Diversity of Human and Macaque Airway Immune Cells at Baseline and during Tuberculosis Infection

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Abstract

Immune cells of the distal airways serve as “first responders” of host immunity to the airborne pathogen *Mycobacterium tuberculosis* (*Mtb*). *Mtb* infection of cynomolgus macaques recapitulates the range of human outcomes from clinically silent latent tuberculosis infection (LTBI) to active tuberculosis of various degrees of severity. To further advance the application of this model to human studies, we compared profiles of bronchoalveolar lavage (BAL) cells of humans and cynomolgus macaques before and after *Mtb* infection. A simple gating strategy effectively defined BAL T-cell and phagocyte populations in both species. BAL from *Mtb*-naïve humans and macaques showed similar differential cell counts. BAL T cells of macaques were composed of fewer CD4⁺ cells but more CD8⁺ and CD4⁺CD8⁺ double-positive cells than were BAL T cells of humans. The most common mononuclear phagocyte population in BAL of both species displayed coexpression of HLA-DR, CD206, CD11b, and CD11c; however, multiple phagocyte subsets displaying only some of these markers were observed as well. Macaques with LTBI displayed a marked BAL lymphocytosis that was not observed in humans with LTBI. In macaques, the prevalence of specific mononuclear phagocyte subsets in baseline BAL correlated with

ultimate outcomes of *Mtb* infection (i.e., LTBI versus active disease). Overall, these findings demonstrate the comparability of studies of pulmonary immunity to *Mtb* in humans and macaques. They also indicate a previously undescribed complexity of airway mononuclear phagocyte populations that suggests further lines of investigation relevant to understanding the mechanisms of both protection from and susceptibility to the development of active tuberculosis within the lung.

Keywords: bronchoalveolar lavage; alveolar macrophages; tuberculosis; macaques; human subjects

Clinical Relevance

The comparison of bronchoalveolar lavage findings in humans and cynomolgus macaques in this study will help optimize the usefulness of studies of nonhuman primates to the understanding of human immunity to *Mycobacterium tuberculosis*. Better understanding of the complexity of bronchoalveolar lavage cell populations in both species may clarify the roles of specific airway cells in the outcome of respiratory infection with *M. tuberculosis*.

(Received in original form April 6, 2016; accepted in final form August 7, 2016)

This work was supported by National Institutes of Health National Heart, Lung, and Blood Institute grants RO1-HL106798, RO1-HL106804 (J.F.), and RO1 HL-106800 (supplement), all of which were components of the National Institutes of Health Tuberculosis Systems Biology Program. Additional funding was provided by the Bill and Melinda Gates Foundation (P.L.L.), the Otis Foundation for Tuberculosis/PNC Trust (P.L.L.), RO1 AI111871 (P.L.L.), and RO1 HL111523 (R.F.S.). Research bronchoscopies were performed in the Dahms Clinical Research Unit, which is a clinical core of the Case Western Reserve University/University Hospitals Case Medical Center Clinical and Translational Science Collaborative, supported by NIH UL1 RR024989 from the National Center for Research Resources.

Author Contributions: R.F.S. and P.L.L.: study design, data analysis, and manuscript preparation and editing; A.J.M. and J.J.: cell staining studies, flow cytometry data acquisition, data analysis, and manuscript preparation; T.B.: study design, flow cytometry data analysis and optimization of methodology, and manuscript review; T.R.: cell staining and T-cell subset analysis for cynomolgus macaque samples; and J.F.: overall design of cynomolgus macaques infection studies, consultation on data analysis, and manuscript review.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 6, pp 899–908, Dec 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2016-0122OC on August 10, 2016

Internet address: www.atsjournals.org

Infection with *Mycobacterium tuberculosis* (*Mtb*), the bacterium that causes tuberculosis (TB), occurs via airborne transmission of droplet nuclei. As a result, immune cells of the distal airways are the first to encounter the pathogen and likely play a key role in determining the outcome of infection. Accordingly, the role of local immunity to *Mtb* within the lung has become an increasingly prominent focus of TB research and vaccine development (1–3). In humans, assessments of pulmonary immune responses are largely limited to evaluation of airway cells obtained via bronchoalveolar lavage (BAL). Regarding this, animal models of TB are advantageous because they allow for serial sampling, evaluation of lung pathology over time, and correlation between specific immune responses and protection from respiratory *Mtb* challenge. However, comparison of these findings with those that can be assessed in humans is essential for validating the applicability of these studies to understanding human immunity to the organism. Low-level respiratory infection of cynomolgus macaques with *Mtb* results in outcomes that, like those of humans, extend from clinically silent latent tuberculosis infection (LTBI) to the development of active TB with extensive and, at times, destructive lung disease (4, 5). Furthermore, these nonhuman primates are large enough to allow for the performance of bronchoscopically directed BAL procedures that are more comparable to human BAL than are the whole-lung lavages used in mice and other small animals. In addition, because macaques are substantially more likely than humans to develop active TB after *Mtb* infection, interspecies differences in BAL findings could be relevant to clarifying mechanisms of susceptibility and resistance to the organism. We therefore sought to characterize and compare BAL cells of naive and *Mtb*-infected humans and cynomolgus macaques as a means of assessing the applicability of this macaque model to understanding local immunity to *Mtb* in the human lung.

