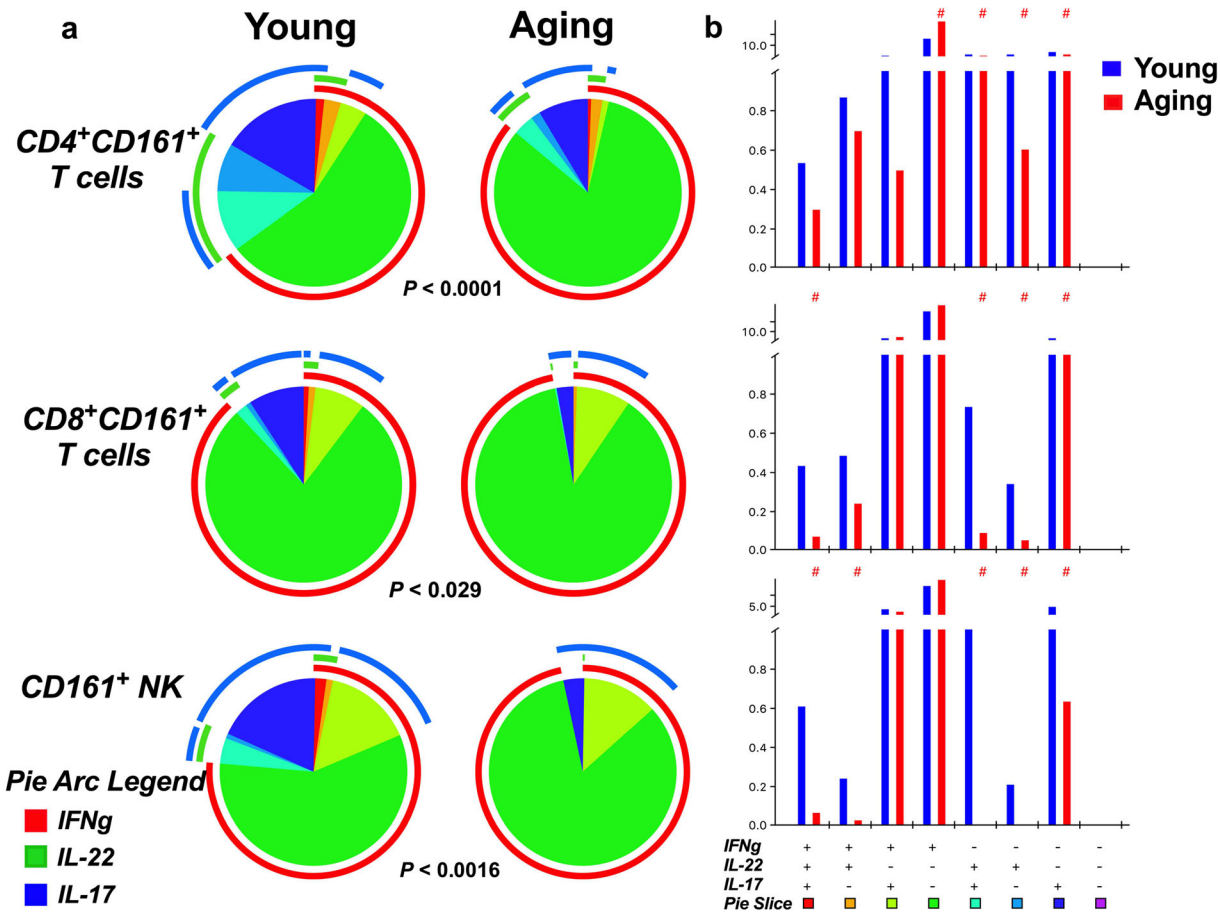

Fig. 8 Skewing of Th1/Th17-type polyfunctional responses towards a dominant Th1-type response in CD161-expressing cells of aging/old macaques (> 15 years old). Cytokine profile of CD161-expressing T and NK cell subsets in young ($n = 12$) and aging ($n = 8$) macaques were represented in regard to the concurrent production of IFN-γ, IL-17, and IL-22. PBMC from young and aging macaques were stimulated for 16 h with mitogen for ICS. **a** Pie charts are shown comparing polyfunctionality of CD161-expressing CD4 T cells, CD8 T cells, and NK cells between young and aging macaques. The pie charts represent the average frequencies of active cytokine-producing cells producing every possible combination of the three cytokines analyzed. The segments within the pie chart denote populations producing different combinations of cytokines and are color coded. The arcs around the circumference indicate the particular cytokine produced by the proportion of cells that lie under the arc. Parts of the pie surrounded by multiple arcs represent polyfunctional cells. The pie arc legend shows I+ in red represents IFN-γ, 22+ in green represents IL-22, and 17+ in blue represents IL-17 production. **b** The abridged bar graph shows frequencies (%) of combinations of cytokines produced by CD161-expressing cells young (blue bars) and aging (red bars). The colors in the bar below correspond to the pie segment colors in (**a**) and indicate the number of cytokines produced. # denotes significant differences. $p < 0.05$ was considered statistically significant.

important in the gut barrier immunity, we next investigated the impact of aging on these functions in PBMC. CD161-expressing T cells and NK cells were more activated as evidenced by greater expression of CD69 in the aging/older macaques > 15 years old) in comparison to the young group of macaques (Fig. 7a). CD161+ CD8 T cell and NK cell subsets were capable of similar levels of IFN-γ production in response to mitogen (PMA/Ca^{2+}-ionophore) stimulation (Fig. 7b). Interestingly, mitogen-stimulated IFN-γ production was higher in the CD4+ T cell subsets of aging macaques (Fig. 7b). On the other hand, the production of two of the Th17 type cytokines IL-17 and IL-22 was significantly impacted in the CD161-expressing CD4 T and CD8 T cell subsets of aging macaques > 15 years old (Fig. 7b). The reduced Th17 cytokine response was particularly significant for IL-22 production and in addition to the T cell subsets;

this function was notably impacted in the NK cell subsets as well (Fig. 7b). However, there was no significant difference in IL-17, or IL-22 cytokine-producing ability by total T cell populations between the young and aging animals (> 15 years old), suggesting that CD161+ T cell subsets were specifically impaired for Th17-type cytokine production in aging and old macaques (ESM_3). The total NK (CD3–CD8α+) cells, on the other hand, displayed a similar decline in IL-22 producing ability to that of the CD161+ subsets besides increased IFN-γ production in response to mitogen stimulation, suggesting an increase in Th1 versus Th17-type functions in aging macaques (ESM_3).

