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Microenvironments in Tuberculous Granulomas Are Delineated by Distinct Populations of Macrophage Subsets and Expression of Nitric Oxide Synthase and Arginase Isoforms

Joshua T. Mattila,* Olabisi O. Ojo,* Diane Kepka-Lenhart,* Simeone Marino,*† Jin Hee Kim,‡ Seok Yong Eum,§ Laura E. Via,* Clifton E. Barry, III,* Edwin Klein,*‡ Denise E. Kirschner,* Sidney M. Morris, Jr.,* Philana Ling Lin,* and JoAnne L. Flynn***

Macrophages in granulomas are both antmycobacterial effector and host cell for Mycobacterium tuberculosis, yet basic aspects of macrophage diversity and function within the complex structures of granulomas remain poorly understood. To address this, we examined myeloid cell phenotypes and expression of enzymes correlated with host defense in macaque and human granulomas. Macaque granulomas had upregulated inducible and endothelial NO synthase (iNOS and eNOS) and arginase (Arg1 and Arg2) expression and enzyme activity compared with nongranulomatous tissue. Immunohistochemical analysis indicated macrophages adjacent to uninvolved normal tissue were more likely to express CD163, whereas epithelioid macrophages in regions where bacteria reside strongly expressed CD11c, CD68, and HAM56. Calprotectin-positive neutrophils were abundant in regions adjacent to caseum. iNOS, eNOS, Arg1, and Arg2 proteins were identified in macrophages and localized similarly in granulomas across species, with greater eNOS expression and ratio of iNOS/Arg1 expression in epithelioid macrophages as compared with cells in the lymphocyte cuff. iNOS, Arg1, and Arg2 expression in neutrophils was also identified. The combination of phenotypic and functional markers support that macrophages with anti-inflammatory phenotypes localized to outer regions of granulomas, whereas the inner regions were more likely to contain macrophages with proinflammatory, presumably bactericidal, phenotypes. Together, these data support the concept that granulomas have organized microenvironments that balance antimicrobial anti-inflammatory responses to limit pathology in the lungs.

The online version of this article contains supplemental material.

Abbreviations used in this article: Arg, arginase; eNOS, endothelial NO synthase; iNOS, inducible NO synthase; L-NIL, N6-(1-iminoethyl)-L-lysine; nNOS, neural NO synthase; NOS, NO synthase; qRT-PCR, quantitative RT-PCR; TB, tuberculosis.

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The high level of stratification in human and nonhuman pri-
mate granulomas (3, 6) suggests that protection depends on microenviron-
ments (7, 8) that promote bacterial clearance while minimizin-
g damage to uninvolved tissues adjacent to the granuloma. Macrophage subsets engaging in anti-inflammatory or proin-
flammatory processes are likely to be important, yet poorly under-
stood, mediators determining the characteristics of these micro-
environments. Activated macrophages are often classified as either clas-
sically activated M1 (proinflammatory) macrophages that engage in bac-
terial activity or alternatively activated M2 (anti-
inflammatory) macrophages that mediate prohealing responses. Expression of inducible NO synthase (iNOS) is the hallmark of proinflammatory macrophages and, in murine systems, is necessary for improved resistance to TB (9–11). iNOS-expressing macro-
phages have been identified in the lungs of humans with TB (12–14), although a correlation between human TB and deficient iNOS ex-
pression has proven difficult (15). The other NO isoforms, endo-
thelial NOS (eNOS) and neural NOS (nNOS), can also be present in granulomas (12), but it is not known whether they have homeostatic or bactercidal functions.

Prohealing anti-inflammatory macrophages are characterized by arginase 1 (Arg1) expression (16, 17), although this is best defined in murine systems. Arginases can compete with NO syntheses for L-arginine and generate urea and L-ornithine, which can be subsequently converted to L-proline (18), an amino acid or clinically latent to undergo necropsy as part of other studies. For immunohistochemistry, granuloma-containing tissues were excised and fixed in 10% neutral buffered formalin prior to placement in histology cassettes and paraffin embedding. Tissues were cut into 5-µm-thick sections by the University of Pittsburgh Medical Center’s in situ histology laboratory and mounted on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA). Formalin-fixed paraffin-embedded human lung and tissue samples containing granulomas were dissected from tissue removed during therapeutic lung resection surgery at the National Masan Tuberculosis Hospital from patients refractory to second line drug therapy. Tissue collection (2003–2007) was approved by the National Masan Tuberculosis Hospital institutional review board, an exemption from National Institutes of Health, and with written consent of the subjects; samples were deidentified when provided for analysis.

Materials and Methods

Tissue processing and sectioning

All animal procedures and husbandry practices were included in protocols approved by the University of Pittsburgh’s Institutional Animal Use and Care Committee. Cynomolgus macaques were infected with low-dose (~25 CFU) Erdman-strain M. tuberculosis as previously described (4). Macrophages with active TB were humanely euthanized and necropsied as previously described (4). Microbial loads of granulomas were measured as CFU/mL using bacterial culture techniques (4). Granulomas were dissected from tissue removed during therapeutic lung resection surgery at the National Masan Tuberculosis Hospital from patients refractory to second line drug therapy. Tissue collection (2003–2007) was approved by the National Masan Tuberculosis Hospital institutional review board, an exemption from National Institutes of Health, and with written consent of the subjects; samples were deidentified when provided for analysis.