Although studies of protective immunity in TB have focused largely on lymphocyte populations (6), mononuclear phagocytes are the predominant immune cell population within the airway. However, strategies for the characterization of these cells are not well standardized. Recent studies in both humans and macaques have

categorized lung and airway phagocytes using a limited number of markers and have focused largely on the anatomic localization of these populations (7–12). In contrast, we hypothesized that *Mtb* infection could induce the migration of cells from peripheral blood or other lung compartments into the alveolar spaces and could alter the activation and maturation states of these populations. We therefore analyzed a panel of receptors that could distinguish between alveolar macrophages and other airway phagocyte populations and that were also potentially relevant to the host response to *Mtb* in particular. As detailed in Table 1, the mannose receptor CD206, long described as a marker of alternatively activated macrophages (13), has more recently been identified as being ubiquitously expressed in all populations of lung macrophages (14). HLA-DR, CD11b, and CD11c receptors are additional well-recognized surface markers of multiple mononuclear phagocyte populations including airway mononuclear cells (11, 15), whereas CD209 is characteristically described as a marker of dendritic cell populations (16), and expression of both CD36 and CD163 is associated with various degrees of phagocyte activation (17–19). In addition, assessment of the coexpression of multiple markers provided a means of assessing the heterogeneity of BAL phagocyte populations in greater detail.

In general, BAL of uninfected cynomolgus macaques and humans displayed similar proportions of cell types, and these were characterized by comparable patterns of receptor expression. However, some differences in receptor expression were observed in both lymphocyte and mononuclear phagocyte subsets. In contrast, BAL cells of macaques with LTBI displayed substantially higher percentages of lymphocytes than did those of humans with LTBI, indicating a need for additional assessments to clarify the relationship between these states as defined in the two species. Importantly, our data also revealed that multiple subpopulations of mononuclear phagocytes can be detected within BAL of both species on the basis of combinations of coexpressed receptors. Variations in the predominance of these subpopulations may play a key role in the initial immune response to infection with *Mtb* and other airborne pathogens.

Materials and Methods

Human Studies

Human subject protocols were approved by the Institutional Board of Review of University Hospitals Case Medical Center. Healthy nonsmokers aged 18–50 years were recruited into two study groups, *Mtb*-naive and LTBI, on the basis of responses to the purified protein derivative of *Mtb* skin testing and/or blood testing with QuantiFERON TB Gold In-Tube (Quest Diagnostics, Madison, NJ). In subjects with positive TB tests, LTBI status was confirmed by a lack of TB symptoms (e.g., cough, fever, weight loss, night sweats) and negative chest X-rays. For BAL procedures, the bronchoscope was wedged into a subsegmental bronchus via direct visualization. Lavage was performed using the instillation of six to eight 30-ml aliquots of saline, as described previously (20).

Animal Studies

Animal protocols were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Adult (>4 yr) cynomolgus macaques (*Macaca fascicularis*; Valley Biosystems, West Sacramento, CA) were infected via bronchoscopic instillation of *Mtb* Erdman (5). Macaques were subsequently observed in BSL-3 housing and categorized as having active TB if they exhibited clinical signs (e.g., cough, anorexia), abnormal X-rays, or growth of *Mtb* by gastric aspirate or BAL beyond 2 months of infection; animals that did not develop any of these findings within 6 months of infection were defined as having LTBI (4, 5). As in human studies, BAL was performed after wedging the bronchoscope into a subsegmental bronchus using direct visualization; however, in consideration of the smaller size of macaques (average weight, 5 kg), lavage used only four 10-ml aliquots of saline.

Immunophenotyping of BAL Cells

In both humans and macaques, total cell counts were determined by light microscopy; after modified Wright staining, differential cell counts of cytospin preparations were also performed microscopically. The BAL cells of each human subject were used to prepare compensation tubes for flow cytometry studies, with relevant voltage adjustments to avoid autofluorescence. Because of more limited cell numbers, compensation for macaque studies was performed

Table 1. Mononuclear Phagocyte Markers Evaluated in this Study

Marker (Other Names)	Function	Receptor Ligands	Cells Reported to Express Marker	Potential Roles in <i>Mtb</i> Infection
CD206 (macrophage mannose receptor) (8, 9, 11, 13, 49)	Phagocytic internalization of fungi and bacteria	Glycoconjugates containing mannose, fucose, or N-acetylglucosamine	Alternatively activated macrophages such as AM	Binding of <i>Mtb</i> to CD206 mediates phagocytosis
HLA-DR (Class II MHC) (11, 15)	Presents antigenic peptides to CD4 ⁺ T cells	T-cell receptor	Multiple mononuclear phagocyte populations, B cells	(See function)
CD11b (ITGAM, CR3A) (11, 15)	Leukocyte adhesive interactions; phagocytosis; chemotaxis	iC3b, CD54 (ICAM-1), CD102 (ICAM-2), CD50 (ICAM-3)	Multiple mononuclear phagocyte populations, neutrophils, NK cells	Binding of <i>Mtb</i> to CD11b mediates phagocytosis
CD11c (ITGAX) (9, 11, 15)	Leukocyte adhesive interactions; phagocytosis	iC3b, fibrinogen, CD54 (ICAM-1)	Multiple mononuclear phagocyte populations, neutrophils, B cells	Binding of <i>Mtb</i> to CD11c mediates phagocytosis; CD11c enhances capacity of phagocytes to stimulate antigen-primed T cells
CD209 (DC-SIGN) (16)	Cellular trafficking; binding of high mannose-containing glycoproteins on viruses and bacteria	CD102 (ICAM-2) CD50 (ICAM-3),	Dendritic cells in mucosal tissues, some macrophage populations	Expression on AM reported in patients with active TB
CD163 (M130, hemoglobin scavenger receptor) (8, 17, 50)	Scavenger receptor	Hemoglobin, haptoglobin	Activated tissue macrophages	Expressed on macrophages observed in the periphery of granulomas from <i>Mtb</i> -infected macaques
CD36 (GPIIB) (18, 19)	Glycoprotein and scavenger receptor; component of the antiinflammatory response initiated by activation of PPAR- γ	Collagen, thrombospondin	Mononuclear phagocytes	Expression of CD36 (and PPAR- γ) associated with increased <i>Mtb</i> survival