To investigate the impact of aging on the functional specialization of CD161-expressing cells, we analyzed the polyfunctional response profiles of these subsets. Boolean gating analysis of the data revealed that the IL-17, IL-22, and IFN-γ polyfunctional profiles expressed by CD161+ CD4 T cells and NK cells accounted for nearly half of the total response in young animals (Fig. 8a). In contrast, the aging macaques (> 15 years old) displayed a dominant IFN-γ response and significantly lesser polyfunctionality (15–20% of the total response), with most notable decline in IL-22 production (Fig. 8b). Thus, the CD161-expressing cells in aging macaques shifted their polyfunctional Th1/Th17 type cytokine production pattern to a primarily Th1-type functional profile. Overall, these findings suggest that aging is associated with increased activation and a specific decline in Th17-type effector functions of the CD161-expressing immune cells along with maintained or increased Th1 cytokine response.

Discussion

Inflammaging is a challenge for vaccine efficacy and protection against infectious and degenerative diseases in older adults. Defining the immune mechanisms underlying sterile inflammation of aging is essential for developing intervention therapies to enhance vaccine responses and delay age-related pathologies in the expanding aging population. In this study, we provide the first comprehensive analysis of the relationship between aging-associated inflammation and the leaky gut phenotype in a nonhuman primate model. Consistent with our hypothesis of a link between inflammaging phenotype and loss of gut mucosal barrier functions, circulating pro-inflammatory cytokines correlated significantly with biomarkers of gut permeability in aging macaques. Namely, plasma concentrations of TNF-α, IL-6, GM-CSF, and IL-1β were highly correlated with I-FABP, LBP, and sCD14 in aging macaques. Additionally, this inflammatory status and leaky gut phenotype was associated with reduced frequencies of circulating CD161-expressing type-17 subsets of T cells, NK cells, and ILCs. Finally, CD161+ immune cells of aging and older macaques exhibited significant lower ability to produce epithelial barrier-protective type-17 cytokines in comparison to young adults. Thus, the inflammaging phenotype in older macaques is characterized by increase in circulating pro-inflammatory cytokines and leaky gut markers and is associated with age-related reduction in frequencies of CD161-expressing immune cells and impairment of type-17 effector functions.

