#### TUBERCULOSIS

# Immune correlates of tuberculosis disease and risk translate across species

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One quarter of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB). Although most infected individuals successfully control or clear the infection, some individuals will progress to TB disease. Immune correlates identified using animal models are not always effectively translated to human TB, thus resulting in a slow pace of translational discoveries from animal models to human TB for many platforms including vaccines, therapeutics, biomarkers, and diagnostic discovery. Therefore, it is critical to improve our poor understanding of immune correlates of disease and protection that are shared across animal TB models and human TB. In this study, we have provided an in-depth identification of the conserved and diversified gene/ immune pathways in TB models of nonhuman primate and diversity outbred mouse and human TB. Our results show that prominent differentially expressed genes/pathways induced during TB disease progression are conserved in genetically diverse mice, macaques, and humans. In addition, using gene-deficient inbred mouse models, we have addressed the functional role of individual genes comprising the gene signature of disease progression seen in humans with *Mtb* infection. We show that genes representing specific immune pathways can be protective, detrimental, or redundant in controlling *Mtb* infection and translate into identifying immune pathways that mediate TB immunopathology in humans. Together, our cross-species findings provide insights into modeling TB disease and the immunological basis of TB disease progression.

#### **INTRODUCTION**

The immune mechanisms that govern risk of progression from infection to tuberculosis (TB) disease remain poorly defined. Animal models have been used to characterize immune mechanisms of Mycobacterium tuberculosis (Mtb) infection outcomes (1). However, not all of the immune correlates identified using animal models have been translated to human studies (2). Similarly, several human blood transcriptomic signatures that differentiate asymptomatic *Mtb* infection and active TB disease have been reported (3–5). Using transcriptional profiling approaches, we recently described genes that were differentially expressed between progressors and nonprogressors in a well-characterized longitudinal adolescent cohort study (ACS) of South African individuals with Mtb infection, some of whom progressed to pulmonary disease (6, 7). We also identified a correlate of risk of TB signature (7), comprising a set of 16 genes (hereafter referred to as the "16-gene ACS signature"), which predicted onset of TB disease more than a year before disease

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was diagnosed. Most of these 16 genes were also differentially expressed between individuals with asymptomatic Mtb infection and TB cases (3–5), suggesting conserved biology during progression and clinical disease. However, a mechanistic understanding of the role of the immune pathways identified in the human transcriptomic studies has not been functionally probed using animal models.

Although animal models, especially mice, are used commonly to study mechanisms of immunity or inflammation in TB (1), disease outcomes seen in inbred mouse models do not fully reflect the heterogeneity observed in human TB (1). We and others have established a mouse model of TB in diversity outbred (DO) mice that demonstrate considerable heterogeneity in inflammatory and protective responses after Mtb infection, reflecting some of the genetic diversity found in humans (8, 9). In addition, the nonhuman primate (NHP) model of TB allows preclinical studies of *Mtb* infection and TB disease progression, as granulomas in rhesus macaques with TB disease recapitulate the morphology and physiology observed in human TB (10). Thus, using a combination of the DO Mtb-mouse model and the macaque model of TB, we recently identified that lung inflammation during TB correlated with increased accumulation of neutrophils and associated production of neutrophilic proteins S100A8/A9 (8). Increased S100A8/A9 protein concentrations in serum of patients with TB also correlated with increased disease severity (8). Thus, a combination of animal models and human clinical samples allowed us to functionally implicate neutrophils in mediating inflammation and disease severity in TB. However, the pace of such translational discoveries could be enhanced by a comprehensive and in-depth understanding of common immune correlates of protection and inflammation across relevant animal models and human TB. To address this gap, we evaluated whether relevant animal models of TB exhibit similar transcriptional profiles or expressed the 16-gene

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ACS signature identified in humans. By comparing transcriptional signatures in lung samples from a spectrum of DO mice infected with *Mtb*, macaques with TB, and blood transcriptomic profiles in patients with TB, we identified immune correlates of TB disease that are prominently shared across animal models and humans. In addition, using gene-deficient mice, we have addressed the functional role of individual genes comprising the 16-gene ACS signature (7).

#### RESULTS

### Common immune correlates of TB disease identified using analysis across animal models and human TB

We compared our existing ACS human blood global transcriptomic profile dataset (6, 7) to lung transcriptional profiles from animal models to identify common patterns of gene expression and biological pathways or mechanisms of immune control or disease of Mtb infection. Between 2005 and 2007, 6363 adolescents were enrolled in Western Cape, South Africa, a setting with a TB incidence of ~1%, and followed up for 2 years (11, 12). Among those with evidence of Mtb infection (tuberculin skin test-positive and/or QuantiFERON Gold In-Tube-positive), 46 adolescents developed culture and/or sputum smear-positive, intrathoracic TB (termed progressors), and we selected 107 matched adolescents by age at enrollment, sex, ethnic origin, school of attendance, and presence or absence of previous episodes of TB disease and remained healthy (termed controllers). We measured mRNA expression by RNA sequencing (RNA-seq) in whole blood ex vivo from progressors and controllers (table S1) and investigated the biological pathways associated with progression to TB disease (table S2). For animal studies, mouse lung samples from DO mice that were left uninfected (naïve), individual DO mice that controlled Mtb infection (controllers), or individual DO mice that progressed to severe TB infection (progressors; Fig. 1A) were included. In the DO infected mice, phenotypic clinical observations were used to determine the severity of the infection. The samples were divided into controller and progressor groups based on a "TB severity score" assessed at euthanasia (log10 bacterial burden of the lung tissue sample)  $\times$  (log<sub>10</sub> percentage lung inflammation, by area), using thresholds of <6.5 and >8.5, respectively (Fig. 1B). These conservative thresholds excluded 15 samples as not classifying as progressor or controller types ("Both" in Fig. 1B), with the rest clustering as either progressors or controllers. Thus, the naïve, controller, and progressor mouse samples were subjected to RNA-seq analysis, and the distinct clustering is shown in Fig. 1C.

Lung samples from uninfected macaques (naïve), macaques that were classified as asymptomatic TB (controllers), or macaques that exhibited clinical symptoms and TB disease (progressors; Fig. 1A) were also analyzed by RNA-seq and showed distinct clustering (Fig. 1D). Macaque lungs from progressors were collected 5 to 8 weeks after *Mtb* infection, whereas those from controllers were collected 22 to 24 weeks after *Mtb* infection.

We performed pairwise differential gene expression analysis between each species group including mice and macaques (Fig. 2, A and B). Adequate power and consistent expression patterns allowed for the identification of significantly differentially expressed genes [using DEseq2 (13) with default settings,  $P \le 0.05$ after FDR correction] in DO mice (fig. S1A) and in macaques (fig. S1B). The key difference between mice and macaques was that, in the latter, the controllers and the naïve groups expressed relatively similar transcriptional profiles. As a result, a smaller number of differentially expressed genes (n = 184) were identified between the controllers and the naïve group in macaques (fig. S1B). Genes were then matched between mouse, macaque, and human using Ensembl (14) orthology matches; 17,044 macaque protein-coding genes and 18,658 human protein-coding genes were matched to the 22,005 proteincoding mouse genes. Differences in expression values of genes between human progressors and controllers from the human progressor study (7) were also aligned to the dataset, facilitating the identification of overlapping, differentially expressed orthologous genes across the three species. The results are presented in Fig. 2A (identifying genes consistently higher in controllers versus both progressors and naïve), fig.S1C (lower gene expression in controllers versus both progressors and naïve groups), fig. 2B (higher expressed in progressor groups versus both controllers and naïve), and fig. S1D (lower expression in progressors versus both controllers and naïve). We further defined genes expressed higher and lower in progressors compared to both naïve and controllers in mice and macaques in relation to human gene expression. These results together define transcriptional profiles from Mtb-infected DO mice and macaques that demonstrate conserved biology in disease processes that overlap with human TB disease. We observed significant overlaps of differentially expressed genes in each of these comparisons across species  $[P < 10^{-10}]$ , false discovery rate (FDR)-corrected negative binomial distribution tests; table S3], with the exception of the macaque comparisons involving controller versus naïve (due to the minimal differences observed for this, as discussed above), and a significant overlap for macaque controller versus progressor and the human negative controller–associated comparison ( $P = 2.0 \times 10^{-4}$ ).