Definition of abbreviations: AM, alveolar macrophage; CD, cluster of differentiation; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; GPIIB, glycoprotein IIB; HLA-DR, human leukocyte antigen-antigen D related; ICAM, intercellular adhesion molecule; IIMHC, class II major histocompatibility complex; ITGAM, integrin α M; ITGAX, integrin submit α X; *Mtb*, *Mycobacterium tuberculosis*; NK, natural killer; PPAR- γ , peroxisome proliferator-activated receptor- γ ; TB, tuberculosis.

using commercially available beads (UltraComp eBeads; eBioscience, San Diego, CA). In both species, cells were gated first on CD45⁺ (clone SK7; BD Biosciences, San Jose, CA) and then on CD3⁺ or CD3⁻ populations (clone SP34-2; BD Biosciences) (see Figure E1 in the online supplement). The following additional antibodies were used in all studies: CD163 (clone EBIOGHI/61; eBioscience), CD209 (clone DCN46; BD Biosciences), CD11c (clone 3.9; eBioscience), CD206 (clone 19.2; BD Biosciences), HLA-DR (clone LN-3; eBioscience), CD36 (clone CB38; BD Biosciences), CD11b (clone ICRF44; BD Biosciences), CD4 (clone L200; BD Biosciences), and CD8 (clone DK25; DAKO,

Carpinteria, CA). All data were acquired on BD LSRII flow cytometers BD Biosciences and analyzed with FlowJo software v9.0 (FlowJo, LLC, Ashland, OR).

Statistical Analysis

After initial assessment for normal distribution using the D'Agostino-Pearson Omnibus normality test, all datasets were evaluated using nonparametric analytical methods. Unpaired, two-sample comparisons were performed with Mann-Whitney rank sum tests, whereas the Wilcoxon rank sum test was used for paired, two-sample comparisons (Prism 6.0; Graphpad Software, La Jolla, CA). Two-way analysis of variance with Sidak's multiple comparison adjustment

was performed for comparisons of marker expression between different groups within one species (e.g., naive human subjects versus human subjects with LTBI). Coexpression analyses were performed using SPICE 5.3 (21).

Results

Characterization of BAL Cells from *Mtb*-Naive Humans and Cynomolgus Macaques

Total and differential cell counts. The total number of cells recovered from BAL of *Mtb*-naive human subjects was greater

than that from BAL of healthy macaques (7.9×10^6 [interquartile range (IQR)₂₅₋₇₅, 7.1×10^6 – 1.0×10^7] versus 2.8×10^6 [IQR₂₅₋₇₅, 1.6×10^6 – 5.1×10^6]), consistent with the larger size of humans and correspondingly greater volume of fluid instilled into the airways during the procedure (see METHODS). When corrected for returned BAL fluid volume, there was no significant difference in total cell counts between *Mtb*-naïve humans (47,900 cells per milliliter [IQR₂₅₋₇₅, 42,850–62,125]) and macaques (114,000 cells per milliliter [IQR₂₅₋₇₅, 47,950–200,500]). Differential cell counts showed a predominance of mononuclear phagocytes with relatively few lymphocytes in both humans (mononuclear phagocyte, 94%; lymphocyte, 5%; neutrophil, 1%) and macaques (mononuclear phagocyte, 82.5%; lymphocyte, 9.5%; neutrophils, 1%).

T-cell populations. T cells were identified as CD45⁺/CD3⁺, SSC low cells (Figure E1). CD4 single-positive T cells (CD3⁺, CD4⁺, CD8[−]) represented the largest BAL T-cell population in both species, but were present to a significantly greater degree in humans than in macaques (median, 60.4% in humans versus 42.9% in macaques; $P < 0.0001$); correspondingly, the percentage of CD8 single-positive T cells was lower in human BAL than in macaque BAL (human, 26.8% versus macaque, 39.9%; $P = 0.004$, Mann–Whitney test). In addition, macaque BAL demonstrated a small but well-defined population of double-positive T cells (DP_T) expressing both CD4 and CD8; no corresponding population was observed in human BAL samples.