Immunofluorescence, immunohistochemistry, imaging, and

image analysis

Formalin-fixed paraffin-embedded tissue sections from M. tuberculosis-infected mice m907, m9209 m1307, m1707, m3809, m9095, m10708, m21802, m15304, and m13207 were selected for study. All animals except m10708 had active TB at the time of necropsy. Tissue sections were deparaffinized in xylene, 100% ethanol, and 95% ethanol. Tissue sections were then placed into an Ag retrieval buffer (20 mM Tris/820 mM EDTA/0.00005% Tween 20 [pH 9]) containing a pressure cooker (Mantra, Pis-
catay, NJ), incubated under pressure for 7 min before removal from the hotplate, and allowed to cool slowly over 30 min. Sections were incubated in blocking buffer (2.5% BSA in PBS) for 30 min at 37˚C, prior to addition of primary Ab diluted in blocking buffer. Abs for immunohistochemistry were against human CD3e (ready-to-use format, 1:2 dilution; DakoCyto-
mation, Carpintera, CA), CD11c (clone 5D11, 1:30 dilution; Leica Microsystems, Buffalo Grove, IL), CD68 (clone KPI, 1:50 dilution; Lab Vision, San Diego, CA), CD163 (clone 10D6, 1:30 dilution; Lab Vision), calprotectin (clone MAC387; 1:100 dilution; Lab Vision), HAM56 (ready-
to-use format, 1:2 dilution; Enzo Life Sciences, Farmingdale, NY), iNOS (rabbit polyclonal; Lab Vision), eNOS (rabbit polyclonal; Lab Vision), Arg1 (clone 19/arginase1, 1:100 dilution; BD Biosciences), arginase 2 (rabbit polyclonal, 1:40 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and nitrotyrosine (rabbit polyclonal, 1:100 dilution; Millipore, Billerica, MA). The specificity of iNOS and eNOS Abs were confirmed by Western blotting of iNOS and eNOS (0.1 µg/lane) in conjuction with 1 µg M. tuberculosis lysate to confirm that the Abs were isofrom specific and not reactive with bacterial proteins (data not shown). We were unable to identify an anti-nNOS Ab that worked for immunohistochemistry in ma-
caque tissues and was not cross-reactive with other NOS isoforms (data not shown). Tissue sections were incubated at room temperature in cocktails of primary Abs for 1 h. Secondary Abs, purchased from either Jackson ImmunoResearch Laboratories (West Grove, PA) or Life Technologies, were diluted in blocking buffer and applied to tissue sections that had been washed three to five times with IHC wash buffer (0.2% Tween-20 in PBS) and incubated for 1 h at room temp in the dark. HAM56 was stained with anti-mouse μ-chain–specific secondary Ab (Jackson ImmunoResearch Laboratories) that was made in donkey and not crossreactive with mouse IgG Abs. The specificity of secondary Abs was confirmed either by isotype or no-primary controls using the same staining and imaging protocol as sections containing stained with primary Abs. The slides were then washed three to five times with IHC wash buffer and directly labeled conjugates applied. Abs for direct labeling were chosen based on their ability to work well following significant dilution when used with a secondary Ab. The unla-
beled Abs were labeled with either Alexa Fluor 488 or Alexa Fluor 647 using the Zenon direct labeling kit (Life Technologies). Tissue sections were incubated with direct conjugates for 2 h at room temperature or overnight at 4˚C. Slides were washed four times with IHC wash buffer, once with PBS, and then coverslips were applied using Prolong Gold mounting medium containing DAPI (Life Technologies). Slides were cured for 24 h at room temperature before imaging. Granulomas were imaged with either an Olympus Fluoview 500 or Fluoview 1000 laser scanning confocal microscope (Olympus, Center Valley, PA) maintained by the University of Pittsburgh’s Center for Biologic Imaging and a Fluoview 1000
laser scanning confocal microscope maintained by the University of Pittsburgh’s Microbiology and Molecular Genetics Department. Individual tissue sections from animals with active TB frequently contained multiple granulomas of various sizes and type; we chose to image granulomas with features that were representative of that particular granuloma type. Three-color images (red, green, and far red [pseudocolored as blue]) were acquired sequentially, followed by a DAPI image (gray) showing nuclei. Images (either single sections or serial Z sections acquired at 1-μm intervals) were acquired and saved as TIFF-format images. Z series images were opened with MacBiophotonics ImageJ (available at www.macbiophotonics.ca/software.htm) or FIJI (available at http://pacific.mpi-cbg.de/wiki/index.php/Downloads) and saved as maximum-intensity projections. At least three fields in the macrophage–lymphocyte region were imaged at ×400–600 original magnification for counting cells in tissues. Images were opened in Photoshop (Adobe Systems), and an overnight image overlay with a grid was used to facilitate analysis, and counted manually by examining each channel separately or in combination for positively stained cells. The number of nuclei per image, which was used to determine the number of cells within an image, was assessed with CellProfiler v2.0 (available at http://www.cellprofiler.org/). Granulomas were too large to be imaged by one ×20 original magnification field; consequently, multiple overlapping fields were acquired and the image of the entire granuloma assembled into a single montage using Photoshop (Adobe Systems). Preliminary work indicated that counting individual cells in granulomas for phenotypic analysis by automated or manual means was not going to be feasible due to the complexity of the environment; consequently, we used a region-based approach to analyze staining (signal) intensity in lymphocyte cusp or epithelioid macrophage regions. For analysis of region-based characteristics (macrophage regions, iNOS/Arg1 expression), nonoverlapping image fields (×200 original magnification) of granulomas containing both epithelioid macrophage and lymphocyte cusp regions were acquired as previously indicated. From these images, regions of interest were drawn around epithelioid macrophage or lymphocyte cusp regions, and the mean pixel intensity of the red, green, and blue channels was determined with Photoshop’s histogram tool (Adobe Systems). The iNOS/Arg1 ratio was calculated by dividing the mean iNOS signal by the mean Arg1 signal. Pairwise comparisons were made between macrophage surface marker signal or iNOS/Arg1 signal ratio were made between the epithelioid macrophage and lymphocyte cusp regions.