The results obtained from this study are in agreement with several prior studies demonstrating age-related increases in inflammatory cytokine levels including IL-6, TNF-alpha, IL-1beta, and GM-CSF. (Cesari et al. 2003; De Martinis et al. 2005; Salvioli et al. 2006; Schaap et al. 2009). We did not find any significant differences in the plasma cytokines/chemokines tested between the young male and female groups of macaques, so the data obtained from the predominantly female aging group of macaques in our study may be representative of older male macaques as well, but this would still need to be corroborated in future studies. Moreover, a previous study in rhesus macaques showed no gender-specific differences in the circulating levels of pro-inflammatory cytokines in aging macaques (Didier et al. 2012). Among the pro-inflammatory cytokines evaluated, IL-12p70, TNF-a, and IL-6 were most notably different between the young and old groups of macaques in our study. IL-6 has emerged as one of the most consistent pro-inflammatory markers associated with age-related degenerative diseases (Forsey et al. 2003; Wei et al. 1992), with higher plasma levels correlating with sarcopenia in mixed gender populations as well as separately in older men and women (Miko et al. 2018). Similarly, increased TNF-α production in blood cells of centenarians and octogenarians with atherosclerosis has been associated with mortality in the elderly population (Bruunsgaard et al. 2003; McNerlan et al. 2002). Additionally, increased levels of serum IL-12p70 in aging adults have been shown to be associated with poor cognitive functions (Trollor et al. 2012). Taken

together, increases in IL-6, IL-12, and TNF-α in elderly humans are strongly correlated with loss of muscle mass, poor cognitive function, and increased risk of cardiovascular disease, all hallmarks of frailty. In this context, our data suggest that aging macaques display similar increases in circulating markers of inflammation that are associated with frailty in the elderly human population characterized by inflammation (Franceschi et al. 2000). Thus, aging macaques likely have similar mechanisms of inflammation underlying increased susceptibility to age-related degenerative diseases. Interestingly, IL-1R antagonist (IL-1Ra), an anti-inflammatory protein, was also high in the old group of macaques. Since IL-1Ra can specifically inhibit the pro-inflammatory cytokines IL-1α and IL-1β, higher levels in the oldest group of macaques in the face of multiple pro-inflammatory mediators suggest a likely response to counteract ongoing sterile inflammation in these animals, albeit unsuccessful.

In our study, circulating monocyte chemoattractant protein-1 (MCP-1) and GM-CSF demonstrated the strongest association with age; this association was highest in the oldest macaques (> 25 years old). MCP-1 is a chemokine responsible for recruiting monocytes and has been indicated as a potential biomarker of biological aging in the murine model (Yousefzadeh et al. 2018). Furthermore, higher circulating MCP-1 has also been proposed as a biomarker of frailty (Yousefzadeh et al. 2018) and cognitive decline in clinically healthy, community dwelling older adults (Bettcher et al. 2019; Lee et al. 2018). Likewise, GMCSF levels have been shown to increase in older subjects (Mansfield et al. 2012; Yasui et al. 2007). Since GM-CSF can induce inflammatory cytokine production by increasing cytokine gene expression (Cannistra et al. 1987; Chantry et al. 1990; Heidenreich et al. 1989) and by priming inflammatory cells to amplify their responses to IFNγ (Hamilton and Anderson 2004), it is likely that increase in GM-CSF levels in the oldest macaques may be contributing to the increased levels of circulating pro-inflammatory cytokines. Overall, cross-sectional comparison of 37 cytokines, chemokines, and growth factors in plasma samples from young, aging, and elderly macaques in our study demonstrates that in addition to IL-6, cytokines such as IL-12, and TNF-α, MCP-1, and GM-CSF represented additional biomarkers of age-related inflammation similar to the findings of several other studies in older humans.