Phenotypic characterization of airway mononuclear phagocytes. Phagocyte populations were evaluated on the basis of the expression of multiple phagocyte markers (identified in a CD45⁺/CD3[−]/SSC hi gate) rather than by categorization into specific populations. Neutrophils were eliminated from this analysis by excluding cells that were CD11b⁺ but negative for all other markers. In both *Mtb*-naïve humans and macaques, single expression of the following markers was observed on most cells within this gate: HLA-DR (human, 97% versus macaque, 81%), CD206 (human, 96% versus macaque, 82%), CD11b (human, 97% versus macaque, 87%), CD11c (human, 95% versus macaque, 74%) and CD163 (human, 71% versus macaque, 85%). In contrast, distinct

differences were observed in the expression of CD209 (human, 2% versus macaque, 11%; $P < 0.05$, Mann–Whitney) and CD36 (human, 14% versus macaque, 77%; $P < 0.001$, Mann–Whitney). In general, greater host-to-host variability in surface receptor expression was observed in macaques than in humans. In *Mtb*-naïve human subjects, separation of CD206⁺ phagocytes into CD206 hi and CD206 lo populations was observed consistently. This correlated with expression of both CD163 and CD36, but not the other markers studied; specifically, CD163 expression was observed on a significantly lower percentage of CD206 lo cells than on CD206 hi BAL phagocytes of *Mtb*-naïve human subjects (54.85% versus 94.25%; $P = 0.002$, Mann–Whitney), whereas the percentage of CD36⁺ cells was significantly higher on CD206 lo phagocytes than on CD206 hi human cells (24.9% versus 7.36%, $P = 0.004$, Mann–Whitney). In contrast, no clear demarcation between CD206 hi and lo phagocyte populations was observed in macaque BAL.

Coexpression of surface markers identified various subpopulations of mononuclear phagocytes within BAL of both humans and cynomolgus macaques. The predominant phagocyte populations in both species displayed simultaneous expression of HLA-DR, CD206, CD11b, and CD11c. These cells accounted for almost all BAL phagocytes of *Mtb*-naïve humans (98%)

and for a majority (58%) of the mononuclear phagocytes in BAL from *Mtb*-naïve macaques; this lower figure primarily reflected the lower frequency of expression of CD11b and CD11c on macaque phagocytes as compared with those of humans.

Changes in Human and Macaque BAL Cells after *Mtb* Infection

Total and differential cell counts. Regardless of infection status, the total number of BAL cells was greater in humans than in macaques (Figure 1). As in baseline studies, however, no significant differences were observed in the cell counts of humans and macaques with LTBI after correction for returned BAL fluid volume (humans with LTBI with 72,700/ml, [IQR₂₅₋₇₅, 41,200–88,500] versus macaques with LTBI with 149,231/ml [IQR₂₅₋₇₅, 95,455–220,219]). Total BAL cell counts were similar between *Mtb*-naïve and LTBI cohorts within individual species, as illustrated; BAL cell counts in humans with LTBI displayed a more bimodal distribution than those observed in control subjects, whereas no such distinction was observed in macaques (Figure 1). Among macaques, the percentage of mononuclear phagocytes was significantly lower in LTBI compared with *Mtb*-naïve animals, whereas no such difference was observed in humans (Figure 2 and detailed in Table E1). The

Figure 1. Total cell counts observed in bronchoalveolar lavage (BAL) of *Mycobacterium tuberculosis* (*Mtb*)-naïve macaques ($n = 21$), macaques with latent tuberculosis infection (LTBI) ($n = 9$), *Mtb*-naïve human subjects ($n = 10$), and humans with LTBI ($n = 11$). More cells were obtained from BAL of humans than of macaques (**** $P < 0.001$, two-way analysis of variance with Sidak's multiple comparison), consistent with differences in the sizes of the hosts as well as the volume of fluid instilled. Differences in BAL cell yields were not significant (N.S.) among *Mtb*-naïve versus LTBI groups within either species. Squares and circles represent cell counts obtained from individual macaques or human subjects, respectively; horizontal lines indicate median values for each group.

Figure 2. BAL differential cell counts of *Mtb*-naïve humans and macaques are similar but differ substantially during LTBI. As detailed in the text, differential cell counts of (A) *Mtb*-naïve humans and (B) macaques are both dominated by mononuclear phagocytes (dark gray) with limited percentages of lymphocytes (black) and fewer neutrophils (white) or eosinophils (light gray). (C) Human subjects with LTBI continue to display this same profile. (D) In contrast, lymphocytes comprise 33% of BAL cells in macaques with LTBI, representing a marked increase from naïve animals; BAL neutrophils are increased in macaques with LTBI as well.

percentage of BAL lymphocytes was increased in macaques with LTBI as compared with *Mtb*-naïve animals; again, no such difference was observed in BAL of human subjects with LTBI versus uninfected human subjects. All other cell types had similar compositions between humans and macaques regardless of infection status.

BAL T-cell subsets. The differences in composition of BAL T-cell subsets of humans and macaques at baseline were maintained after *Mtb* infection. Specifically, although CD4⁺ T cells were the predominant T-cell population in *Mtb*-naïve and infected subjects of both species, BAL of humans had a greater frequency of CD4 T cells but fewer CD8 T cells compared with that of macaques, regardless of infection status. As in uninfected cohorts, DP_T were observed in the BAL of macaques with LTBI, but not in corresponding human samples (Figure E2).

Phenotypic characterization of BAL mononuclear phagocytes. For the most part, the percentage of BAL phagocytes displaying single expression of the surface markers evaluated did not differ significantly between *Mtb*-naïve and LTBI

groups for either species (Table 2); the only exception was CD11b, which was expressed with significantly lower frequency on BAL phagocytes of humans with LTBI compared with those of *Mtb*-naïve subjects. In contrast, CD11b expression was similar on the phagocytes of *Mtb*-naïve macaques and macaques with LTBI. In general, the receptors CD206, CD11b, CD11c, HLA-DR, and CD163 were expressed in the majority of phagocytes from both humans and macaques of both *Mtb*-naïve and LTBI status.