Auramine-rhodamine staining

Granulomas were stained with auramine-rhodamine reagents to visualize the mycobacterial cell wall component mycolic acid. Tissue sections were deparaffinized as previously indicated and equilibrated in distilled H2O for 5 min in between each wash. With auramine staining, sections were first washed off with distilled H2O and decolorized in multiple washes of isopropanol with 5% HCl (v/v) before being counterstained with potassium hydroxide. The red, green, and blue channels was determined with Photoshop’s histogram tool (Adobe Systems). The iNOS/Arg1 ratio was calculated by dividing the mean iNOS signal by the mean Arg1 signal. Pairwise comparisons were made between macrophage surface marker signal or iNOS/Arg1 signal ratio were made between the epithelioid macrophage and lymphocyte cusp regions.

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Results

Arg1, Arg2, iNOS, and eNOS are expressed and functional in the granuloma

Factors initiating macrophage polarization and phenotypes of classically or alternatively activated macrophages have not been fully resolved in primates. In mice, NOS expression defines classically activated macrophages, whereas arginase expression delineates alternatively activated macrophage populations; consequently, we used Arg1, Arg2, iNOS, or eNOS (Fig. 1) as markers for macrophage function with the caveat that these markers may not fully describe macrophage polarization. Moreover, because of the difficulty assigning an activation state to primate macrophages, we will use the term proinflammatory to indicate macrophages with dominant NOS expression and anti-inflammatory to characterize macrophages with dominant arginase expression. Arg1 was abundant in macrophages, whereas the most intense Arg2 signal occurred as discrete granules in cells with segmented nuclei and at much lower levels in macrophages (Fig. 1A). iNOS and eNOS were identified in macrophages, each with a characteristic staining pattern. Macrophage iNOS had a punctate appearance and appeared to be scattered throughout the cytoplasm (Fig. 1A), whereas eNOS staining in epithelioid macrophages was more intense and associated with the cell membrane (Fig. 1A). We also found substantial iNOS and Arg1 coexpression, particularly in macrophages (Fig. 1B) but also in neutrophils (Fig. 1B). These results demonstrate that macrophage activation is not binary but occurs along a spectrum.

qRT-PCR and biochemical assays for arginase and NOS activity confirmed that arginase and NOS are expressed and functional in granulomas. qRT-PCR indicated Arg1, Arg2, eNOS, and iNOS expression was upregulated in granuloma-containing tissues relative to uninfected control tissues (Fig. 2A), with Arg1 and iNOS upregulated more than Arg2 and eNOS, respectively. Although nNOS expression was identified, its expression in granulomas was not strongly upregulated above uninfected lung. Biochemical assays for enzyme activity in tissue lysates demonstrated that granuloma-containing tissues had significantly more arginase and NOS activity than uninvolved tissues (Fig. 2B–D). The NOS activity associated with individual granulomas within a monkey was highly variable but generally higher than the NOS activity from uninvolved lung from that same monkey (Fig. 2C). When these data were aggregated and compared, granulomas showed significantly more NOS activity than uninvolved tissue from infected animals (Fig. 2D). Although the iNOS activity of many granulomas was inhibited by L-NIL, it was not possible to inhibit the NOS activity in some tissues, suggesting other NOS isoforms contribute to NO production (data not shown). Finally, we identified nitrotyrosine, the nitrosylated tyrosine residues produced after NO–protein interactions, in granuloma macrophages and neutrophils (Fig. 2E), supporting that the NOS species present in granulomas are functional.

Identification of macrophage- and neutrophil-specific Abs for cynomolgus macaques

Despite the abundance of macrophages in granulomas, little is known about their molecular identities. Two categories of macrophages found in human granulomas are described morphologically (33) as epithelioid macrophages and foamy macrophages. Epithelioid macrophages are defined by their high cytoplasm/nucleus ratios and diffusely eosinophilic cytoplasm (34, 35) and are particularly abundant in granuloma regions adjacent to caseous necrosis. Foamy macrophages are also associated with tuberculous granulomas and are identifiable by their foamy, lipid-rich cytoplasm (2). To better characterize the presence and location of these cells in granulomas, we used Abs against human macrophage Abs that we validated for use in macaque tissues. These Abs, including CD11c, CD68, CD163, HAM56, and calprotectin (Mac387), reliably stained cells in macaque tissues, whereas we excluded other commonly used macrophage markers, including CD11b and CD14 because of their broad myeloid cell expression. Dendritic cell–specific ICAM-3-grabbing nonintegrin (CD209), a marker associated with dendritic cells, was expressed by macrophages in throughout granulomas (data not shown) and, consequently, was not included in this study. We also excluded F4/80, a commonly used macrophage marker in murine systems because the human homolog (EMR1) is an eosinophil-associated protein (36) and CD15, a marker for human neutrophils because it expression on a subset of lymphocytes and lung epithelial cells (data not shown). These Abs can react with other structures (e.g., HAM56 and CD163 with endothelium and CD68 with fibroblasts), but endothelium is not abundant in granulomas and fibroblasts associated with granulomas did not stain positively for CD68; data not shown). Staining patterns for CD11c+, CD68+, CD163+, and HAM56+ cells were morphologically consistent with macrophage-like cells (Supplemental Fig. 1). The Ab Mac387 is often described as a macrophage marker; yet in cynomolgus macaques, the morphology of calprotectin-expressing cells was more neutrophil-like. Subsequent experiments identified calprotectin–bright cells in cynomolgus macaques as neutrophils (Supplemental Fig. 2).