A growing body of evidence in small animal models (Clark et al. 2015; Rera et al. 2012; Thevaranjan et al. 2017), as well as in older humans with no overt signs of disease (Liu et al. 2019; Steele et al. 2014), indicates that systemic inflammation is caused by loss of gastrointestinal barrier functions and microbial translocation. With increased plasma levels of sCD14, I-FABP, and LBP, and significant correlation with IL-6, GM-CSF, and TNF-α, our results demonstrate a similar relationship between higher inflammation and impaired gut barrier function in aging macaques. Indeed, age-associated breaches in gastrointestinal barrier functions have been reported in other nonhuman primate species including baboons (Tran and Greenwood-Van Meerveld 2013) and African green monkeys (Mitchell et al. 2017; Wilson et al. 2018), where it is associated with increase in microbial translocation. Although sCD14 alone is not a specific marker for microbial translocation, concomitant higher levels of I-FABP in the old macaques above 20 years of age indicated that higher circulating sCD14 was indeed related to increased permeability of the epithelial barrier due to enterocyte apoptosis. This was further supported by significantly higher levels of LBP, which chaperones LPS from gut bacteria to both membrane-bound and soluble CD14 to elicit immune response in monocytes and epithelial/endothelial cells respectively. It is interesting to note that unlike the leaky gut markers LBP and I-FABP that increased drastically in the oldest group of animals in contrast to the young and aging groups, sCD14 displayed a gradual increase from young adults to aging and older macaques. The upregulation of sCD14 before the signs of leaky gut and microbial translocation in aging group of macaques suggests a contribution of other factors to monocyte activation and release of sCD14 with biological aging. Although the underlying factors remain elusive, higher expression of CD14 on circulating monocytes from aging individuals (66–72 years old) was shown to correspond to greater TNF-a production both at baseline and following LPS-stimulation, indicating that inflammatory monocytes increase with aging (Hearps et al. 2012).

Inflammaging and immunosenescence are closely related features of biological aging, since the functional perturbations of immune cells with advancing age can fuel inflammation and vice versa (Fulop et al. 2017). Immunosenescence has been most studied in the context of the T cell compartment, which displays changes in the cellular composition with decrease in the number of naive cells and increase in the number of memory phenotype cells, resulting in a persistent pro-inflammatory state and

characterized by a diminished capacity of the immune system to respond to new antigens present in vaccines or infectious agents. As previously shown by others (Asquith et al. 2012; Pitcher et al. 2002), we found a similar decrease in naïve T cells and increase in memory T cells in the macaques in our study (data not shown). Several studies have demonstrated the importance of CD161 expression in the Th17-type effector functions of human T cells and regulation of tissue-specific immune responses (Billerbeck et al. 2010; Fergusson et al. 2016; Maggi et al. 2010; O'Keeffe et al. 2004). Given the significance of CD161 expression on Th17-type effector functions and gut barrier-protective role, and our observation of elevated levels of leaky gut markers in older macaques, we focused on the impact of aging on CD161-expressing immune cell functions. An important finding of this study is the striking impact of aging on the frequency of CD161-expressing subsets across multiple immune cell types including T cell subsets, NK cells, and ILC. A recent study in human subjects showed that aging was associated with decline of circulating CD161high CD8+ T cells, and decreased production of IFN-γ and IL-17 cytokines by CD161+ CD4+ T cells (van der Geest et al. 2018). Further, in accordance with prior reports (Lee et al. 2014; Novak et al. 2014; Walker et al. 2014), the numbers of MAIT cells, a subset of CD161+ CD8+ T cells that express TCR-Vα7.2, also declined with age in our study animals. Prior reports have indicated that CD161+ CD8+ T cells are potent producers of Th1 and Th17 cytokines (Fergusson et al. 2016; Fergusson et al. 2014; Rout 2016). In the current study, we show that CD161+ CD8+ T cells of young and old macaques showed similar ability to produce IFN-γ, but the cells of older macaques (> 15 years) had significant lower ability to produce IL-17, and IL-22. Thus, while the Th1 effector function of CD161+ CD8+ T cells was not affected by aging, the Th17 effector functions were significantly impacted in older macaques. IL-17 and IL-22 are critical for promoting mucosal barrier function and protection from pathogens (Lo et al. 2019; Ouyang et al. 2008; Sugimoto et al. 2008), and paucity of IL-17 has been shown to increase epithelial injury and compromise barrier function in mouse models of colitis (Lee et al. 2015; Maxwell et al. 2015). Similarly, loss of IL-22 in the mouse model of burn injury under the influence of alcohol was associated with increased intestinal permeability, and treatment with exogenous IL-22 prevented the leaky gut phenotype in this model (Rendon et al. 2013). Thus, despite a pathogenic role in certain disease settings, IL17 and IL-22 have key roles in gut mucosal homeostasis, suggesting that the decreased production in older macaques could contribute to the leaky gut phenotype based on plasma biomarkers of epithelial breach and microbial translocation. Future studies to explore the relationship of leaky gut-mediated inflammation with the impaired cognitive functions in older macaques as demonstrated by previous studies (Justice et al. 2017; Robinson et al. 2018; Shobin et al. 2017) will provide key insights into the role of imbalance in gut mucosal immunity and the gut-brain axis in morbidity of aging individuals.

Besides T cells, the decline in CD161-expressing NK cells and ILC with aging in macaques and loss in NK cell production of IL-22 underscores the dysfunction of a broader group of innate immune cells that have a shared gut barrier-protective function independent of lineage and phenotype. Human CD161-expressing cells, including TCRαβ+ CD4 and CD8, MAIT, and γδ T cells, appear to be related by a shared transcriptional signature and innate-like function (Fergusson et al. 2014). Further, functional similarities between CD8+ T cell and NK cell subsets have been reported including cytotoxic functions and enrichment in tissues (Kurioka et al. 2018) suggesting that diverse cell types can operate utilizing common pathways towards a shared immune function. Little is known regarding the impact of age-related changes in the expression of CD161 on NK cells and ILCs. Considering the inhibitory role of CD161 on IFN-γ secretion by NK cells (Aldemir et al. 2005), our results suggest that age-associated decline of CD161 expression may further contribute to the pro-inflammatory environment observed in older individuals. Notably, in addition to the shrinking of the CD161-expressing subsets of CD4 T, CD8 T, and NK cells in older macaques, our results demonstrated a loss in polyfunctionality and skewing of the overall cytokine response from a shared Th1/Th17 type function towards a dominant Th1 type function owing to significant decrease in production of IL-17 and/or IL-22 cytokines. Considering the increased activation of CD161-expressing T cells and NK cells in older macaques in comparison to young adults, the loss of polyfunctionality can result in dysfunctional pro-inflammatory innate immune responses in vivo. Thus, the age-related loss of epithelial barrier-protective type-17 effector functions of CD161-expressing immune cells along with a skewing of the residual cells towards pro-inflammatory cytokine production likely contribute to the leaky gut phenotype and inflammation in older macaques. Future longitudinal studies with interventions aimed at modulating CD161 expressing immune cell populations via microbiome

modulation or cytokine therapy are required to investigate the role of innate type-17 immune cells in the maintenance of healthy gut barrier, and thus preventing/reducing the inflammaging phenotype.

Overall, our results support the concept that chronic low-grade inflammation of aging is associated with compromised intestinal epithelial barrier function, and we propose the use of macaques as a model to further delineate the mechanisms of inflammaging that underlie impaired immune responses to vaccines and infections in the elderly people. In summary, this study characterizes the inflammatory and leaky-gut phenotype of aging macaques based on plasma biomarkers of inflammatory cytokines/chemokines, and LBP, I-FABP, and sCD14 as markers for loss of gut barrier integrity and microbial translocation. Furthermore, our data suggest that a loss of CD161 expression may contribute to decline in gut barrier functions, and thereby underlie low-grade systemic inflammation in older macaques in the absence of overt clinical infection or disease. Further studies to assess the immune functions of CD161-expressing innate cells in tissues, particularly the gastrointestinal tract, are essential to define their role in the persistent inflammation of aging.

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Author contributions Namita Rout was responsible for the study design, data analysis and interpretation and wrote the manuscript. Edith Walker coordinated the overall work including sample collection procedures and conducting the flow cytometry data analysis and in vitro functional assays and helped with manuscript preparation. Nadia Slisarenko and Giovanni Gerrets helped with sample collection and processing and data analysis. Elizabeth S. Didier and Marcelo J. Kuroda provided samples and contributed to the study design. Patricia Kissinger performed the statistical data analyses. Ronald S. Veazey and S. Michal Jazwinski helped with overall data interpretation. All authors read and approved the final manuscript. All authors helped edit and reviewed the final manuscript.

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Compliance with ethical standards

Ethics statement This study was performed using samples collected from nonhuman primates. All procedures were approved by the TNPRC Institutional Animal Care and Use Committee, Animal Welfare Assurance A-4499-01, and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, *National Research Council*, *2011*. The TNPRC maintains an AAALAC-I accredited animal care and use program.

Conflict of interest The authors declare that they have no conflict of interest.

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