Although total expression of CD206 did not differ between *Mtb*-naïve human subjects and human subjects with LTBI, a higher frequency of CD206 lo phagocytes was observed in BAL of individuals with LTBI than in BAL of uninfected subjects (Figure E3). As in *Mtb*-naïve humans, CD206 lo phagocytes displayed decreased expression of CD163 in individuals with LTBI. In contrast, CD206 lo phagocytes of subjects with LTBI demonstrated biphasic expression of CD36, so that the correlation of the CD206 lo population with increased prevalence of CD36⁺ phagocytes seen in *Mtb*-naïve individuals was not observed in LTBI. As was the case in *Mtb*-naïve

animals, macaques with LTBI did not demonstrate demarcation of CD206 expression into hi and lo subsets. Lo expression of CD206 has been reported previously in interstitial macrophage populations in humans, which were primarily defined by lack of expression of CD169 (11). Accordingly, we also evaluated CD169 expression on BAL phagocytes of a limited number of human subjects ($n = 5$ naïve, 3 LTBI). In contrast to the previous findings, however, in each subject we observed that fewer than 10% of CD206 lo BAL phagocytes were CD169⁺ (data not shown).

As was the case prior to *Mtb* infection, BAL mononuclear phagocytes in LTBI displayed multiple subpopulations on the basis of surface receptor coexpression. In both humans and macaques, the most abundant subpopulation of airway mononuclear phagocytes displayed coexpression of CD206, CD11b, CD11c, CD163, and HLA-DR (but not CD209); the population expressing all six of these receptors (CD206, CD11b, CD11c, CD163, HLA-DR, and CD209) was the second-most prevalent for each species (not shown). Limiting the analysis to the four most commonly coexpressed markers (HLA-DR, CD206, CD11b, CD11c), various combinations were observed on BAL mononuclear phagocytes from *Mtb*-naïve and LTBI cohorts of both species (Figure 3). In all groups, the majority of cells coexpressed all four markers (“4⁺” phenotype). Reductions in the frequency of this population were observed in both humans and macaques with LTBI compared with *Mtb*-naïve subjects, although this difference did not reach statistical significance in either species. Macaques with LTBI had a reduced proportion of HLA-DR⁺/CD206⁺/CD11b⁺/CD11c⁺ (3⁺/CD11c⁺) phagocytes compared with *Mtb*-naïve animals. In contrast, no significant differences were observed between *Mtb*-naïve human subjects and human subjects with LTBI regarding the prevalence of phagocytes expressing any of the “3⁺” combinations of these markers.

Evaluation of BAL Findings in *Cynomolgus* Macaques with Latent versus Active TB

Comparison of macaque BAL phagocytes in LTBI versus active TB. The range of

Table 2. Frequency of Alveolar Macrophages that Stain for Common Macrophage Markers in *Mtb*-Naive Humans and Cynomolgus Macaques before Infection (*Mtb*-Naive) and after Development of LTBI

Phagocyte Marker	Macaque		Human	
	<i>Mtb</i> -Naive (n = 16)	LTBI (n = 8)	<i>Mtb</i> -Naive (n = 10)	LTBI (n = 11)
CD206	82.1 [70.9, 88.7]	72.5 [63.7, 89.5]	95.8 [93.4, 97.1]	93.6 [89.9, 95.5]
CD11b	86.6 [80.4, 90.8]	93.0 [85.3, 95.0]	97.0 [86.4, 98.9]*	81.9 [73.4, 87]*
CD11c	73.5 [65.2, 79.2]	72.5 [65.2, 81.8]	95.0 [90.3, 98.2]	91.9 [79.4, 96.9]
HLA-DR	80.8 [69.6, 87.0]	76.2 [61.4, 85.0]	96.7 [89.0, 97.9]	92.6 [83.9, 96.4]
CD163	85.2 [71.1, 90.6]	77.8 [68.6, 90.1]	70.6 [62.0, 76.9]	53.3 [46.6, 77.5]
CD36	77.0 [55.0, 86.8]	73.3 [64.2, 86.1]	13.7 [9.8, 16.1]	7.4 [5.3, 24.4]
CD209	10.8 [4.5, 18.8]	11.0 [9.7, 24.3]	2.0 [1.0, 2.1]	2.0 [0.4, 2.4]

Definition of abbreviations: CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; IQR, interquartile range; LTBI, latent tuberculosis infection; *Mtb*, *Mycobacterium tuberculosis*. Data are presented as median [IQR₂₅, IQR₇₅].