Myeloid cell populations in macaque and human granulomas

In this study, we imaged granulomas representing the types commonly seen in cynomolgus macaques (5) with active TB to determine whether different, antigenically defined populations of

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Immunohistochemical identification of arginase and NOS isoforms in cells from cynomolgus macaque granulomas. (A) Epithelioid macrophages, as depicted by H&E staining (H&E) were stained for Arg1, Arg2, iNOS, and ENOS (green) and nuclei (blue) and imaged at ×600 original magnification to represent the unique staining patterns associated with each enzyme. Each panel presents an independent set of epithelioid macrophages in the macrophage region. Scale bar, 20 μm. (B) Coexpression of Arg1 (red) and iNOS (green) in CD163+ macrophages (blue, top panel) and in cells with segmented neutrophil-like nuclei (arrowheads, bottom panel). Image acquired at ×600 original magnification.
macrophages exist in different microenvironments and how the position of these cells correlate with bacterial localization. In addition to markers of different macrophage populations, we examined the NOS and arginase expression by region to determine how microenvironments correlate with macrophage function. Macrophage activation states in primates are complex and poorly understood, so although these markers may reflect a functional capacity, we realize they cannot fully describe the full spectrum of macrophage polarization. We also imaged fibrocalcific granulomas associated with clinically latent TB to compare the organization and population structure of macrophage subsets in healed lesions with active lesions from animals that poorly controlled TB. To compare the nonhuman primate data with human data, we obtained deidentified samples from patients undergoing lung resection for calcific TB; these patients are likely to have had long courses of infection and may have undergone several rounds of treatment. Under these circumstances, the pathology can be more complex than in macaques and can include extensive fibrosis. In fact, lung tissue samples obtained contained multiple granulomas that were often highly fibrotic; we selected nonnecrotic and necrotic granulomas with the lowest amount of fibrosis for imaging and were mindful that these lesions represent those from chronic, poorly controlled disease hosts.

Nonnecrotic granulomas
Nonnecrotic granulomas were characterized by dense macrophage populations without necrotic areas (Fig. 3A). CD68<sup>+</sup> and CD163<sup>+</sup> macrophages were most abundant at the periphery, although macrophages with lower expression of these markers could be found throughout these granulomas (Fig. 3B, 3D). Neutrophils were randomly distributed throughout nonnecrotic granulomas (Fig. 3B). HAM56<sup>+</sup> foamy macrophages were present but were especially abundant in central regions of granulomas with higher levels of organization (Fig. 3B). The position of iNOS<sup>+</sup> cells was similar to that observed for HAM56 expression; less organized, nonnecrotic granulomas had randomly distributed iNOS expression (Fig. 3C), whereas increasingly organized granulomas had foci of iNOS expression that was consistent with the position of epithelioid macrophages (data not shown). Similarly, eNOS expression most strongly correlated with the presence and location of epithelioid macrophages (Fig. 3D). Arg1-expressing cells were identified but did not localize to any particular region in these granulomas. Relatively few Arg2-expressing cells were identified and most of these appeared to be in macrophages at the granuloma’s outer edge and in neutrophils dispersed throughout the granuloma (Fig. 3E).

Caseous necrotic and suppurative granulomas
Necrotic and suppurative granulomas (Fig. 4) have similar features (e.g., well-defined lymphocyte cuffs and epithelioid macrophage-rich regions), but caseous granulomas have noncellular necrotic centers and suppurative granulomas have centers nearly completely infiltrated by neutrophils. Although we consider necrotic and suppurative granulomas to be defined morphotypes, they are grouped together in this study because of their similarities.