Many of the most significant pathways that were expressed more abundantly in controller versus progressor samples in animal models were associated with lung tissue repair after injury (Fig. 2C) such as bone morphogenetic protein signaling pathways (15), insulin-like growth factor 1 receptor (16), and fibroblast growth factor receptor (17). Although no enriched pathways were identified among the 109 of 777 genes that are higher in controller versus progressor in all three species (Fig. 2C and table S2), there are several genes related to cellular repair including (i) latent transforming growth factor beta binding protein 4 (*LTBP4*) (18), (ii) myosin IXA (*MYO9a*) (19), and (iii) v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ERB2*), required for epithelial cell repair after neutrophil elastase exposure (20). These data suggest that lung tissue damage is being actively repaired in controllers, without triggering a substantial inflammatory response.

Many general immune response pathways including those linked to inflammation and lung damage were associated with disease progression in all three species (Fig. 2D). Among the top progressionassociated pathways is "neutrophil degranulation," which is consistent with the role of neutrophils in TB pathology (8, 21-23) and the prominence of neutrophils in published TB disease gene signatures (3).

There also are enriched pathways related to cytokines and chemokines, which initiate and coordinate immune cell recruitment and activation in *Mtb*-infected lungs (24). Overall, the pathways associated with progressors suggest an active and broad inflammatory response, with the most significantly enriched pathway ( $P = 3.9 \times 10^{-11}$ ) being neutrophil pathways.

Upon further analysis, we identified 10 genes with significantly higher expression in controllers across species ( $P \le 0.05$  for the comparisons in each species), which suggests possible immune



Fig. 1. Overview of the samples used and the computational analysis. (A) Workflow of cross-species immune correlates of TB disease. (B) Mouse samples were divided into progressor and controller sample groups based on (log<sub>10</sub> bacterial burden) × (log<sub>10</sub> percentage lung inflammation), using thresholds of <6.5 and >8.5, respectively. (C) PCA clustering of the mouse RNA-seq samples based on the default DESeq2 method (top 500 most variable genes) and (D) PCA clustering of the macaque RNA-seq samples.

parameters that may limit TB disease. This did not take into consideration controllers versus naïve groups in macaque, because there was minimal difference in their gene signatures (Fig. 3A). Among this set are the genes, PH domain and leucine-rich repeat protein phosphatase 2 (*PHLPP2*), which dephosphorylates AKT1 (25), and HECT and RLD domain containing E3 ubiquitin protein ligase family member 1 (*HERC-1*), which regulates the ERK signaling pathway (26), suggesting a potential mechanism by which ERK signaling is altered during TB infection to promote *Mtb* control. In comparison, there were 13 genes that were consistently lower in controllers across species (Fig. 3B). Among these are *SCARF1*, one

of the genes in the 16-gene ACS signature previously identified to be up-regulated during TB infection in humans (7), and intercellular adhesion molecule 1 (*ICAM1*).

Of the 837 genes more highly expressed in progressor versus controller and naïve groups in both mouse and macaque, the top 19 genes also included 9 of the 16 genes in the 16-gene ACS signature, including *BATF2*, *Etv6* (the mouse ortholog of human *ETV7*), *FCGR1*, *GBP2*, *GBP2B*, *GBP3*, *GBP5*, *STAT1*, and *TRAFD1* (Fig. 3C). Among the remaining 10 of the top 19 genes, several were interferon (IFN)–stimulated genes, namely, IFN-induced protein with tetratricopeptide repeats 2 (*IFIT2*). A total of 777 genes had lower expression





Reactome pathway	# Genes			genes)			Reactome pathway	# Genes		(257 gene	es) 1
			1	2	3	4	Immuno system (169256)	00		i	_
Signaling by BMP (201451)	9			-			Inimule system (166256)	39		_	-
Import of palmitoyl-CoA into the mitochondrial matrix (200425)	7			_			Innate Innune system (166249)	11		_	=
IRS-related events triggered by IGF1R (2428928)	11						Neutrophil degranulation (6798695)	41		_	=
IGF1R signaling cascade (2428924)	11						Antigen processing-cross presentation (1236975)	17		_	-
Signaling by type 1 IGF1R (2404192)	11						Adaptive immune system (1280218)	38		_	
Insulin receptor signaling cascade (74751)	11						Cytokine signaling in immune system (1280215)	27			
PI3K cascade (109704)	10		_				Signaling by interleukins (449147)	23		-	
IRS-mediated signaling (112399)	10						Class I MHC-mediated antigen processing & presentation (983169)	23			
Signaling by receptor tyrosine kinases (9006934)	35						C-type lectin receptors (CLRs) (5621481)	12			
Signaling by insulin receptor (74752)	12						Interleukin-1 family signaling (446652)	12			
Signaling by TGF-β family members (9006936)	13						ER-phagosome pathway (1236974)	7			
Downstream signaling of activated FGFR2 (5654696)	8		_				Fc epsilon receptor (FCERI) signaling (2454202)	12			
Downstream signaling of activated FGFR3 (5654708)	8		_				TCR signaling (202403)	11			
Downstream signaling of activated FGFR4 (5654716)	8						Antigen presentation: Folding, assembly and peptide (983170)	7			
Negative regulation of the PI3K/AKT network (199418)	14						CLEC7A (Dectin-1) signaling (5607764)	10			
Signaling by FGFR4 (5654743)	9						Interleukin-1 signaling (9020702)	10			
Axon guidance (422475)	25						FCERI mediated NF-kB activation (2871837)	9			
Signaling by FGFR3 (5654741)	9						Interleukin-4 and interleukin-13 signaling (6785807)	5			
Downstream signaling of activated FGFR1 (5654687)	8		_				Classical antibody-mediated complement activation (173623)	4			
PI-3K cascade:FGFR2 (5654695)	6						Initial triggering of complement (166663)	5			
Signaling by FGFR1 (5654736)	9		_	]			Toll-like receptor cascades (168898)	12			
Tight junction interactions (420029)	4			]			Cross-presentation of soluble exogenous antigens (1236978)	7			
FGFR2b ligand binding and activation (190377)	4			]			Endosomal/vacuolar pathway (1236977)	5			
Rho GTPase cycle (194840)	15						Creation of C4 and C2 activators (166786)	4			
Intracellular signaling by second messengers (9006925)	22						Interleukin-27 signaling (9020956)	4			
PI-3K cascade:FGFR3 (5654710)	5						Interleukin-6 signaling (1059683)	4			
Cell-cell junction organization (421270)	8						Regulation of IFNG signaling (877312)	4			
SUMOylation of intracellular receptors (4090294)	6						TNFR2 noncanonical NF-KB pathway (5668541)	9			
PI5P, PP2A, and IER3 regulate PI3K/AKT signaling (6811558)	12						Dectin-1-mediated noncanonical NF-KB signaling (5607761)	7			
Signaling by EGER2 (5654738)	10						NIK>noncanonical NE-KB signaling (5676590)	7			

Fig. 2. Conserved differentially expressed genes in controllers and progressors. The counts of overlapping significantly differentially expressed genes between mouse, macaque, and human genes (7) are shown for (A) genes higher in controller and (B) genes higher in progressor. Shaded areas represent gene sets used for Reactome enrichment testing, as shown in (C) for genes higher in controller and in (D) for genes higher in progressor. \*Gene sets visualized in Fig. 3. TCR, T cell receptor; IRS, insulin receptor substrate; ER, endoplasmic reticulum.

in progressors in both animal models versus both naïve and controller samples (the top 10 lower expressed genes are listed in Fig. 3D). The most significantly expressed genes among these include (i) phospholipase C (PLCE1) and (ii) glypican 3 (GPC3), which regulates macrophage recruitment into tissues (table S3) (27). Collectively, our results suggest that highly expressed genes in Mtb-infected DO mouse and macaque recapitulate the IFN-inducible gene expression pattern previously reported in human TB progressors (3).