* $P < 0.0001$, two-way analysis of variance with Sidak's multiple comparison *post hoc* test. This reflects within-host comparisons (i.e., CD11b proportions differ among *Mtb*-naive humans and humans with LTBI).

outcomes after bronchoscopic *Mtb* infection of cynomolgus macaques allowed us to compare BAL profiles in LTBI and active TB in these macaques (Table 3). BAL from animals with active TB displayed lower frequencies of phagocytes expressing CD11c than did that from macaques with

LTBI. Trends toward lower expression of CD36 and CD163 were observed among animals with active TB compared with LTBI, but these were not statistically significant ($P = 0.07$ in both cases, Mann-Whitney). In contrast, mononuclear phagocytes expressing CD209⁺ were

Figure 3. Heterogeneous populations of BAL phagocytes exist in *Mtb*-naive and LTBI populations in both humans and macaques. The prevalence of BAL phagocytes defined by coexpression of receptors CD206, HLA-DR, CD11b, and CD11c is illustrated for human subjects ($n = 10$ *Mtb*-naive and $n = 11$ LTBI) and cynomolgus macaques ($n = 16$ *Mtb*-naive and $n = 8$ LTBI). As detailed in the text, the “4⁺” population is less common in LTBI BAL than in *Mtb*-naive BAL from both species (but approaches significance only for human subjects). The prevalence of the “3⁺/CD11c⁻” population is decreased significantly in macaques with LTBI compared with *Mtb*-naive animals, whereas no significant differences in the prevalence of any of the “3⁺” combinations were observed between *Mtb*-naive human subjects and humans subjects with LTBI. ($^{\$}P = 0.06$, $^*P < 0.05$, Mann-Whitney.) Results for individual subjects as well as median values are displayed for each group. CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related.

observed with greater frequency in BAL of macaques with active TB compared with BAL of animals with LTBI, as has been noted in human BAL during active TB (16).

Correlation between baseline BAL cell profile and outcomes of *Mtb* infection.

Because of the notable heterogeneity of BAL phagocytes in *Mtb*-naive macaques, we also assessed the correlation between baseline surface marker profiles and the subsequent outcome of *Mtb* infection. Preinfection BAL from animals that ultimately developed active TB demonstrated a significantly lower percentage of phagocytes that coexpressed all four of the predominant surface markers (HLA-DR, CD206, CD11b, and CD11c) compared with that of animals that would later develop LTBI. In contrast, a greater proportion of phagocytes expressing the 3⁺/CD11c⁻ phenotype were observed in baseline BAL of animals that developed active disease compared with that of those who developed LTBI (Figure 4). For better distinction of these subpopulations, we also evaluated preinfection coexpression of all six phagocyte markers studied (CD206, HLA-DR, CD11b, CD11c, CD209, CD36) and correlated the subsets thus identified with the subsequent outcome (Figure E4). A greater frequency of 6⁺ coexpressing cells was observed in preinfection BAL of animals that would remain latently infected compared with those that ultimately displayed signs of active TB. Conversely, subpopulations of airway mononuclear cells that coexpressed CD206⁺, HLA-DR⁺, CD11c⁺, and CD36⁺, but were CD11b⁻ and CD209⁻ were less common in animals that would later develop LTBI compared with those that subsequently developed active TB.

Discussion

Cynomolgus macaques offer an attractive model of human TB through their unique capacity to spontaneously develop a range of infectious outcomes after low-level infection with virulent strains of the *Mtb*. More specifically, these animals develop both clinically silent LTBI and active disease of various degrees of severity, including advanced and cavitary pulmonary TB. Like humans, macaques with LTBI have a limited number of granulomas in the lungs and mediastinal lymph nodes (4, 21), a

Table 3. Differential Expression of Surface Markers of BAL Phagocytes Obtained from Cynomolgus Macaques with LTBI and with Active TB

BAL Monocyte Marker	LTBI (n = 9) (%)	Active TB (n = 10) (%)	P Value
CD206	72.5 [63.4, 89.5]	60.3 [49.4, 78.3]	NS
CD11b	93.0 [85.3, 95.0]	86.6 [79.2, 91.6]	NS
CD11c	72.5* [65.2, 81.8]	50.5* [24.8, 66.6]	0.02
HLA-DR	76.2 [61.4, 85.0]	68.7 [50.7, 82.9]	NS
CD163	77.8 [68.6, 90.1]	60.5 [48, 78.3]	NS
CD36	73.3 [64.2, 86.1]	51.5 [34.6, 76.7]	NS
CD209	11† [9.7, 24.3]	31.4† [24.6, 53.4]	0.009

Definition of abbreviations: BAL, bronchoalveolar lavage; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; IQR, interquartile range; LTBI, latent tuberculosis infection; NS, not significant; TB, tuberculosis.

Data are presented as median [IQR₂₅, IQR₇₅].

(* $P < 0.05$, † $P < 0.01$, Mann-Whitney.)

spectrum of granuloma types seen both by histopathology (e.g., caseous, fibrotic, nonnecrotizing) and bacterial burden (4, 21, 22), and a range of radiographic patterns and inflammatory responses in the lung seen by modern 2-deoxy-2-[¹⁸F]-fluoro-D-glucose positron emission tomography/computed tomography (PET/CT) imaging of the thorax (23–25). Importantly, both humans and macaques develop a spectrum of latent infection (4, 22, 26). In the macaque model, the

spectrum of LTBI can be distinguished by PET/CT characteristics that are associated with the risk of reactivation (27). Although this range of manifestations is similar to the diverse outcomes of human *Mtb* infection, cynomolgus macaques are nevertheless more susceptible to *Mtb* than are humans, in that ~50% of infected animals develop active disease within months of initial infection, whereas only 5% of otherwise healthy humans do so. Direct comparison with studies of human airway cells obtained

by BAL is required to clarify how these animals can best be used to illuminate the mechanisms by which local immune responses contain, or fail to contain, respiratory infection with the organism. To the best of our knowledge, this study provides the first systematic comparison of BAL cell phenotypes in humans and cynomolgus macaques in the presence and absence of *Mtb* infection.