CD163<sup>+</sup> macrophages were most abundant in peripheral regions of granulomas adjacent to, or inside, the lymphocyte cuff (Figs. 4B, 4G, 5A, 5D). Clusters of strongly CD11c<sup>+</sup>CD68<sup>+</sup>CD163<sup>+</sup> alveolar macrophage-like cells were often immediately adjacent to the lymphocyte cuff (Fig. 5A, arrowheads). Epithelioid macrophages were CD163<sup>−</sup> or CD163<sup>dim</sup> but often strongly expressed CD68 (Fig. 4D, 4I, 5A, 5C) and CD11c (Fig. 5B). HAM56<sup>+</sup> foamy macrophages were most abundant on the rim of the caseous or neutrophilic center (Fig. 4B, 4G) with more elongate cellular morphology extending perpendicular to the center of the granuloma. Calprotectin<sup>+</sup> neutrophils were present in the lymphocyte cuff and...
macrophage-rich regions but were most abundant in areas near the HAM56+ foamy macrophages adjacent to the caseum (Fig. 4B) and centers of suppurative granulomas (Fig. 4G). Arg1 expression was observed throughout the cellular regions of granulomas (Fig. 4C, 4H), but was most strongly expressed by cells in the lymphocyte cuff (Fig. 5F). Epithelioid macrophages and lymphocyte cuff macrophages also expressed iNOS (Figs. 4C, 4H, 5E), with more iNOS signal observed in lymphocyte cuff region. The lymphocyte cuff region is significantly more cellular than the epithelioid macrophage region (mean density, 6373 nuclei/mm² versus 4459 nuclei/mm², respectively; \( p = 0.0008 \), Mann–Whitney \( U \) test; \( n = 9 \) granulomas) and contains a residual population of epithelial cells that are iNOS rich; consequently, this figure may overrepresent macrophage iNOS expression in the lymphocyte cuff region. When the ratio of iNOS/Arg1 signal intensity from epithelioid macrophages and lymphocyte cuff regions was calculated, epithelioid macrophages expressed significantly more iNOS relative to Arg1 than cells in the lymphocyte cuff region (Fig. 5G), suggesting that Arg1 expression differentiates NO-generating capacity of macrophages in the epithelioid macrophage and lymphocyte cuff regions.

Strong eNOS expression was also noted in epithelioid macrophages, with the majority of eNOS associated with the plasma membrane (Fig. 4D, 4I). We also observed smaller cells in the lymphocyte cuff that were strongly positive for cytoplasmic eNOS but did not stain for either macrophage or endothelial markers (CD31, von Willebrand factor; data not shown). Arg2 expression was largely associated with neutrophils at the border of the caseum and in the center of suppurative granulomas (Fig. 4E, 4J).

**Fibrocalcific granulomas**

Small numbers of fibrocalcific granulomas are commonly found in latently infected hosts, but also in active TB, and are likely to represent the successful outcome of an effective immune response. These granulomas commonly contain mineralized material surrounded by fibrotic tissue and limited numbers of lymphocytes and macrophages (Fig. 6A). Many of the cells in the fibrotic region surrounding mineralized centers expressed CD163 (Fig. 6B). These granulomas contained very few neutrophils (Fig. 6B), HAM56+ (Fig. 6B) and CD68+ (Fig. 6D) macrophages, or eNOS-expressing macrophages (Fig. 6D). iNOS and Arg1 were expressed in the cells closest to the mineralized material (Fig. 6C). Arg2 expression was minimal, but strong signal was noted at the fibrosis–mineral interface (Fig. 6E).

**Bacteria are present in multiple areas in the granuloma**

The localization of bacterial populations is poorly understood for primate granulomas. We stained granulomas for the presence of mycobacterial cell wall components with auramine-rhodamine with overlaid H&E-stained features to correlate the positions of bacteria and different macrophage populations. Ongoing studies in our laboratory where homogenized granulomas are plated for bacterial culture indicate that numbers of bacteria per granuloma are variable and generally low, ranging from sterile granulomas to \( 10^6 \) bacteria/granuloma (J.L.F. and P.L.L., manuscript in preparation). \( M. \) tuberculosis were often not abundant in granulomas, with most granulomas having either no or very small numbers of visible bacteria per 5-μm tissue section. In granulomas in which bacteria were visible, bacilli were present in a variety of locations, including in epithelioid macrophages (Fig. 7A), intermixed with neutrophils at the caseum–macrophage interface (Fig. 7A), and in giant cells at the granuloma’s periphery (Fig. 7B). Bacteria were most commonly present as small groups of individual bacilli associated with necrotic appearing cells at the caseum–macrophage interface. Small numbers of individual bacilli were also occasionally visible deep in the necrotic regions of caseous lesions (Fig. 7B). Auramine-rhodamine–stained objects that did not ap-

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**FIGURE 3.** Macrophage phenotypes and distribution in nonnecrotic granulomas from macaques with active TB. Individual panels show serial 5-μm-thick sections of a representative nonnecrotic granuloma. (A) H&E staining (top panel) with a pseudocolored representation (bottom panel) indicating lymphocyte-rich (cyan) and epithelioid macrophage-rich (purple) regions. Black box indicates the region depicted at higher magnification in (C)–(E). (B) Macrophage-specific stains including HAM56 (red), CD163 (green), and calprotectin-stained neutrophils (blue). White box indicates the region depicted at higher magnification in (C)–(E). (C) iNOS (green) and Arg1 (red) expression with nuclei (blue). (D) CD68 (red) and eNOS (green) expression with nuclei (blue). (E) Arg2 (green) expression with nuclei (blue). Scale bars, 100 μm.
Pear to be intact bacteria were also occasionally visible in epithelioid macrophages adjacent to foamy macrophages with elongated morphologies consistent with foamy HAM56+ cells (Fig. 7C). Interestingly, fibrotic granulomas from latent disease contained a ring of auramine-rhodamine–stained mycolic acid at the fibroblast-collagen–transformed caseum interface (Fig. 7D), suggesting persistence and diffusion of bacterial Ag after most of the bacteria appeared to have been cleared.