#### The previously identified human 16-gene ACS signature is enriched in progressors across animal models

We analyzed whether genes in the previously identified human 16-gene ACS signature were enriched in mouse and macaque progressors. Of the 16 genes, 13, including BATF2, Etv6 (the mouse ortholog of human ETV7), FCGR1, SERPING1, GBP family members (p65 guanylate-binding proteins GBP1, GBP2, GBP4, and GBP5), STAT1, TRAFD1, TAP1 (macaque ortholog absent), APOL1 (mouse ortholog absent), and ANKRD22 (mouse ortholog absent), were expressed more highly in progressors than in naïve groups in both mouse and macaque DESeq2 (13) results (Fig. 4A). In addition, expression of these 11 genes in mice was also increased in controllers compared to the naïve group, suggesting a potential role in controlling Mtb infection (Fig. 4A). Expression of 14 genes was increased in macaque progressors but not in controllers, relative to the naïve group (Fig. 4, A to C). Of interest was SCARF1, which is involved in apoptotic cell clearance (28). Scarf1 gene expression in mice was lower in controllers

-Log FDR-adjusted P value

10

		Z score of expression      TB severity score        Relative gene expression per sample      <-1 -1      0      1      >1      >2      0      5      10      15      20						
	Mouse gene	Meuroe						
		Naive	Controller	Progressor	Naive	Con-	Progressor	
Α	POGZ Pogo transposable element with ZNF domain					troller	i regreeeer	
	MYO9A  Myosin IXa    PHLPP2  PH domain and leucine-rich phosphatase    TBC1D32  TBC1 domain family, member 32    RALGAPA1  Ral GTPase activating protein, alpha subunit 1    SUPT3  suppressor of Ty 3    MGA  MAX gene associated    HERC1  HECT and RLD domain containing E3 ubiquitin    LRBA  LPS-responsive beige-like anchor    ZFP182  Zinc finger protein 192	8					l,	
В	HIST2H2BB    Histone cluster 2, H2bb      PRAM1    PML-RAR alpha-regulated adaptor molecule 1      ICAM1    Intercellular adhesion molecule 1      KCTD12    Potassium channel tetramerisation domain      NUB1    Negative regulator of ubiquitin-like proteins 1      H2AFJ    H2A histone family, member J      FOLR2    Folate receptor 2 (fetal)      H3F3A    H3 histone, family 3A      DNTTIP1    Deoxynucleotidyltransferase, terminal, interacting      LTBR    Lymphotoxin B receptor      MRPL23    Midochondrial ribosomal protein L23      MFSD148    Major facilitator superfamily domain conatining 14B      SCARF1    Scavenger receptor class F, member 1							
C	BATF2    Basic leucine zipper transcription factor, ATF-like 2      ETV6    ets variant 6      FCGR1    Fc receptor, IgG, high affinity I      GBP2B    Guanylate binding protein 2b      GBP2    Guanylate binding protein 3      GBP3    Guanylate binding protein 3      GBP5    Guanylate binding protein 5      STAT1    Signal transducer and activator of transcription 1      TRAFD1    TRAF type zinc finger domain containing 1      IFIT2    Interferon-induced protein with tetratricopeptide repeats 2      C1RA    Complement component 1, r subcomponent A      CASP7    Caspase 7      LAP3    Leucine aminopeptidase 3      PSMB8    Proteasome (prosome, macropain) subunit, beta type 8      GCH1    GTP cyclohydrolase 1      SNX10    Sorting nexin 10      MLKL    Mixed lineage kinase domain-like      NMI    N-myc (a							
D	PLCE1    Phospholipase C, epsilon 1      GPC3    Glypican 3      OSGEPL1    O-sialoglycoprotein endopeptidase-like 1      MUM1    Melanoma associated antigen (mutated) 1      MYRF    Myelin regulatory factor      WDR35    WD repeat domain 35      IFT172    Intraflagellar transport 172      LRRC1    leucine-rich repeat containing 1      PTPDC1    Protein tyrosine phosphatase domain containing 1      PPP1R9A    Protein phosphatase 1, regulatory (inhibitor) subunit 9A							

Fig. 3. Differentially expressed genes that are significantly different in TB controllers across species. (A) Higher in controller versus progressor and naïve in mouse and higher in controller versus progressor in macaque and human; (B) lower in controller versus progressor and naïve in both mouse and macaque; (C) higher in progressor versus controller and naïve in both mouse and macaque and higher in progressor versus controller in mouse (includes ACS signature genes and top 10 most significant other genes); (D) lower in progressor versus controller and naïve in both mouse and macaque and lower in progressor versus controller in mouse (top 10 most significant are shown). IgG, immunoglobulin G.

than in naïve but induced in progressors (Fig. 4B). Similarly, the expression of *SCARF1* in macaques was higher in progressors than in naïve and controllers (Fig. 4B), as also observed in humans (6, 7). Another gene of interest was *SEPT4*, which belongs to a family of nucleotide binding proteins involved in cytoskeletal orga-

nization (29). Whereas SEPT4 expression was induced in macaque progressors when compared with naïve or controllers as observed in human TB progressors, the reverse was true in mice where Sept4 expression was lower in controllers and further decreased in progressors (Fig. 4C).

In addition to analyzing the differences between the groups, the correlation of the gene expression of these ACS signature genes was compared to the percentage lung inflammation and the bacterial burden (table S4) across the controller and progressor samples (n = 29 for mouse, n = 12 for macaque). Significant correlations  $(P \le 0.05, after FDR correction)$  were observed for all ACS signature genes in macaque and in all genes except for SCARF1 and bacterial burden in mouse (P = 0.07), although it was significantly correlated with lung inflammation (P = 0.002). In both mouse and macaque, the ortholog of Fc Fragment Of IgG Receptor Ia (FCGR1a)/FCGR1b was the most significantly associated with the metadata ( $P = 4.9 \times$  $10^{-5}$  with lung inflammation and  $P = 8.3 \times 10^{-5}$  with bacterial burden in mouse,  $P = 1.2 \times 10^{-6}$  with lung inflammation and  $P = 1.8 \times 10^{-6}$ 10<sup>-14</sup> with bacterial burden in macaque). In macaques, correlations were higher with bacterial burden than with lung inflammation in all genes except GBP4 and SEPT4, although this was only true for half of the mouse genes. These correlation results suggest that the expression of these ACS signature genes correlates with disease severity even within the controller and progressor groups. Collectively, our results suggest that the majority of human immune pathways induced in blood during TB disease progression are similarly induced in the lungs of the animal models in this study. However, pathways involving Sept4 may function differentially in TB disease in mice when compared with macaques or humans. Thus, our studies provide an in-depth understanding of the conserved and diversified immune pathways in animal models and human TB.

#### Delineating the functional relevance of genes in the 16-gene ACS signature using gene-deficient mouse models

Using the well-established inbred mouse model of TB, we conducted mechanistic studies to delineate the functional relevance of genes in the 16-gene ACS signature that were increased in progressors in mice, macaques, and humans. Of the 16 human genes, APOL1 and ANKRD22 lack a mouse ortholog, and FCGR1a and FCGR1b only have one mouse ortholog (Fcgr1), leaving 13 potential genes for study. *Etv6<sup>-/-</sup>* mice are not viable and die between days 10 and 11 of embryonic development (30) and could not be evaluated. Therefore, we focused on the remaining 12 genes in this study. Accordingly, Scarf1<sup>-/-</sup>, Stat1<sup>-/-</sup>, Sept4<sup>-/-</sup>, Tap1<sup>-/-</sup>, Trafd1<sup>-/-</sup>, Batf2<sup>-/-</sup>, Serping1<sup>-/-</sup>, and  $Fcgr1^{-/-}$  mice and mice deficient for five murine GBP genes located on chromosome 3 (*Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and *Gbp7''*), namely, GbpChr3<sup>-/-</sup>, were inoculated with low doses of aerosolized Mtb clinical W-Beijing strain, HN878. At acute [30 days postinfection (dpi)] and chronic stages (60 dpi or more) of Mtb infection, lungs were harvested, and accumulation of immune cells, disease inflammation in the lungs, and Mtb burden were measured. In these studies, lung cellular immune responses such as CD4<sup>+</sup>, CD8<sup>+</sup>, accumulation of myeloid dendritic cells, alveolar macrophages, monocytes, recruited macrophages, and neutrophils were all enumerated and are shown in table S5. Only cellular responses in gene-deficient mice that were significantly different from control Mtb-infected control mice are included in the figures below. On the basis of the Mtb burden in the lung during acute, chronic, or both stages of infection, we classified the gene-deficient mice into three groups: (i) gene-deficient mice that did not exhibit any effects on Mtb control, suggesting that these genes do not contribute to protective outcomes in TB, at least in mice; (ii) gene-deficient mice that were more susceptible to Mtb infection and sustained higher Mtb burden, suggesting that these genes are important for

protection; and (iii) gene-deficient mice that were resistant to *Mtb* infection and had decreased burden in the lung, suggesting that these genes were involved in mediating disease progression and TB susceptibility.