Our studies of BAL cells from *Mtb*-naïve subjects of both species indicate that overall, airway cells in humans and cynomolgus macaques are similar. More specifically, we found that a simple gating strategy that was based on side scatter and staining with anti-CD45 and anti-CD3 effectively identified T-cell and mononuclear phagocyte populations in both species. In the absence of *Mtb* infection, the differential BAL cell counts of the species were similar as well; likewise, CD4⁺ cells were the predominant T-cell population in both humans and macaques. The surface markers expressed by BAL phagocytes were generally similar between the two species, and the most commonly observed phagocyte phenotype in human subjects and macaques was characterized by the coexpression of surface markers CD206, HLA-DR, CD11b, and CD11c.

Some differences in baseline BAL cells of the two species were observed; in some cases, these may be associated with the greater susceptibility of macaques than humans to the development of more active TB. For example, CD36 expression was observed on a significantly greater percentage of BAL phagocytes in macaques than in humans. CD36 expression has been associated with an overall antiinflammatory phenotype that can be induced by peroxisome proliferator-activated receptor- γ (28) and is also associated specifically with increased intracellular survival of *Mtb* (19, 29, 30). Likewise, peroxisome proliferator-activated receptor- γ expression/activity is itself associated with *Mtb* survival in human macrophages (31). The greater frequency of CD11c⁺ phagocytes observed in macaques could also be associated with *Mtb* susceptibility, because CD11c⁺ airway mononuclear cells have a greater capacity to stimulate antigen-primed T cells than do their CD11c⁺ counterparts (reviewed in Reference 9). It is unclear if the increased expression of CD209 on macaque BAL phagocytes with active TB (as reported

Figure 4. Prevalence of BAL phagocyte subsets in *Mtb*-naïve cynomolgus macaques correlates with subsequent outcome of *Mtb* infection. In preinfection BAL phagocytes of macaques that ultimately developed active tuberculosis ($n = 11$), the “4⁺” subpopulation coexpressing CD206, CD11b, CD11c, and HLA-DR was less common compared with those of macaques that would develop LTBI. In contrast, the “3⁺/CD11c⁺” population was more prevalent in animals that would later develop LTBI ($n = 5$) compared with active tuberculosis. (* $P = 0.07$, * $P < 0.05$, Mann-Whitney). Results for individual macaques as well as median values are displayed.

previously in humans in Reference 16) represents a potentially protective phenotype developed in the context of a high bacterial burden or, conversely, an immunosuppressive population that contributes directly to the progression of TB. Regardless, the presence of large proportions of these cells in BAL of macaques with active TB provides a potential means of investigating this issue.

In contrast to recent studies that focused on distinguishing alveolar macrophages, interstitial monocytes, and/or extra- or intravascular monocytes from whole-lung homogenate (11, 12, 32), our study focused on the assessment of coexpression of a range of markers on airway phagocytes obtainable via BAL. This evaluation revealed multiple subpopulations of alveolar macrophages in both humans and cynomolgus macaques. The potential significance of this heterogeneity regarding *Mtb* infection is suggested by our observations that the frequency of these subpopulations within BAL appears to differ between humans and macaques, and also between the preinfection BAL samples of macaques that ultimately developed LTBI or active TB. Specifically, the “4⁺” phenotype (coexpressing CD206, HLA-DR, CD11b, and CD11c) was more prevalent in uninfected humans than in macaques and also more common in animals that develop LTBI versus those found to have active TB, suggesting that this subpopulation could contribute to the development of effective immunity to *Mtb*. Conversely, the “3⁺/CD11c[−]” phenotype (CD206⁺, HLA-DR⁺, CD11b⁺, and CD11c[−]) was more prevalent in baseline BAL of macaques than that of humans and was observed more frequently in animals that developed active TB than in those whose infection remained latent, and therefore could contribute to TB susceptibility. The recent demonstration of distinct functional profiles of sorted lung phagocyte populations (32) indicates the potential feasibility of assessing how the responses of these subpopulations to *Mtb* infection may differ. Further investigation is warranted to determine if these subpopulations of airway phagocytes of humans and cynomolgus macaques can be exploited to further clarify the mechanisms of immune protection (and evasion) in response to *Mtb*.

The capacity of cynomolgus macaques to develop LTBI after infection is critical to

making a comparison of their pulmonary immune responses with those of humans. In contrast, no similar paucibacillary state is observed in standard murine models of *Mtb* infection. The potential applicability of this finding to understanding human immunity to *Mtb* is emphasized by the ability of relevant insults to the immune system, such as infection with simian immunodeficiency virus and treatment with tumor necrosis factor- α blocking agents, to induce reactivation TB in macaques with LTBI (33–35). Despite these clinical similarities, however, our results demonstrate different BAL findings in humans and macaques with LTBI. Specifically, dramatic changes in airway cell composition (i.e., an increase in lymphocytes and a decrease in mononuclear phagocytes) were observed in macaques with LTBI, but not in humans with LTBI. This observation suggests that ongoing pulmonary inflammation may be less characteristic of human LTBI than that of macaques. This possibility is likely to be addressed by the anticipated publication of studies using 2-deoxy-2-[¹⁸F]-fluoro-D-glucose PET/CT scanning to evaluate immune activity in human LTBI (36), as has been reported previously for macaques (27). In any case, however, the characteristics of the two LTBI cohorts being examined in this study must be considered. Specifically, in the cynomolgus macaque model, LTBI status was assigned after observation for 6 months after *Mtb* infection. “Early” LTBI in macaques may therefore still reflect the ongoing development of specific immunity to *Mtb*; this may be distinct from the more established immune status of our human subjects with LTBI from the United States, most of whom had been identified as having *Mtb* infection several years prior to their study participation. LTBI in cynomolgus macaques may therefore be more comparable to the immune events observed in humans in which recent *Mtb* infection is well documented, such as those individuals whose tests for LTBI convert after household contact with patients with active pulmonary TB. No BAL studies of such cohorts have yet been published, although such assessments are currently being initiated in Uganda (W.H. Boom, personal communication). An additional possibility is that the more “quiescent” immune profile of human LTBI reflects the impact of antibiotic treatment of LTBI, as had been completed by most of our human subjects. An assessment of BAL profiles in

both humans and macaques before and after antibiotic treatment for LTBI would therefore be of great interest in clarifying this issue.

We also observed previously undescribed differences between human and macaque BAL that were observed in both *Mtb*-naïve and -infected conditions. Previous studies of human BAL cells have used lymphocyte percentage and BAL CD4:CD8 T-cell ratios as basic measures of immune activity (37, 38). Comparison of CD4:CD8 ratios in BAL of humans and macaques was confounded by the observation that DP_T expressing both CD4 and CD8 were observed in BAL of macaques, but not of humans. DP_T populations have been reported in multiple animal models (39–41) and in previous human studies (42–44). In humans, DP_T have been described in the peripheral blood as differentiated antigen-specific cells developed in response to viral pathogens (44) and in the affected tissues of suspected autoimmune diseases (reviewed in Reference 39); DP_T were also identified in BAL of HIV-infected patients with chronic obstructive pulmonary disease but not in HIV infection or chronic obstructive pulmonary disease alone (45). In both humans and macaques, peripheral blood DP_T display distinct functional responses to both pathogen-specific and nonspecific stimulation (44, 46–48); the mycobacterial-specific responses of these cells have not yet been published. However, determination of the distinct antigen specificities and functional responses of this population is needed to determine whether its presence alters the overall BAL T-cell response in macaques in a way that is not recapitulated in humans. In terms of phagocyte populations, although a CD206 lo phagocyte population was observed in human BAL, no such population was identified in BAL of macaques. This population was observed to a greater extent in individuals with LTBI than in *Mtb*-naïve human subjects. Furthermore, CD206 lo phagocytes were less likely to express CD163 but more likely to express CD36 than were CD206 hi cells. Our finding that CD206 lo BAL phagocytes do not generally express CD169 would indicate that these cells do not share the recently described phenotype of interstitial macrophages (11). Nevertheless, our observations raise the question as to whether CD206 lo phagocytes may represent a population that

has been recruited recently (from the interstitium or elsewhere) or that is otherwise not fully differentiated or activated; in either case, their increased presence in LTBI may be indicative of ongoing local immune activity in this condition. Our inability to detect CD206 lo phagocytes in BAL of macaques could reflect the different methods of fluorochrome compensation used in the two species, in that our greater capacity to adjust flow cytometry parameters for individual samples in humans than in macaques (given the larger numbers of BAL cells obtained) could have facilitated the detection of a CD206 lo population. Greater attention to this issue in future studies could therefore clarify whether this observation represents a true interspecies difference relevant to host susceptibility, or simply a technical aspect of flow cytometry assessment.

Additional factors could also contribute to cross-species differences in the phagocyte receptor expression we observed. We cannot rule out the possibility that differences in the results of human versus macaque studies could be caused by unrecognized differences in our protocols, because samples from the two species were processed and analyzed at two different institutions. Although

imperfect cross-reactivity between the human antibodies and macaque epitopes is a possibility, this seems less likely, given the similarity in overall patterns of receptor expression observed between species. This consideration could explain the lower expression of CD11b and CD11c observed on macaque phagocytes than on those of humans; however, the finding that differences in the expression of these receptors are also seen among the various macaque groups suggests that these findings are indeed biologically valid. Furthermore, decreased antibody affinity for macaque epitopes could not account for the differences in the expression of several markers (e.g., CD36 and CD209) that were present to a greater extent on BAL phagocytes of macaques than on human cells.

Conclusions

To the best of our knowledge, our observations provide the first extensive comparative immune phenotyping of BAL cells from *Mtb*-naïve and *Mtb*-infected cynomolgus macaques and humans. Our findings support the relevance of the cynomolgus macaque model in providing BAL findings that are readily comparable to those of humans. These

cross-species observations provide new information about subsets of alveolar T cells and mononuclear phagocytes that may reveal mechanistic factors of host susceptibility and suggest further avenues of investigation to better characterize the evolution of the LTBI state in both humans and macaques. Importantly, the finding of different subpopulations of airway mononuclear cells before infection and their correlation to *Mtb* infection outcome supports the notion that mucosal immune responses in the airway may play a critical role during infection. Further evaluation of these populations may lead to novel insights into the optimal application of macaque models for understanding protective pulmonary immune responses to *Mtb* in humans. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Drs. Larry S. Schlesinger of Ohio State University and W. Henry Boom of Case Western Reserve University for helpful discussions and manuscript review. They also thank Melanie O'Malley, Dan Fillmore, Mark Rodgers, Catherine Cochran, Chelsea Chedrick, and Pauline Maeillo for their efforts in veterinary, laboratory, and statistical assistance.

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