**FIGURE 4.** Macrophage phenotypes and distribution in necrotic and suppurative granulomas from macaques with active TB. Individual panels show serial 5-μm–thick sections of representative necrotic and suppurative granulomas. H&E staining (top panel) of necrotic (A) and suppurative (F) granulomas with pseudocolored representation (bottom panel), indicating lymphocyte-rich (cyan), epithelioid macrophage-rich (purple), and necrotic [yellow, (A)] or [suppurative yellow, (F)] regions. Black boxes indicate the regions depicted at higher magnification in (C)–(E) and (G)–(J). (B and G) Macrophage-specific stains including HAM56 (red), CD163 (green), and calprotectin-stained neutrophils (blue). White box indicates the region depicted at higher magnification in (C)–(E). (C and H) iNOS (green) and Arg1 (red) expression with nuclei (blue). (D and I) CD68 (red) and eNOS (green) expression with nuclei (blue). (E and J) Arg2 (green) expression with nuclei (blue). Scale bars, 100 μm.

Nonnecrotic and necrotic granulomas from humans are similar to cynomolgus macaques with respect to macrophage subset, NOS, and arginase localization

The organization of macrophage subsets in nonnecrotic and necrotic human granulomas was grossly similar to macaque granulomas. In nonnecrotic human granulomas (Fig. 8A), CD163+ macrophages were present throughout the granuloma, with cells in the outer regions commonly coexpressing HAM56 (Fig. 7B).
Necrotic granulomas (Fig. 8F) had large numbers of CD163+ HAM56+ macrophages in the peripheral tissue, whereas cells in the lymphocyte cuff were more likely to be CD163+HAM56 (Fig. 8G). Although HAM56 staining adjacent to the caseum was observed, it was not as intense or as distinct as it was in macaque granulomas. Epithelioid macrophages in nonnecrotic and necrotic granulomas strongly expressed CD68 (Fig. 8D, 8I). Clusters of macrophages in the peripheral tissue surrounding these granulomas were HAM56+CD68+CD163+ (arrows, Fig. 8G, 8I), whereas these clusters in macaques were HAM56+CD68+CD163+ (data not shown). Calprotectin-expressing neutrophils were present, and their distribution in nonnecrotic granulomas was similar to that in fibrocalcific granulomas from macaques with latent TB. Individual panels show serial 5-μm-thick sections of a representative necrotic granuloma. (A) H&E (top panel) with pseudocolored representation (bottom panel) indicating the outer fibrotic region (cyan), fibrocalcific interface (purple), and central region containing mineralized material (yellow) that shattered during cutting. Dashed line indicates portion of the fibrocalcific interface that is separated from the rest of the granuloma and is present as an artifact in (A). Black box indicates the region depicted at higher magnification in (C)–(E). (B) Macrophage-specific stains including HAM56 (red), CD163 (green), and calprotectin-stained neutrophils (blue). Gray line outlines the tissue edge denoted by the dashed line in (A) where the fibrocalcific interface has separated from the surrounding tissue and reflected over the other side. White box indicates the region depicted at higher magnification in (C)–(E). (C) iNOS (green) and Arg1 (red) expression with nuclei (blue). (D) CD68 (red) and eNOS (green) expression with nuclei (blue). (E) Arg2 (green) expression with nuclei (blue). Scale bars, 100 μm.
observed in macaque granulomas (Fig. 8B). Although we observed neutrophils in necrotic granulomas, they were not as numerous as they were in macaque granulomas and did not appear to accumulate at the macrophage–caseum interface (Fig. 8G).

iNOS was expressed at low levels by macrophages distributed throughout human nonnecrotic granulomas or adjacent to caseum in necrotic granulomas (Fig. 8C, 8H). Unidentified cells expressing high levels of iNOS were present in regions of nonnecrotic granulomas and in the lymphocyte cuff of necrotic granulomas. Similarly stained cells were observed in some macaque granulomas. CD68+ macrophages in human granulomas frequently expressed eNOS (Fig. 8D, 8I), and clusters of HAM56+CD68+CD163+ macrophages in the tissue adjacent to granulomas stained particularly strong for eNOS (arrows). Arg1 expression was similar that seen in macaque granulomas: Arg1 staining was visible throughout cellular regions of granulomas with little evidence for region-specific expression and Arg1 expression frequently occurred in cells that also expressed iNOS (Fig. 8C, 8H). As has been previously reported (25), there was little Arg2 expressed in human granulomas. Human granulomas contained scattered Arg2-expressing cells, but Arg2 staining did not appear to be as tightly correlated with neutrophils as it was in macaque granulomas (Fig. 8E, 8J).

**Discussion**

Large populations of macrophages are a prominent feature of tuberculous granulomas, yet there are many unanswered questions surrounding the spatial organization of macrophage subsets in granulomas and whether macrophages have microenvironment-specific homeostatic or bactericidal functions. Much of what we know about granuloma macrophages comes from animal models that may not represent the spectrum of pathology seen in humans or has been derived from cells removed from the context of the granuloma. To address these questions, we used immunohistochemistry to clarify the interplay of microenvironment and macrophage biology by identifying macrophage subsets and arginase and NOS expression in granulomas from cynomolgus macaques, a nonhuman primate that recapitulates human TB (4). We found that granulomas have macrophage subsets that are stratified into pro- and anti-inflammatory regions with the implication that this organization may limit immunopathogenic antimicrobial activity to bacteria-rich microenvironments by surrounding them with a layer of cells with anti-inflammatory phenotypes.