### Serping1 of the 16-gene ACS signature mediates no effect on Mtb control in mice

SERPING1 (serpin family G member 1) is a highly glycosylated plasma protein that inhibits activated C1r and C1s and, thus, negatively regulates complement activation (31). Although SERPING1 mRNA expression is up-regulated in Mtb-infected lungs (32, 33), its contribution to Mtb control and inflammation has not been assessed. Mtb-infected wild-type C57BL/6 mice expressed Serping1 mRNA within granulomas (fig. S2A). Upon Mtb infection, no differences in lung bacterial burden were observed between Serping1<sup>-/-</sup> and wild-type mice with no major differences in myeloid or T cell populations, except recruited macrophages at 30 dpi (fig. S2B and table S5). In contrast, we observed more inflammation in the lungs of Serping  $1^{-/-}$  mice than wild-type mice at multiple time points (fig. S2B), which may be due to the activation of the complement cascade (34, 35) in the absence of Serping1. Thus, Serping1 had no effect on Mtb control yet still regulated inflammation. Although no role in Mtb control was observed, a deficiency of Serping1 led to dysregulation of inflammation.

### Genes in the 16-gene ACS signature that mediate protective responses in mice

Of the gene-deficient mice tested, those with an absence of Stat1, Tap1, Gbps, Trafd1, and Sept4 displayed increased Mtb susceptibility. STAT1 is critical for type I, II, and III IFN signaling pathways (36), and mice deficient in Stat1 are extremely susceptible to Mtb (37). Accordingly,  $Stat1^{-/-}$  mice exhibited high lung Mtb burden during acute infection and severe lung inflammation, when compared with control Mtb-infected C57BL/6 mice (Fig. 5A). TAP1 (Transporter associated with Antigen Processing 1) facilitates loading of peptide antigens onto major histocompatibility complex (MHC) class I molecules for presentation to CD8<sup>+</sup> T cells, and Tap1<sup>-/-</sup> mice exhibit defects in MHC class I antigen presentation (38, 39). We found a significant increase in lung Mtb burden of  $Tap1^{-/-}$  mice during the acute and chronic stages of infection (Fig. 5B) and decreased numbers of activated CD8<sup>+</sup> T cells compared to infected wild-type mice (Fig. 5B and table S5). However, differences in the cytokine secretion profile of CD8<sup>+</sup> but not CD4<sup>+</sup> T cells were observed in  $Tap1^{-/-}$  mice when compared with controls (fig. S3, A and B).  $Tap1^{-/-}$ mice also had no changes in accumulation of neutrophils and recruited macrophages (fig. S3C) but exhibited decreased accumulation of alveolar macrophages at acute (30 dpi) phase of infection (fig. S3, D and E). Consistent with the increased lung Mtb burden, greater lung inflammation was observed in  $Tap1^{-/-}$  than in wild-type mice at all the time points assessed (Fig. 5B). GBPs are IFN-inducible proteins with documented key antiviral and antibacterial functions (40, 41). The mouse genome has 13 Gbp genes (2 of which are pseudogenes) organized in specific clusters on chromosomes 3 and 5 (42). To elucidate the contribution of the GBPs to host immunity to Mtb, we used mice lacking the entire cluster of Gbps on chromosome 3 (GbpChr3<sup>-/-</sup>). In contrast to results with Mycobacterium bovis BCG infection in  $Gbp1^{-/-}$  mice (41),  $GbpChr3^{-/-}$  mice demonstrated increased susceptibility only during chronic infection and not during the acute phase, and this coincided with decreased accumulation of lung neutrophils and recruited macrophages, without affecting inflammation (Fig. 5C). TRAFD1 (TRAF-Type Zinc



Fig. 4. Expression of the 16-gene ACS signature genes across species. (A) Ortholog gene expression of 13 of 16 human ACS signature genes (7) in progressor versus controller, progressor versus naïve, and controller versus naïve in mouse and macaque. Note that both *FCGR1A* and *FCGR1B* are represented by a single gene in mice and macaques, and human *ETV7*'s ortholog in mouse is called *Etv6*. (B) *SCARF1* expression in progressor and controller versus naïve in mouse and macaque. All *P* values shown on the expression swarm plots represent FDR-corrected significance values for differential expression calculated by DESeq2.



**Fig. 5. Genes in the 16-gene ACS signature that mediate protective immune responses during infection.** Control or gene-deficient mice were infected with *Mtb* HN878 (100 CFU) by aerosol route. On 30, 60, and 100 days postinfection (dpi), lungs were harvested to assess bacterial burden by plating or inflammatory cell infiltration by flow cytometry or histologically by H&E staining in (**A**)  $Stat1^{-/-}$  mice, (**B**)  $Tap1^{-/-}$  mice, (**C**)  $GbpChr3^{-/-}$  mice, (**D**)  $Trafd1^{-/-}$  mice, and (**E**)  $Sept4^{-/-}$  mice, and relevant controls are shown. The data points represent the means (±SEM) of one of two separate experiments. Significance for bacterial burden analysis for C57BL/6 and genedeficient mice was determined using unpaired two-tailed Student's *t* test at different dpi.

Finger Domain Containing 1) belongs to the tumor necrosis factor (TNF) family–like genes and is a negative feedback regulator of the Toll-like receptor (TLR) signaling pathway, which results in the control of excessive immune responses (43, 44). Similar to  $GbpChr3^{-/-}$  mice,  $Trafd1^{-/-}$  mice displayed increased susceptibility only during chronic infection and not during the acute phase (Fig. 5D). This susceptibility coincided with a significant decrease (P = 0.001) in neutrophil accumulation at 60 dpi, without affecting recruited macrophage accumulation (Fig. 5D). Unexpectedly, we observed a decrease in lung inflammation in  $Trafd1^{-/-}$  mice during acute and chronic infections (Fig. 5D). Trafd1 deficiency is known to regulate type I IFN responses (43), which could have an impact on the reduced inflammation observed.

Sept4 originally was described in yeast as a cell division cycle regulatory protein (45). Upon *Mtb* infection, *Sept4<sup>-/-</sup>* mice showed enhanced susceptibility to *Mtb* at early stages, which correlated with reduced accumulation of lung recruited macrophages but not neutrophils and increased inflammation, but this was not maintained during chronic stages of infection (Fig. 5E). Together, our results show that *Stat1* and *Tap1* contribute to protection through acute and chronic stages, whereas *Gbps*, *Trafd1*, and *Sept4* have disease stage–dependent protective roles during *Mtb* infection.

### Genes in the 16-gene ACS signature that mediate detrimental effects during infection of mice

Of the 16-gene ACS signature genes analyzed for functional relevance in mice, we identified three genes (Batf2, Fcgr1, and Scarf1) that contribute to *Mtb* susceptibility at distinct phases of infection. BATF2(Basic Leucine Zipper ATF-Like Transcription Factor 2) is a basic leucine zipper transcription factor that is closely related to BATF and BATF3 (46), which together regulate dendritic cell differentiation and development.  $Batf2/3^{-/-}$  mice were more resistant to Mtbinfection and unexpectedly showed improved Mtb control upon infection during the transition from acute to chronic stages at 60 and 100 dpi (Fig. 6A). Because  $Batf3^{-/-}$  mice are more susceptible to Mtb infection at 60 dpi, improved Mtb control likely depends on the loss of Batf2 expression (fig. S4), as also suggested recently (46). The increased resistance to Mtb infection was associated with diminished neutrophil and recruited macrophage accumulation in the lungs at 60 dpi (Fig. 6A). However, the enhanced protection against Mtb infection observed in the lungs of  $Batf2/3^{-/-}$  mice was associated with enhanced proportion of CD4<sup>+</sup> interleukin-17-positive (IL-17<sup>+</sup>) cells and CD4<sup>+</sup> IL-17<sup>+</sup> IFN $\gamma^+$  cells (fig. S5). *Batf2/3<sup>-7-</sup>* mice demonstrated increased lung inflammation at 60 and 100 dpi (Fig. 6A), suggesting that although Batf2 expression was detrimental to Mtb control, its expression limits inflammatory responses during Mtb infection, possibly mediated by T helper 17 ( $T_H$ 17) cells.

FCGR1 inactivation affects nitric oxide production by neutrophils (47), cytokine responses and antigen presentation (48, 49), and antibody-dependent killing of pathogens by macrophages (50). We used  $Fcgr1^{-/-}$  mice to assess the functional significance of this gene in protective immunity to *Mtb*. We observed improved *Mtb* control in the lungs of  $Fcgr1^{-/-}$  mice compared to the control mice at 60 dpi (Fig. 6B). The decrease in lung *Mtb* burden in  $Fcgr1^{-/-}$  mice was associated with reductions in neutrophils, but not in recruited macrophages, in the lung at both 60 and 100 dpi (Fig. 6B) but no major effect on inflammation in the lung tissue at 60 dpi (Fig. 6B), which coincided with increased IFN $\gamma$ /IL-17 coproducing CD4<sup>+</sup> T cell during early disease (fig. S6). Thus, both *Batf2* and *Fcgr1* are detrimental for *Mtb* control but likely required to limit inflammation in the lung.

SCARF1 is involved in the uptake of modified low-density lipoprotein (LDL) or acetylated LDL (51, 52). We assessed the functional relevance of this gene for *Mtb* using *Scarf1<sup>-/-</sup>* mice. We observed a significant reduction (P = 0.002) in bacterial burden in the lungs of  $Scarf1^{-/-}$  mice during the chronic phase (120 dpi) but not earlier (Fig. 6C). This was associated with reduced accumulation of neutrophils but not of recruited macrophages in the lungs of Scarf1<sup>-/-</sup> *Mtb*-infected mice compared to wild-type *Mtb*-infected mice (Fig. 6C). Although Scarf1 has been associated with clearance of apoptotic cells by macrophages (28) and impaired apoptotic cell clearance is associated with chronic inflammation (53), we observed reduced inflammation in the lungs of  $Scarf1^{-/-}$  mice (Fig. 6C). To further characterize the functional role of Scarf1 in TB, we performed RNA-seq profiling of lung tissue from C57BL/6 mice and Scarf1<sup>-/-</sup> mice at 120 dpi, the time point at which  $Scarf1^{-/-}$  mice exhibited better Mtb control and reduced inflammation. Differential gene expression analysis identified 218 higher and 394 lower expressed genes in infected Scarf1<sup>-/-</sup> compared to infected wild-type mice (table S6). While there was no functional enrichment among the 218 induced genes (Fig. 6D), analysis of the reduced genes suggested that Scarf1 might affect neutrophil-related and other downstream immune signaling pathways.

To investigate whether similar biological pathways may be associated with SCARF1 in humans, we sought to identify plasma proteins quantified in a previous study (6, 54) that correlate with SCARF1 mRNA abundance in ACS progressors and controllers. Because soluble mediators of immune responses produced in inflamed tissues more readily enter the blood than intracellular RNAs, we hypothesized that plasma protein concentrations more likely reflect Mtb disease in the lungs than transcriptomic data from blood leukocytes. Pearson correlation coefficients were calculated for SCARF1 mRNA abundance in whole blood (measured by RNA-seq) and proteomics abundance data for 2872 plasma proteins (measured with SOMAscan, a highly multiplexed proteomic assay) (6, 54) across 311 samples from 30 progressors (65 samples) and 102 nonprogressors (209 samples). These Pearson correlations per protein were used as a ranked input list for gene set enrichment analysis (GSEA) (55), performed using WebGestalt (56) to identify Reactome (57) pathways enriched among the highest- or lowest-ranking proteins. Because the GSEA considers the ranked positions of proteins within the list rather than the total number, any functional bias among the 2872 proteins relative to the entire proteome will not affect the results. This analysis identified four significantly enriched pathways with SCARF1 gene expression in humans: neutrophil degranulation, IL-10 signaling (comprising primarily innate cytokines and chemokines), the complement cascade, and the innate immune system (Fig. 6E and table S6). Genes in the neutrophil degranulation and innate immune system pathways were enriched as down-regulated genes in Mtb-infected Scarf1<sup>-/-</sup> mice when compared to infected wild-type control mice (table S6). This coincided with significantly decreased (P = 0.0007) IL-10 production in Mtb-infected Scarf1<sup>-</sup> macrophages when compared with C57BL6 Mtb-infected macrophages, whereas other cytokines such as TNFa levels were comparable between B6 and  $Scarf1^{-/-}$  macrophages (Fig. 6F). These data align with the human proteomics data, although genes in the IL-10 signaling pathway were not enriched among down-regulated genes in the  $Scarf1^{-/-}$  mice (table S6). Compared to the well-defined large granulomas with enhanced collagen expression in the lungs of infected wild-type mice, the lung granulomas in Scarf1<sup>-/-</sup> mice were Fig. 6. Genes in the 16-gene ACS signature that mediate detrimental effects during infection. Wild-type or genedeficient mouse strains were infected with Mtb HN878 (100 CFU) by aerosol route. On 30, 60, 100, and 120 dpi, lungs were harvested to assess bacterial burden by plating, or inflammatory cell infiltration by flow cytometry, or histologically by H&E staining in (A)  $Batf2/3^{-/-}$  mice, (**B**) *Fcqr1<sup>-/-</sup>* mice, (**C**) *Scarf1<sup>-/-</sup>* mice, and relevant controls. (D) DESeq2 differential gene expression comparisons identified in infected Scarf1<sup>-/-</sup> mice compared to wild-type mice at 120 dpi. ECM, extracellular matrix; MET, MET receptor tyrosine kinase. (E) Gene set enrichment analysis (GSEA) for Reactome pathways significantly enriched among human proteins with high correlation to SCARF1. Enrichment plots are shown for each of the terms, showing the relative rank of genes from the enriched pathways (across the x axis), and (F) IL-10 and TNFa production was measured in culture supernatants from uninfected and Mtb HN878-infected bone marrow-derived macrophages (MOI, 1) from C57BL/6 and *Scarf1<sup>-/-</sup>* mice after 2 dpi. The data points represent the means (±SEM) of one of two separate experiments. Bacterial burden in gene-deficient mice compared to control mice and cytokine production from macrophages were performed using unpaired two-tailed Student's t test at different dpi.

not well formed, with little to no collagen expression (fig. S7, A and B). *Mtb*-infected wildtype C57BL/6 mice ex-



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pressed *Scarf1* mRNA within granulomas (fig. S7C). Furthermore, Scarf1 protein localized to granulomas within the lungs of *Mtb*infected wild-type mice and progressor macaques (fig. S7D). Five of the 394 genes lower in the *Scarf1<sup>-/-</sup>* mice also overlapped with the 17 proteins correlated with SCARF1 expression in humans (IL-1 $\beta$ , tissue inhibitor of metalloproteinase 1, IL-1 receptor antagonist, TNF receptor superfamily member 1a, and prostaglandinendoperoxide synthase 2) (table S6). Together, these results implicate SCARF1 as a key regulator of the innate immune response that possibly can contribute to immunopathology and tissue destruction during the chronic stages of TB disease in mice and humans.

#### DISCUSSION

The pace of translational discoveries from animal models to human TB for many platforms including vaccines, therapeutics, biomarkers, and diagnostics is suboptimal because of a poor understanding of shared immune correlates of disease and protection across species. In this study, we provide an in-depth identification of the conserved and diversified immune pathways in animal models and human TB. Our results suggest that most of the prominent differentially expressed genes induced during TB disease progression are conserved in mice, macaques, and humans. However, some pathways ([e.g., Septin 4 (SEPT4)]) may function differently in TB disease in mice or be regulated differentially when compared with macaques or humans. Using the well-established inbred mouse model of TB, our mechanistic studies begin to define the functional relevance of a suite of genes strongly associated with disease progression (6, 7, 58) and TB (4, 5) in humans. We classified the gene-deficient mice infected with Mtb into groups that were more susceptible to Mtb infection, suggesting that these host genes contribute to protection. In addition, gene-deficient mice that had increased resistance to Mtb infection suggest that these genes likely mediate disease progression and TB susceptibility. One such pathway is the Scarf1 pathway, which we identified as a driver of TB immunopathogenesis across species. Our studies in mice also identified genes that did not exhibit any direct effects on *Mtb* control but regulated inflammation. Last, we identified that genes expressed during control of Mtb infection across species are related to the repair of lung injury. Together, these results identified cross-species correlates of disease and annotated the functional role of specific genes within the 16-gene ACS signature. These findings may allow more effective use of appropriate animal models for translation to human TB.

Animal models are critical for testing vaccines and therapeutics for clinical development for human use. The two most widely used animal models of TB are mice and NHPs. Although the former are most extensively used, the latter are universally accepted to recapitulate key aspects of the human TB disease. However, some cellular phenotypes and immune signaling events occurring in uninfected hosts are different between mice, NHPs, and humans (59). Animal models have been predictive of some human mechanisms of immunological protection, such as the roles of the cytokines IFN $\gamma$  and TNF $\alpha$ in protective immunity to TB (60-62). Mice deficient in IFNy and TNF $\alpha$  are vulnerable to *Mtb* infection in inbred mouse models (60–62). In humans, deleterious mutations in the IFN-IFNAR-STAT1 pathway give rise to Mendelian susceptibility to TB (63), and humans with Mtb infection who undergo therapeutic TNFα blockade are at increased risk of TB reactivation (64). However, these examples are limited, as it is not known if immune correlates of TB disease are similar or distinct in mice, macaques, and humans. In addition, most mouse studies have used inbred strains with the major limitation that genetic diversity, a key driver of variability in infection outcome and immune responses in macaques and humans, is not reflected in the inbred mouse model. Although the mouse model of TB has been useful in identifying some key immune mechanisms responsible for the control of TB (65) and assessment of drugs and therapeutics, its utility in effectively modeling the progression of *Mtb* infection and pathology has been questioned (66, 67). Recently, two mouse models with the ability to offer maximal allelic variation, thus mimicking human populations, have been generated. One, the Collaborative Cross model, is a large panel of inbred mouse strains that display a broad range of susceptibility to Mtb infection (68).

The other, the DO mouse model, is produced by an outbreeding strategy such that each DO mouse is genetically unique, and their groups approximate the genetic diversity found in human populations. When DO mice were infected with standard doses of Mtb via aerosol, supersusceptible, susceptible, and resistant phenotypes were observed (8, 9). In the current study, we show that with the exception of Sept4 and orthologs absent in mice, 12 of the ACS signature (7) genes are up-regulated in the lungs of progressor DO mice, when compared with controller DO mice. Therefore, vaccines or therapeutics targeting immune pathways in mice, excluding the Sept4 pathway, may reflect outcomes occurring in human TB. Upon aerosol infection with Mtb, macaques typically either progress to TB disease or, unlike mice but similar to humans, remain asymptomatic. Our study demonstrates that the rhesus macaque progressors faithfully express the 16-gene ACS signature, with the exception of TAP1, for which there is no known macaque ortholog. This is consistent with a study of the cynomolgus macaque model of lowdose Mtb infection, which showed that a subset of genes comprising the 16-gene ACS signature prospectively discriminated progressor from controller animals 3 to 6 weeks after infection (69). One limitation of the study is that the number of NHP animals per group was lower (naïve and controller) than the number of mice and human samples per group owing to associated expense of experimentation. In addition, another limitation was in the cross-species RNA-seq comparison analysis of the human ACS gene signature, as it was based on predefined orthologous protein family groups by Ensembl. Also, if no ortholog was identified in the other two model species (mice or macaques), then only the topmost homologous gene match was accepted (above the set threshold).

In our NHP experiments, the challenge dose of 10 colony-forming units (CFU) versus 100 CFU was used as the key determinant of disease outcome as either "controller" or "progressor." However, the DO mice were all challenged with the same Mtb dose, and therefore in the DO mouse model, the dose of Mtb is not the primary determinant of disease progression where the same infectious dose leads to a range of bacterial burdens and inflammation in the lungs. We interpret this spectrum of clinical outcome as a significant advantage of the DO model given that such a spectrum is observed in human Mtb infection outcomes. Bacterial burden and inflammatory responses are interdependent, and some gene expression changes may be a response to heightened bacteria, not the cause. We were not able to rule out from these correlative data that the differentially expressed genes are the consequence of the different bacterial loads, which may very well be the case and is a limitation of the current study.

Consistent with humans who progress to TB, IFN response pathways, innate immune system, cytokine signaling, complementation activation, myeloid cell activation pathways such as TLR cascades, and signaling by ILs were elevated in progressor mice and macaques. When we considered pathways that were up-regulated in controllers compared to progressors in macaques and mice, those associated with lung remodeling and repair were prominent, thus providing important insights into protective immune parameters that limit TB disease. The identification of several genes associated with controlled *Mtb* infection projects these genes as potential candidates for drug targeting or biological experimentation to better understand their role in *Mtb* control.

Our work suggests that molecular mechanisms that govern the equilibrium between lung repair and inflammatory immune responses

are the key determinants of progression of Mtb infection. This result was remarkable in light of recent work demonstrating that IL-22, a mediator of epithelial repair, was protective against chronic infection of mice with the virulent HN878 strains of Mtb (70). The biological pathways associated with control of Mtb infection can thus engender hypotheses of protective immunity. One limitation of this study is that we were unable to follow up on specific mechanisms uncovered by our findings in DO mice as these studies will require gene knockouts to enable functional studies.

Despite the limitation that genotype-phenotype relationships are complex to infer by studying a single genetic background (71), our studies with gene-deficient mice show that greater susceptibility to Mtb infection is likely due to underlying deficiencies in distinct immunological mechanisms. For example, an absence of Stat1 in mice, and humans with mutations in STAT1, results in greater susceptibility to Mtb infection (37). Accordingly, Stat1<sup>-/-</sup> mice had increased Mtb burden and increased inflammation due to defects in  $T_{\rm H1}$  immunity, which is critical for control of *Mtb* replication (37). By contrast, the up-regulation of STAT1 transcripts in TB progressors reflects Mtb-induced inflammation and type I and II IFN signaling, which is indicative of elevated *Mtb* replication. In *Tap1*<sup>-</sup> mice, cell surface expression of MHC class I is impaired, and thus,  $CD8^+$  T cell priming is defective as shown here and before (39). GBPs are guanosine triphosphatases induced in response to IFNs and regulate activation of macrophages in response to many pathogens. GBPs function through induction of autophagy, activation of inflammasomes, and formation of vacuolar compartments (72). GBPs have important roles in controlling several pathogens, including Toxoplasma gondii and M. bovis (41, 73). In light of this, our finding that increased *Mtb* lung burden was observed only at late time points after Mtb aerosol infection in GbpChr3<sup>-/-</sup> mice was unexpected. We acknowledge that gene deficiency in multiple GBP proteins (*GbpChr3<sup>-/-</sup>* mice lack functional *Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and Gbp7), only some of which may be implicated in immunity to Mtb, may underlie this finding. However, it is also possible that although GBPs are critical for immunity to *M. bovis* (41, 73), the role of GBPs in Mtb infection may be more redundant. Because TRAFD1 is associated with negative regulation of inflammation (43, 44), it would be expected that a genetic deficiency might result in enhanced inflammation in the lungs. Unexpectedly, a deficiency of Trafd1 resulted in reduced lung inflammation during late stages of Mtb infection. It has been reported that cells lacking TRAFD1 secrete more IFN $\beta$  than wild-type cells (43). Because IFN $\beta$  has anti-inflammatory effects by promoting a switch from T<sub>H</sub>1 to T<sub>H</sub>2 responses in blood cells (74), this might explain the reduced inflammation observed in our study. Although there is as yet no published data on interaction between Trafd1 and Gbp genes, they belong to the category of IFN-stimulated genes whose expression occur in response to type 1 or type II IFN stimulation (75). Therefore, it is interesting to observe a similar pattern in the bacterial burden in the lungs of  $GbpChr3^{-/-}$  and  $Trafd1^{-/-}$ -infected mice, which could possibly implicate a role for *Stat* signaling. SEPT4 has been implicated in diverse cellular functions, including cytokinesis, apoptosis, and tumor suppression (76, 77). Sept $4^{-/-}$  mice have improved wound healing and regeneration of hair follicles and are protected against apoptosis (76), and males are sterile due to immotile and structurally defective sperm (77). In our study,  $Sept4^{-/-}$ mice exhibited transient susceptibility due to reduced macrophage recruitment.

In the absence of Serping1, mice undergo greater activation of the complement pathway and sustain increased inflammation (*34*, *35*). It remains to be determined if this mechanism explains the increased inflammation seen in Serping1 deficiency during *Mtb* infection.

In a third group of gene-deficient mice, the absence of *Batf2*, Scarf1, and Fcgr1 improved Mtb control and was associated with reduced neutrophil recruitment. Neutrophil accumulation is associated with increased disease severity in human TB (3, 8) and in mouse models of TB (21, 78). Although these three genes were directly associated in Kyoto Encyclopedia of Genes and Genomes (79) or Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (80) functional protein databases, identification of pathways overlapping in the absence of these genes may allow us to define targets that can limit neutrophil accumulation and improve Mtb control. Our studies show that plasma proteome markers of innate immune activation, neutrophil degranulation, and complement activation pathways correlate with SCARF1 mRNA expression of human TB progressors. These pathways were down-regulated in Scarf1<sup>-/-</sup>-infected mice, suggesting a previously unknown functional role for SCARF1 in modulating neutrophil-mediated immunopathology during TB. A number of mediators of lung matrix destruction, the process that leads to cavitary TB in humans, were among the proteins and genes associated with SCARF1 expression; this suggests a possible role for SCARF1 in regulating pulmonary caseation, necrosis, and cavitation, which are associated with poor treatment outcome (81). Moreover, the IL-10 signaling pathway was the top enriched reactome in the human proteomics analysis conducted. IL-10-producing macrophages preferentially clear apoptotic cells (82), and Scarf1 has been associated with clearance of apoptotic cells (28). However, IL-10 also aids immune evasion strategies of mycobacteria, and IL-10 overexpression in mice fails to control *Mtb* infection and mice develop severe lung pathology (83). In summary, we investigated transcriptional signatures in lung samples from DO mice and NHPs infected with Mtb that reflect a spectrum of infection and disease outcomes. Comparison of these transcriptional signatures with those from human TB led to identification of common immune correlates of TB disease that not only implicate overlapping biology but also highlight distinct processes between animal models and humans. Our results and data provide a resource that will be valuable to others in the field. Furthermore, our functional studies using gene-deficient mouse models have addressed the functional role of TB disease risk signatures and demonstrate that specific immune pathways associated with TB progression can be protective or detrimental, or can regulate infection and inflammation during Mtb infection. Together, our crossspecies findings provide insights into modeling TB disease and the immunological basis of TB disease progression.

#### MATERIALS AND METHODS

#### **Study design**

Our objective was to compare the common immune correlates of TB disease and risk across species. Whole-blood RNA-seq data from human subjects were aligned to the hg19 human genome using Genomic Short-read Nucleotide Alignment Program (GSNAP), as performed previously (7). Normalized gene-level expression estimates were derived from mapped read pairs. Briefly, mapped read pairs were assigned to genes by collapsing all transcripts into a single gene model and counting the number of reads that fully overlap the resulting exons using htseq (v.0.6.0) (84), with strict intersection and including strand information. Gene models for protein-coding genes were downloaded from Ensembl (GRCh37.74). Reads that mapped to multiple locations were only counted once, and those mapping to ambiguous regions were excluded. Log<sub>2</sub>-transformed values of counts normalized by adjusted library counts were computed using the cpm function of the edgeR package (85). All mouse strains were bred in-house, used at 6 to 8 weeks of age, and sexmatched, and both sexes were used for all experiments. Specific pathogen-free (SPF), adult Indian rhesus macaques of both sexes were obtained from the Tulane National Primate Research Center. Animals were infected with Mtb via the aerosol route, and tissues were collected for RNA-seq analysis. All animal experiments were performed in accordance with national and institutional guidelines for animal care under approved protocols (animal protocol numbers: mice, 20160129; macaque, P0295R). Primary data are reported in data file S1.

#### Mice

C57BL/6J, DO (86), and 129SvEv (87) mice were obtained from the Jackson Laboratory and bred at Washington University in St. Louis.  $Fcgr1^{-/-}$  (88),  $Scarf1^{-/-}$  (28),  $Batf3^{-/-}$  (89),  $Batf2^{-/-}$  (90),  $Batf2/3^{-/-}$ , and  $GbpChr3^{-/-}$  (73) mice were obtained from J. Ravetch (Rockefeller University), T. Means (Sanofi), K. Murphy (Washington University in St. Louis), and M. Yamamoto (Osaka University, Japan). The 129SvEv mice were used as relevant controls for Batf3<sup>-/-</sup>, Batf2<sup>-/-</sup>, and *Batf2/3<sup>-/-</sup>* mice, whereas C57BL/6J mice were controls for all other gene-deficient mice. CRISPR-Cas9 gene editing was used to generate mice with an out-of-frame 7-nt deletion in Serping1. The sequence of the Serping1 locus was retrieved from the National Center for Biotechnology Information (NCBI), and a single guide RNA (sgRNA) targeting the third exon was designed on the basis of the RefSeq annotation. The sgRNA was selected on the basis of its low off-target profile and the absence of single-nucleotide polymorphisms: 5'-AGCACTGAGCAAGCGGCTCCGGG-3'. The gRNA containing the scaffold was generated by in vitro synthesis (MEGAscript T7 Transcription Kit, Invitrogen), followed by cleanup (MEGAclear Transcription Clean-Up Kit, Invitrogen). Cas9 protein and gRNA were complexed and electroporated into C57BL/6J zygotes. After next-generation sequencing of founders and two generations of mice backcrossed to C57BL/6J mice, a mouse line with a 5'-GCCCGGA-3' deletion at 383 to 389 nt in the Serping1 complementary DNA (cDNA) was generated. After an additional round of backcrossing to C57BL6/J mice, homozygous Serping1<sup>-/-</sup> mice were produced and used for experiments. Sanger sequencing of a polymerase chain reaction amplicon [5'CCTGGCCAACAACCAGTGTAGC-3' (forward) and 5'-GGCACAGCCTCGCAGAAG G-3' (reverse)] was used for genotyping. Serping1<sup>-/-</sup> mice were born in normal Mendelian frequencies and showed no apparent defects in development, growth, or fecundity. Lack of expression of Serping1 mRNA expression was confirmed in lungs of Serping1<sup>-/-</sup> Mtb-infected mice. Cryopreserved sperm from  $Trafd1^{-/-}$  (43) mice was obtained from the Riken Resource Center (Tokyo, Japan), and in vitro fertilization was performed in the Micro-Injection Core at Washington University in St. Louis. All mouse strains were bred in-house, used at 6 to 8 weeks of age, and sex-matched for all experiments.

#### Infection and tissue harvest

Inbred and outbred mice were cohoused but segregated by sex in an ABSL3 facility and infected via aerosol route with  $\sim$ 100 CFU of *Mtb* 

strain HN878 using a Glas-Col airborne infection system as described previously (91). At given time points after infection, lungs were collected and homogenized to assess bacterial burden. Indian rhesus macaques were housed in an ABSL3 facility and verified to be free of Mtb infection by tuberculin skin test. Mtb-naïve, SPF Indian rhesus macaques were exposed to aerosols of Mtb strain CDC1551 (92). Animals were exposed to either a high (93) or a low (94) dose designed to inoculate 100 or 10 CFU in their lungs. Although both groups of animals developed positivity to mammalian old tuberculin after experimental aerosol infection, animals exposed to the higher dose developed TB, as characterized by fever, rapid weight loss, elevated serum complement-reactive protein (CRP) levels, high chest x-ray scores, and detection of viable Mtb CFUs in the bronchoalveolar lavage fluid (93). The animals exposed to low dose were deemed to have asymptomatic TB based on the absence of clinical signals but evidence of TB exposure (tuberculin skin test, PRIMAGAM-IGRA). The animals were subjected to weekly physical examinations by board-certified clinicians, including evaluation of body temperature and weight, and complete blood chemistries, including serum CRP levels (94, 95). Lung tissue from both mice and NHPs were homogenized and snap-frozen in Qiagen Tissue Lysis Buffer (RLT), and deoxyribonuclease (DNase)-treated total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen) for RNAseq analysis.

#### In vitro Mtb infection of bone marrow-derived macrophages

Bone marrow cells from the femur and tibia of C57BL/6J and genedeficient mice were extracted, and  $1 \times 10^7$  cells were plated in 10 ml of complete Dulbecco's modified Eagle's medium (cDMEM) supplemented with mouse recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF; 20 ng/ml) (PeproTech) (8). Cells were then cultured at 37°C in 5% CO<sub>2</sub>. On day 3, 10 ml of cDMEM containing rmGM-CSF (20 ng/ml) was added. On day 7, adherent cells were collected as macrophages (8). Macrophages were infected with *Mtb* strain HN878 [multiplicity of infection (MOI), 1] in antibiotic-free cDMEM for 2 dpi, after which culture supernatants were assayed for IL-10 and TNF $\alpha$  using enzyme-linked immunosorbent assay (R&D Biosystems) according to the recommended standard protocols.

### Generation of single-cell suspensions from tissues and flow cytometry staining

Single-cell suspensions of lungs from untreated or Mtb-infected mice were isolated as previously described (91). Briefly, mice were euthanized with CO<sub>2</sub>; the right lower lobe was isolated and perfused with heparin in saline. Lungs were minced and incubated in collagenase/DNase for 30 min at 37°C. Lung tissue was pushed through a 70-µm nylon screen to obtain a single-cell suspension. After lysis of erythrocytes, the cells were washed and resuspended in complete DMEM (DMEM high glucose/10% fetal bovine serum/1% penicillin/streptomycin) for flow cytometry staining. The following fluorochrome-conjugated antibodies were used for cell surface staining: CD11b APC (clone: M1/70; Tonbo Biosciences), CD11c-PeCy7 (clone: HL3; BD Biosciences), GR-1 Phycoerythrin (PE) (clone: RB6-8C5; Tonbo Biosciences), CD3 AF700 (clone: 500A2; BD Biosciences), CD4 Pacific Blue (PB) (clone: RM4.5; BD Biosciences), CD44 PeCy7 (clone: 1M7; Tonbo Biosciences), and CD8 APC Cy7 (clone: 53-6.7; BD Biosciences). For intracellular staining, fixation/ permeabilization concentrate and diluent (eBioscience) were used

to fix and permeabilize lung cells following the manufacturer's instructions. Intracellular staining with IFN $\gamma$  APC (clone: XMG1.2; Tonbo Biosciences) and IL-17A PE (BD Pharmingen) or the respective isotype control antibodies (APC rat IgG1 k and PE rat IgG1 k isotype, BD Pharmingen) was performed for 30 min. Samples were acquired on a 4 laser BD Fortessa Flow cytometer, and the analysis was performed using FlowJo (Treestar LLC). As before (70), alveolar macrophages were gated on CD11c<sup>+</sup>CD11b<sup>-</sup> cells, neutrophils were defined as CD11b<sup>+</sup>CD11c<sup>-</sup>Gr-1<sup>hi</sup> cells, monocytes were defined as CD 11b<sup>+</sup>CD11c<sup>-</sup>Gr-1<sup>low</sup> cells (fig. S8). Total numbers of cells within each gate were back-calculated on the basis of cell counts per individual lung sample.

#### **Histological analysis**

The left upper lobe was collected for histological analysis of inflammation. The lobes were infused with 10% neutral buffered formalin and embedded in paraffin. Lung sections (5  $\mu$ m) were cut using a microtome, stained with hematoxylin and eosin (H&E), and processed for light microscopy. Formalin-fixed paraffin-embedded (FFPE) lung sections were stained for collagen and muscle using Masson's Trichrome 2000 stain procedure kit (American MasterTech), as per the manufacturer's instructions. Images were captured using the automated NanoZoomer digital whole slide imaging system (Hamamatsu Photonics). Regions of inflammatory cell infiltration were delineated using the NDPview2 software (Hamamatsu Photonics), and the percentage of inflammation was calculated in relation to the total lung area of each section. All scoring was conducted in a blinded manner using n = 3 to 5 per group of mice.

#### In situ hybridization

FFPE lung tissue sections were subjected to in situ hybridization (ISH) with the RNAscope Probes, *Scarf1* cat. no. 535551) or *Serping1* cat. no.535071), using the RNAscope 2.5 HD Detection Reagent-RED kit or the RNAscope 2.5 HD Detection Reagent-BROWN kit, as per the manufacturer's recommendations (Advanced Cell Diagnostics). Representative images were acquired with the Leica Application Suite V4.5 microscope and software (Leica Microsystems).

#### **RNA** sequencing

Mouse or macaque lung tissues were homogenized and snap-frozen in RLT buffer, and DNase-treated total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen). RNA-seq libraries were generated using the Clontech SMART-Seq v4 Ultra Low Input RNA Kit for sequencing and the Illumina Nextera XT DNA Library preparation kit following the manufacturer's protocol. The cDNA libraries were validated using the KAPA Biosystems primer premix kit with Illumina-compatible DNA primers, and quality was examined using Agilent Tapestation 2200. The cDNA libraries were pooled at a final concentration of 1.8 pM. Cluster generation and 75-base pair paired-read dual-indexed sequencing was performed on Illumina NextSeq 500. After adapter trimming using Trimmomatic v0.36 (96), RNA-seq reads were aligned to their respective genome assemblies [Mus musculus GRCm38 and Macaca mulatta Mmul\_8.0.1-Ensembl release 91.0 (14)] using HISAT2 v2.1.0 (97). All raw RNA-seq fastq files were uploaded to the NCBI Sequence Read Archive [SRA (98)], and complete sample metadata and accession information are provided in table S1. Read fragments (read pairs or single reads) were quantified per gene per sample using feature Counts (version 1.5.1)

(99). FPKM (fragments per kilobase of gene length per million reads mapped) normalization was also performed. Gene annotations and matches across species were assigned by retrieving the top hit macaque and human gene to each mouse gene using data retrieved from Ensembl (14) (release 91.0). Human data for the blood RNA signature were retrieved from a previous study (7), and significance values for the association with progressors or controllers were aligned to mouse genes using these human gene matches to the mouse genome. Because Ensembl annotations did not provide a direct ortholog for several key signature genes, these were instead matched by identifying the top mouse hit for the human genes: GBP4 to ENSMUSG0000028268 (reciprocal top hit), GBP-1 to ENSMUSG00000040264 (mouse to human second-best hit; GBP-2 was the top hit), and ETV-7 to ENSMUSG0000030199 (mouse to human second-best hit; ETV-6 was the top hit). Also, note that FCRG1a and FCRG1b both matched to the same mouse gene (ENSMUSG00000015947; mouse to human top two hits are to these two genes). Significantly differentially expressed genes between naïve, controller, and progressor sample sets were identified using DESeq2 (version 1.4.5) (100) with default settings and a minimum P value significance threshold of 0.05 [after FDR (101) correction for the number of tests]. Principal components analysis was also calculated using DESeq2 output (default settings, using the top 500 most variable genes). Read counts, relative gene expression levels, gene annotations, and differential expression data for the mouse genes and their corresponding macaque genes, and the alignment to the human dataset (7) are available in table S2. Pathway enrichment analysis for Reactome (57) pathways among gene sets of interest was performed using the WebGestalt (56) web server (table S6).

#### Statistical analysis

Where applicable, comparisons between two groups were performed using the unpaired two-tailed Student's *t* test, using the GraphPad Prism 5 software. In all instances,  $P \le 0.05$  was considered as significant. DESeq2 (version 1.4.5) (100) with default settings and a minimum P value significance threshold of 0.05 [after FDR (101) correction for the number of tests] were used for all RNA-seq comparisons of differential expression. Associations between ACS signature gene expression levels and TB infection metadata (presented as significance P values for Pearson correlations; table S4) were tested using the Student's "t" distribution and FDR correction was carried out for the number of tests performed.

#### SUPPLEMENTARY MATERIALS

- stm.sciencemag.org/cgi/content/full/12/528/eaay0233/DC1
- Fig. S1. Differential gene expression (DESeq2) across species.
- Fig. S2. Serping1 regulates inflammation but not Mtb control in mice.
- Fig. S3. T cell cytokine profile and myeloid cell recruitment in *Mtb*-infected *Tap1<sup>-/-</sup>* mice.
- Fig. S4. Protection from *Mtb* HN878 infection is mediated by *Batf2*.
- Fig. S5. IL-17–producing CD4<sup>+</sup> T<sub>H</sub>17 cell cytokines are increased in *Mtb*-infected *Batf2/3<sup>-/-</sup>* mice.
- Fig. S6. T cell cytokine profile in *Mtb*-infected *Fcgr1<sup>-/-</sup>* mice.
- Fig. S7. SCARF1 expression drives inflammation within TB granulomas.
- Fig. S8. Hierarchical gating strategy used to identify myeloid populations in mice lungs. Table S1. RNA-seq sample metadata and accessions.
- Table S2. FPKM gene expression values and differential expression statistics for the top 1000 mouse genes, including information about ortholog matches in human and macaque.

Table S3. Significance testing for overlaps of differentially expressed genes between species. Table S4. Correlation analysis between ACS signature genes and TB metadata (lung inflammation and bacterial bacteria) among mouse and macaque controller and progressor samples. Table S5. Flow cytometry quantification of lung cellular subsets in control and gene-deficient models infected with *Mtb*. Table S6. Significantly enriched Reactome pathways among gene sets of interest. Data file S1. Primary data.

View/request a protocol for this paper from Bio-protocol.

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## **Science** Translational Medicine

#### Immune correlates of tuberculosis disease and risk translate across species

Mushtaq Ahmed, Shyamala Thirunavukkarasu, Bruce A. Rosa, Kimberly A. Thomas, Shibali Das, Javier Rangel-Moreno, Lan Lu, Smriti Mehra, Stanley Kimbung Mbandi, Larissa B. Thackray, Michael S. Diamond, Kenneth M. Murphy, Terry Means, John Martin, Deepak Kaushal, Thomas J. Scriba, Makedonka Mitreva and Shabaana A. Khader

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#### **Translating TB**

Mycobacterium tuberculosis can cause tuberculosis (TB) disease in humans, although the majority of infected individuals naturally control infection and never show symptoms. To better understand immune control of *M. tuberculosis*, Ahmed *et al.* compared gene expression data from infected humans and different animal models. Gene expression differing between disease progression and control identified potentially beneficial shared pathways. Some pathways were followed up by examining *M. tuberculosis* infection in gene-deficient inbred mice. These results highlight how focusing on commonalities in TB infection can lead to new insights for treating TB.

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