The importance of NOS in human TB is controversial (36–38). In mice, elimination of NO leads to higher bacterial numbers and decreased survival time (9, 38). *M. tuberculosis* has limited sensitivity to NO-mediated killing (39–41), and mice with functional iNOS still die of TB, demonstrating that nitrogen radical production alone does not correlate with protection. Humans with TB also express iNOS (12–14), but even though they do not generate NO as vigorously as mice, the human immune system is better at containing *M. tuberculosis*, and most infections do not progress to active TB. Our data demonstrate that macaque macrophages can
express NOS, and granulomas have higher eNOS and iNOS expression than uninfected lung tissue. Epithelioid macrophages can be associated with *M. tuberculosis* bacilli in granulomas and iNOS and eNOS expression, with low levels of Arg1 expression, suggests that NO production by these cells is an active component of the anti-*M. tuberculosis* response in macaques. Fibrocalcific granulomas associated with latent *M. tuberculosis* infection also had iNOS-positive cells, implying that even successful immune responses during latency retain low levels of iNOS expression. This population of iNOS-expressing cells remains in place, presumably either because stimulus is provided by residual mycobacterial Ags or the low numbers of viable bacilli that may be present in these lesions. The surprising amount of eNOS in granulomas in active disease brings up the question of whether it participates in protection against *M. tuberculosis*. Its localization to plasma membranes instead of phagosome-like structures suggests it may not be appropriately positioned to target intracellular bacteria. Alternatively, eNOS produces superoxide when uncoupled if tetrahydrobiopterin or l-arginine are limiting (42, 43) or through protein kinase Cζ-mediated processes following exposure to hypochlorous acid (44). Superoxide in the presence of NO generates peroxynitrite, which is lethal to *M. tuberculosis* (45). There remains much to be learned about epithelioid macrophage biology in situ, and eNOS expression may serve alternative functions that are not directly bactericidal but still contribute to protection. However, NO may also downregulate immune responses in the granuloma; for example, by inhibiting T cell functions as has been demonstrated in other experimental systems (20, 46–48) or by limiting inflammasome activation and subsequent IL-1β secretion (49).

Arginases can compete with NOS for l-arginine, thereby downregulating NO production and generating l-ornithine, an amino acid that can be used for proline synthesis (18), which is used for synthesis of proline-rich proteins such as collagen in granulomas, wound healing, and fibrotic tissues or for polyamine synthesis (18). In mice, Arg1 expression occurs in M2-polarized (anti-inflammatory) macrophages (16, 50, 51) and in wound healing macrophages (52). It has been demonstrated that conversion of arginine to proline in anti-inflammatory macrophages is dependent on Arg1 (53), consistent with the notion that arginase-derived ornithine may promote synthesis of collagen (leading to fibrosis) in tuberculous granulomas. However, ablation of macrophage Arg1 expression resulted in increased fibrosis in mice infected with *Schistosoma mansoni* (20), indicating that fibrosis may not be enhanced by Arg1 in all circumstances. Arginase expression is correlated with decreased protection against acute *M. tuberculosis* infection in mice (23, 24), likely reflecting depletion of l-arginine substrate for NO synthesis. Protective immune responses against *M. tuberculosis* may require both proinflammatory macrophages with bactericidal activity and pro-healing anti-inflammatory macrophages to limit immunopathology. Moreover, primate granulomas are highly organized, and protection would be contingent upon appropriate spatial expression of NOS and arginase expression. Because of this, we hypothesized Arg1′NOS′ macrophages would be present in bacilli-rich microenvironments and Arg1′NOS′ macrophages along the outer margins. Instead, we found substantial coexpression of NOS and arginase throughout necrotic granulomas and elevated Arg1 expression in the lymphocyte cuff region, indicating the ratio of NOS/arginase expression is most likely to be a factor determining functional macrophage polarity in primates. The paucity of Arg1 expression in epithelioid macrophages also suggests that competition for l-arginine by Arg1 may be a critical posttranscriptional determinant of macrophage polarity and NOS activity in primate tuberculous granulomas. The abundance of l-arginine–using enzymes in granulomas may also lead to l-arginine depletion. Consequences of this depletion on T cell function may include downregulation of T cell TCR-ζ expression (46, 47), activation (47, 48), proliferation (47, 48) and cytokine secretion (20, 47). Thus, arginase and NOS expression may decrease proinflammatory T cell responses and modulate macrophage function.

Macrophage diversity and spatial organization of cells within granulomas are significant yet underappreciated aspects of the biology of TB. Alveolar macrophages in cynomolgus macaques are
predominately CD11c+CD68+CD163+, but macrophage phenotypes in the granuloma are considerably more complex. CD163 expression delineated two macrophage subsets: CD11c+CD68+CD163+/CD68+CD163+ cells and CD68+CD163+ arginase+ macrophage cases. CD163 expression has been identified as a marker of alternative activation (M2) (51, 54), and the abundance of these cells in outer regions of granulomas are phospho-STAT3 positive (data not shown), an indicator of IL-10 signaling in alternatively activated macrophages (55, 56), and have a lower ratio of iNOS/Arg1 expression relative to epithelioid macrophages suggests these cells may not be actively bactericidal. In contrast, CD11c+CD68+CD163+ epithelioid macrophages are present in regions with the largest number of bacteria or bacterial Ags and had classically activated NOS-expressing phenotypes. HAM56, a reported marker for foamy macrophages (57–59), was also associated with cells at the epithelioid macrophage–caseum interface, a position similar to the location of Oil Red O–stained macrophages in human granulomas (2, 60). In addition to acting as mediators of L-arginine metabolism in granulomas, granuloma macrophages may be important sources of proinflammatory and anti-inflammatory cytokine production that may also influence how successful a granuloma is at containing bacterial replication and dissemination.

The epithelioid macrophage–caseum interface also contained significant numbers of neutrophils. The role of neutrophils in TB is controversial (61). Murine TB models indicate neutrophil infiltration is enabled by impaired adaptive immune responses and leads to increased pathology and accelerated disease progression (62), and a neutrophil transcriptional signature in human blood differentiated persons with active TB or latent infection (63). Similarly, granulomas from cynomolgus macaques with poorly controlled TB can contain large numbers of neutrophils (5), oftentimes in close proximity to bacilli. M. tuberculosis is resistant to neutrophil-mediated killing (61, 64), and neutrophils in the airways of TB patients commonly contain replicating M. tuberculosis (65). Moreover, neutrophil cytosol is rich in calprotectin, an antimicrobial peptide that is supportive of M. tuberculosis growth in vitro (66), further suggesting they are not restricting survival and replication. Moreover, the proximity of degenerating neutrophils with bacilli suggests it is also possible that these cells may provide a nutrient source for M. tuberculosis. Neutrophils in granulomas can express iNOS and stain positively for nitrotyrosine, demonstrating that they generate NO but it is unknown whether this influences bacterial containment. In addition to commonly considered neutrophil effector mechanisms, neutrophils can express cytokines including TNF, IL-1β, IL-12, and vascular endothelial growth factor (67, 68) and participate in cross-priming of CD8+ T cells (69), suggesting neutrophils may exert unappreciated and unknown effects on protection.

These data on macrophage phenotypes, neutrophil distribution, and immune function present a dynamic granuloma–scale picture of immune function in human and primate granulomas. Active lesions display a gradient of anti- and proinflammatory phenotypes, with anti-inflammatory CD163+ iNOS+Arg1high macrophages on outer margins and proinflammatory CD11c+CD68+CD163+ iNOS eNOS+Arg1low macrophages toward the center, thus making it possible to mount antibacterial responses safely away from uninvoluted tissue. Fibrocalcific granulomas retain aspects of this gradient, but the macrophage populations are biased toward anti-inflammatory phenotypes (CD68+CD163+), possibly due to lower numbers of bacilli in these granulomas (70). iNOS expression still occurs in these granulomas, suggesting some continued presence of antimycobacterial activity is required for controlling any bacteria remaining in these lesions. The concept of a cell and effector gradient is also supported by mathematical modeling (28). A better understanding of what constitutes a protective macrophage phenotype in a particular microenvironment and techniques to promote specific macrophage phenotypes may provide important avenues for immunotherapeutic treatment of TB.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Phenotypes of CD11c+, CD68+, CD163+, HAM56+ and calprotectin+ cells in macaque granulomas. Macrophage antigens in macaque granulomas were stained for macrophage antigens and imaged at 600x to show the representative staining patterns associated with each cell type. Scale bar represents 10 μm.

Supplemental Figure 2. Strong expression of calprotectin can be used to identify neutrophils in the peripheral blood and tissues of cynomolgus macaques. A. PBMCs from whole blood were centrifuged onto slides with a Cytospin (Thermo Scientific) and stained for CD3 (red), calprotectin (green) and CD163 (blue). Nuclei (grey) were stained with DAPI and the nuclear morphology was used to identify neutrophil-like cells having segmented nuclei. B. Quantification of CD3+, CD163+ and calprotectin+ cells indicates that neutrophil-like cells with segmented nuclei and calprotectin-positive cells are the most abundant cells in whole blood. C. Nearly all calprotectin-expressing PBMCs have segmented nuclei, suggesting these cells may be neutrophils. D. Percoll isolation of PBMCs from whole blood (inset) yields two fractions, a buffy coat rich in T cells and monocytes, and an RBC pellet enriched in calprotectin-expressing neutrophils that do not co-express CD163 (inset). Inset images show the enrichment for calprotectin+ cells in the RBC pellet after sedimentation Red - CD3+ T cells, green - calprotectin+ cells, blue – nuclei. E. CD163 and calprotectin-stained cells in granulomas demonstrate calprotectin+ cells have segmented nuclei whereas CD163+ cells have oval-shaped nuclei consistent with macrophage and monocyte nuclei. Arrows indicate cells with segmented nuclei. F. High frequencies of calprotectin+ cells have segmented nuclei when compared to CD163+ cells.
Supplemental Figure 2.

A. H&E staining of whole blood cytospins with cell markers, nuclei, and merge images.

B. Analysis of cells from whole blood cytospins:
- Segmented nuclei
- Calprotectin+
- CD3+
- CD163+
- Unstained

C. Calprotectin+ cells with segmented nuclei

D. Flow cytometry analysis:
- Whole blood
- RBC pellet
- SSC vs calprotectin
- CD163 vs calprotectin

E. Immunofluorescence images:
- Calprotectin (green)
- Nuclei (blue)
- Merge + CD163 (red)

F. Percentage of cells with segmented nuclei: