Title page

The TB-specific CD4⁺ T cell immune repertoire in both cynomolgus and rhesus macaques largely overlap with humans¹

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22 **Abstract**

23 Non-human primate (NHP) models of tuberculosis (TB) immunity and pathogenesis, especially rhesus
24 and cynomolgus macaques, are particularly attractive because of the high similarity of the human and
25 macaque immune systems. However, little is known about the MHC class II epitopes recognized in
26 macaques, thus hindering the establishment of immune correlates of immunopathology and protective
27 vaccination. We characterized immune responses in rhesus macaques vaccinated against and/or
28 infected with *Mycobacterium tuberculosis* (Mtb), to a panel of antigens currently in human vaccine
29 trials. We defined 54 new immunodominant CD4⁺ T cell epitopes, and noted that antigens
30 immunodominant in humans are also immunodominant in rhesus macaques, including Rv3875 (ESAT-
31 6) and Rv3874 (CFP10). Pedigree and inferred restriction analysis demonstrated that this phenomenon
32 was not due to common ancestry or inbreeding, but rather presentation by common alleles, as well as,
33 promiscuous binding. Experiments using a second cohort of rhesus macaques demonstrated that a pool
34 of epitopes defined in the previous experiments can be used to detect T cell responses in over 75% of
35 individual monkeys. Additionally, 100% of cynomolgus macaques, irrespective of their latent or active
36 TB status, responded to rhesus and human defined epitope pools. Thus, these findings reveal an
37 unexpected general repertoire overlap between MHC class II epitopes recognized in both species of
38 macaques and in humans, showing that epitope pools defined in humans can also be used to
39 characterize macaque responses, despite differences in species and antigen exposure. The results have
40 general implications for the evaluation of new vaccines and diagnostics in NHPs, and immediate
41 applicability in the setting of macaque models of TB.

42 Keywords: Non-human primate; epitopes; CD4+

43 **Introduction:**

44 Biomedical research and vaccine testing depends in large part on the use of Non-human Primate
45 (NHP) models for testing and development of immunotherapeutic drugs, diagnostics and vaccines. In
46 this context it is desirable to accurately measure immune responses by modern techniques, such as
47 ELISPOT, intracellular cytokine staining (ICS) and tetramer staining assays. This quantification in turn
48 requires definition of MHC class I and class II restricted epitopes recognized in the most widely used
49 NHP species, such as the rhesus and cynomolgus macaques.

50 MHC class I and II genes from rhesus macaques of Indian and Chinese origin have been
51 sequenced and several MHC molecules characterized for their binding specificity¹⁻³⁹. The specificity
52 and epitope motifs associated with class I molecules expressed have been intensely studied, and
53 revealed specific motifs, that in several cases overlapped in terms of epitope binding specificity with
54 the motifs expressed by human HLA class I molecules^{34, 35, 40}. However, in general the binding
55 repertoires of rhesus and human class I appear to be for the most part distinct and non-overlapping^{3, 16,}
56 ^{17, 23, 31, 41-45}. By contrast, little is known about the specific epitopes presented by class II molecules
57 expressed in NHP. In humans, a greater degree of promiscuity and repertoire overlap is associated with
58 HLA class II molecules, as compared to class I molecules⁴⁶. Until this study, it was not clear whether
59 this repertoire overlap would also apply to NHP MHC class II, and whether any repertoire overlap
60 could be exploited in practical terms for evaluation of NHP MHC class II restricted responses.

61 TB remains one of the greatest public health threats with over 2 billion people latently infected
62 with *Mycobacterium tuberculosis* (Mtb)⁴⁷. Approximately 10% of Mtb-infected individuals develop
63 active TB, either primary disease or reactivation of latent infection⁴⁸⁻⁵⁰. Bacille Calmette–Guérin
64 (BCG) is the only vaccine available against TB. BCG vaccination was developed about a century ago
65 and its capacity to protect against TB is highly variable, with efficacy estimates ranging from 80% to
66 no protection⁵¹. Development of alternative more efficacious vaccines is a complex task⁵²⁻⁵⁷.
67 Significant hurdles include the complexity of efficacy trials that are of considerable duration⁵⁸, and
68 since only a fraction of the individuals at risk do actually develop disease, require enrollment of large
69 numbers of subjects⁵⁴.

70 In this context, reliable animal models to study TB pathogenesis, and to evaluate vaccine
71 candidates and vaccination regimens are of significant importance. While humans are the only natural
72 hosts of Mtb, several different animal models of TB vaccination have been extensively utilized,
73 including murine, guinea pig, rabbits, cattle and NHPs⁵⁹⁻⁷¹. Each animal species possesses advantages
74 and caveats as each animal model reacts in its own particular fashion to Mtb infection⁷² and differs in
75 its ability to model human disease progression. Herein, we will point to some overarching

characteristics of each. The murine model has advantages with its in-depth characterized immune system. However, mice are relatively resistant to infection with Mtb, and the disease process and pathology differs extensively⁷¹. Rabbits are also resistant to infection and only limited cytokine reagents are available^{71,72}. Guinea pigs, on the other hand, are extremely susceptible to Mtb, as most infected animals succumb to disease, thus limiting the similarities to human disease process.

NHPs, *e.g.* rhesus and cynomolgus macaques, are appealing models of human disease and vaccination as the physiology, genetics, pathology and immunology are most similar to humans. In addition, there are many cross-reactive antibodies or macaque-specific antibodies, which allow for the characterization of specific cellular subsets, cytokines and chemokines^{73,74}. The aerosol model of Mtb infection in rhesus macaques closely mimics human infections, allowing a more natural distribution of infecting bacilli onto the pulmonary surfaces^{72,75}. Macaques develop latent infection characterized by the absence of any clinical signs of infection, similar to humans, but can be reactivated into an acute form⁷². However, despite their importance in Mtb infection⁷⁶⁻⁸⁶, significant gaps in knowledge exist, particularly regarding CD4⁺ T cell responses. Only one Ag85B (Rv1886c)-specific macaque MHC class II tetramer has been described⁸⁷ and only two class II motifs has been characterized⁹.

Thus, not surprisingly, the characterization of immune responses following Mtb infection and vaccination of macaques is incomplete. In this study, we characterized the MHC immunogenetic background and immunological response to antigens that have been used in human vaccine trials in NHPs that had been vaccinated against and/or infected with Mtb. We defined to an unprecedented level of detail epitopes recognized by macaque CD4⁺ T cells, including patterns of immundominance and inferred restriction. The results suggest that while the CD4⁺ T cell response of NHPs to Mtb is broad and heterogeneous, epitope sets can be defined to broadly follow and characterize these responses in genetically heterogeneous cohorts of NHP. Furthermore, we reveal a striking repertoire overlap between epitopes recognized in NHPs and in humans, and show that epitopes previously defined in humans can be used to characterize NHP responses, despite these species differences, as well as, varying routes of antigen exposure. These results enable broad and effective characterization of immune responses in NHPs in general, and in particular in models of Mtb infection and vaccination.

103 **Materials and methods**

104 **Infections and vaccinations of rhesus macaques**

105 A total of 34 rhesus macaques were utilized in this study, 23 of which were infected and/or
106 vaccinated as detailed in **Table I**. All infections and vaccinations were performed and the animals
107 housed in the Biosafety level 3 (BSL 3) Regional Biosafety Laboratory facility at Tulane National
108 Primate Research Center in Covington, LA. The 23 animals described in **Table I** were vaccinated and
109 challenged in support of two studies. All infected animals were positive for TST (tuberculin skin test)
110 at weeks 3-4 post-experimental exposure. None of the naïve animals were positive for TST. Every
111 animal prior to designation to these studies was checked and was TST negative. One of these studies
112 was aimed at understanding the effectiveness of the *Mtb*: Δ -*sigH* mutant, delivered via aerosol, in the
113 *Mtb* CDC1551 background^{88, 89} in response to lethal aerogenic challenge with homologous *Mtb* in
114 rhesus macaques (Kaushal D et al, manuscript in review, Nature Medicine). In this experiment, we
115 used aerosolized BCG as a control. As shown by David Edwards and colleagues⁹⁰, BCG exhibits
116 higher levels of protection when introduced via the inhalation route, relative to the intradermal route, in
117 the context of guinea pigs. In a seminal study in 1973, aerosolized BCG was shown to be protective in
118 monkeys⁹¹. Another study was aimed at assessing the effectiveness of a 3D-BCG recombinant strain,
119 described by Douglas Kernodle and colleagues⁹², where genes involved in both the secretion of
120 antigens as well as anti-oxidant defense, including *sigH*, were deleted from the parental BCG strain; as
121 a prime vaccine vehicle against aerogenic *Mtb* challenge in rhesus macaques. Animals that did not
122 receive any immunization, were however challenged with comparable *Mtb* to serve as negative
123 controls. Animals were euthanized and necropsied at least six weeks after the last infection. Spleen and
124 whole blood samples obtained at necropsy were shipped to La Jolla Institute for Allergy and
125 Immunology (LJI).

126

127 **Infections of cynomolgus macaques**

128 Blood samples from 16 cynomolgus macaques from Chinese breeding facilities (*Macaca*
129 *fascicularis*) (Valley Biosystems, Sacramento CA) were included in this study. These macaques were
130 *Mtb*-infected and enrolled in unrelated ongoing studies in the Flynn laboratory. These animals were not
131 vaccinated, undergoing anti-mycobacterial treatments, or experiencing immunomodulatory therapies.
132 The macaques were infected with low dose (<25 CFU) or moderate dose (60-150 CFU) *M. tuberculosis*
133 strain Erdman via bronchoscope, as described⁹³. Samples were chosen from 8 animals that eventually
134 developed active TB and 8 animals that developed latent infection, according to previously described
135 criteria⁹³. All macaques were at least 4 years of age and between 4-8 kg. The blood draws used for the

136 samples were obtained at 12 weeks post-infection. Cynomolgus macaques were housed and maintained
137 by the University of Pittsburgh's Department of Laboratory Animals. All procedures were performed
138 in accordance with protocols approved by the University of Pittsburgh's Institutional Animal Care and
139 Use committee.

140

141 **Cell isolation**

142 PBMCs were obtained by density gradient centrifugation (Ficoll for rhesus macaques, Percoll
143 for cynomolgus macaques) from whole blood ⁵ and spleens were homogenized to a single cell
144 suspension. Cells were isolated according to standard protocols in originating labs. All isolated cells,
145 consisting of PBMCs and splenocytes, were cryopreserved in liquid nitrogen ⁹⁴.

146 **Peptides**

147 Sets of 15mer peptides overlapping by 10 residues, were synthesized by A&A (San Diego,
148 CA). The antigens studied correspond to antigens currently considered as vaccine candidates, or
149 included in the QuantiFERON-TB Gold In-tube test [as shown in **Table II**; including Rv0125
150 (Mtb32a), Rv1813c, Rv0288 (TB10.4), Rv1886c (Ag85B), Rv1196 (Mtb39A), Rv2608 (PPE42),
151 Rv3619c (EsxV), Rv3620c (EsxW), Rv3874 (CFP-10) and Rv3875 (ESAT6)]. Pools of previously
152 defined human epitopes were generated based on the information described in Lindestam Arlehamn *et*
153 *al.* ⁹⁵. These pools therefore do not represent the entire TB proteome. Specific information regarding
154 the patients included in this study includes the following⁹⁵:

155 Human LTBI subjects (age range 20-65 years) had a history of a positive tuberculin skin test
156 (TST). LTBI was confirmed by a positive QuantiFERON-TB Gold In-Tube (Cellestis), as well as a
157 physical exam and/or chest X-ray that was not consistent with active tuberculosis. None of the study
158 subjects endorsed vaccination with BCG, or had laboratory evidence of HIV or Hepatitis B.

159 **Ex vivo IFN- γ ELISPOT assay**

160 IFN- γ ELISPOT assays were performed as previously described ^{96,97}. Briefly, macaque PBMCs
161 or splenocytes at a density of 2×10^6 cells/ml were stimulated with peptide pools (2-10 μ g/ml) or
162 individual peptides (20 μ g/ml), ConA (20 μ g/ml) or medium in flat-bottom 96-well nitrocellulose plate
163 (Immobilon-P membrane; Millipore, Bedford, MA), coated with 15 μ g/ml anti-human/monkey IFN- γ
164 (GZ-4; Mabtech, Inc., Mairmont, OH). Each peptide or pool was tested in triplicate. After 20 h
165 incubation at 37 °C, plates were washed with PBS/0.05% Tween 20 and incubated with 2 μ g/ml
166 biotinylated anti-human IFN- γ (7-B6-1; Mabtech) for 2 h at 37 °C. After additional washes with PBS–
167 0.05% Tween 20, spots were developed using Vectastain ABC peroxidase (Vector Laboratories,

168 Burlingame, CA) followed by 3-amino-9-ethylcarbazole solution (Sigma-Aldrich, St. Louis, MO) and
169 counted by computer-assisted image analysis (Zeiss KS ELISPOT reader).

170 Responses are expressed as mean net spots per million cells for each peptide pool or individual
171 peptide. Responses were considered positive if (i) the number of spot-forming cells (SFCs)/10⁶
172 splenocytes or PBMCs exceeded the absolute value of the mean negative control wells (effectors
173 without peptide) by twofold, (ii) the value exceeded 20 SFCs/10⁶ splenocytes or PBMCs, and (iii) were
174 significantly different from the negative (no peptide) control by either a Poisson distribution test or a
175 Student's t-test with $p \leq 0.05$.

176 The response frequency was calculated by dividing the no. of donors responding with the no. of
177 donors tested. The magnitude of response (total SFC) was calculated by summation of SFC from
178 responding donors⁹⁵.

179

180 **MHC sequencing**

181 Genomic DNA (gDNA) was extracted using a QIAmp blood mini kit from splenocytes from the
182 rhesus macaque samples and used as templates for PCR with a series of primer mixes that allow for
183 parallel amplification of MHC class I, DRB, DQA, DQB, DPA & DPB sequences with the Fluidigm
184 Access Array. These amplicons targeted the highly polymorphic peptide binding domain encoded by
185 exon 2 of the MHC class I and II loci. Next, the PCR products were purified using Ampure beads and
186 sequenced using Illumina MiSeq. Mamu-A, Mamu-B, Mamu-DR, Mamu-DQ and Mamu-DP
187 haplotypes were determined by aligning the resulting sequence reads from each animal against a
188 custom database of macaque MHC class I and class II alleles sequences.

189 **Relative Frequency (RF) calculations to infer MHC restriction**

190 Standard Odds Ratios and Relative Frequency (RF) allele frequency calculations were used to
191 infer MHC restriction. RF values were calculated as the ratio of the response in donors expressing a
192 specific allele to the response in all donors and was calculated according to the formula:
193
$$RF = (A^+R^+ / (A^+R^+ + A^+R^-)) / ((A^+R^+ + A^-R^+) / \text{Total donors})$$

194 Where: A⁺ = Subject expresses a specific allele; A⁻ = Subject not expressing the specific
195 allele; R⁺ = Subject has a positive immune response against the specific peptide; R⁻ = Subject does not
196 have a positive immune response against the specific peptide; A⁺R⁺ indicates number of Subjects
197 expressing the specific allele AND having a positive response against a specific peptide. RF values
198 greater >1.0 indicate a positive association, *i.e.* expressing the specific allele increases the “odds” of
199 having positive immune response. Significance is calculated by standard Fisher's exact test.

200 **Intracellular cytokine analysis**

201 Splenocytes were stimulated for 6 hours in complete RPMI medium at 37°C in 5% CO₂ in the
202 presence of 2µg/ml peptide pool and 4µg/ml Brefeldin A (last 4 hours). Unstimulated cells were used
203 to assess nonspecific/background cytokine production. After 6 hours, cells were harvested and stained
204 for cell surface markers anti-CD4-PE (L200), anti-CD3-BV421 (SP34-2, both from BD Biosciences),
205 anti-CD8-APC (RPA-T8), and Live/Dead Aqua (both from eBioscience). After washing, cells were
206 fixed and permeabilized using 4% paraformaldehyde (PFA) and saponin buffer and then stained for
207 cytokine using anti- IFN-γ-FITC (4S.B3, eBiosciences). Samples were acquired on a BD LSR II flow
208 cytometer. The frequency of cells responding to the Mtb-specific peptides was quantified by
209 determining the total number of cytokine⁺ cells and background values subtracted (as determined from
210 the medium alone control) using FlowJo software (Tree Star). Lymphocytes, gated based on forward
211 and side scatter, were gated for live CD3⁺ T cells. Live CD3⁺ T cells were then gated based on CD4
212 and CD8 expression. Then the IFN-γ⁺ cells were identified from these populations.

213 **Results:**

214 **NHP ex vivo T cell responses to TB antigens used in human vaccine trials vary in magnitude and**
215 **frequency**

216 A set of fifteen rhesus macaques was utilized in a first survey of immune responses to TB
217 antigens. These macaques were associated with a rather diverse immunization and infection regimens,
218 reflecting cell sample availability, detailed in **Table I**. These samples enable the characterization of
219 responses in a diverse set of conditions . Splenocytes from each of the fifteen animals were tested in ex
220 vivo IFN- γ ELISPOT against pools of overlapping peptides spanning various TB antigens currently
221 being tested for vaccines in clinical trials or used in diagnostic assays (**Table II**).

222 A wide spectrum of reactivity was apparent (**Figure 1**). Several antigens were
223 immunodominant both in terms of total magnitude, measured by total SFC/ 10^6 across all animals, and
224 frequency, measured by percentage of animals yielding positive responses, of responses. Rv3875 was
225 the most dominant, with a total magnitude of 2343 SFC/ 10^6 and 93% of the animals responding to this
226 antigen, followed by antigen Rv3874, with a total SFC/ 10^6 of 1955 and 80% of the animals responding.
227 Rv2608, was associated with a magnitude of 1350 SFC/ 10^6 and a 60% response frequency. Two
228 additional antigens, Rv0288 (613 SFC/ 10^6 , 47%) and Rv1196 (673 SFC/ 10^6 , 33%), were associated
229 with intermediate immunogenicity. The remaining antigens had lower magnitudes and frequencies of
230 less than 30%.

231

232 **Identification of specific epitopes**

233 Next, positive pools were deconvoluted to identify individual epitopes. **Figure 2 a-h** shows the
234 magnitude and frequency of responses associated with each individual peptide contained within an
235 antigen. Antigen Rv3875, which was the most immunogenic, contained two overlapping peptides,
236 Rv3875₁₋₁₅MA15 and Rv3875₆₋₂₀WG15, with magnitudes of 2868 and 2655 SFC/ 10^6 , respectively.
237 These peptides were positive in 57% of the animals tested for this antigen. Overlapping peptides
238 Rv3875₅₁₋₆₅YL15 and Rv3875₅₆₋₇₀QQ15 were positive in 29% of the animals tested with a magnitude
239 of 392 and 150 SFC/ 10^6 , respectively (**Figure 2a**).

240 A number of epitopes were also identified from antigen Rv3874 (**Figure 2b**), which also
241 yielded a strong response with the corresponding peptide pools. The Rv3874₅₁₋₆₅AQ15 epitope was
242 associated with positive responses in 21% of the subjects tested (total SFC/ 10^6 of 1198). The Rv3874₇₁₋₈₅ER15
243 epitope elicited a response in 36% of individuals with a magnitude of 780 SFC/ 10^6 , and
244 overlapped with Rv3874₇₆₋₉₀IQ15, which also elicited appreciable responses (465 SFC/ 10^6 in 29% of
245 the animals tested).

246 In the case of antigen Rv2608, deconvolution experiments identified positive responses in two
247 animals. Specifically, animal GP21 was positive for sequences Rv2608₂₈₆₋₃₀₀PP15, Rv2608₂₉₁₋₃₀₅AT15,
248 Rv2608₃₀₁₋₃₁₅GP15,, Rv2608₃₂₆₋₃₄₀VM15, and Rv2608₃₆₆₋₃₈₀RD15. Animal IN85 responded to peptides
249 Rv2608₄₈₆₋₅₀₀LA15, Rv2608₄₉₆₋₅₁₀VD15, and Rv2608₅₆₁₋₅₇₅DA15 (**Figure 2c**). The remainder of the
250 antigens, including Rv1886 (**Figure 2d**), Rv1196 (**Figure 2e**), Rv3619c (**Figure 2f**), Rv0125 (**Figure**
251 **2g**) and Rv0288 (**Figure 2h**) yielded between 1-3 positive peptides, with magnitudes between 27 and
252 277 SFC/10⁶, and response frequencies of 7% to 21%. No specific peptide was identified from
253 Rv1813c (not shown).

254 In summary, deconvolution of specific peptide pools identified 54 epitopes (**Supplemental**
255 **Table I**). Elimination of redundant/overlapping sequences by selecting the best response in the case of
256 overlapping peptides, reduced this list to 37 epitopes. Of these, 13 were positive in two or more
257 animals, and encompassed 81% of the overall sum of SFC per million, indicating their
258 immunodominant nature (**Table III**).

259

260 **Cohort inbreeding does not explain epitope dominance in the animal cohort**

261 As presented above, a total of 13 epitopes were recognized in multiple animals. Frequently
262 recognized peptides may be uniquely presented by certain MHC class II allele(s), abnormally frequent
263 in the specific cohort tested, because of common pedigrees and inbreeding. To address this possibility
264 we assessed the pedigree of these animals to determine the level of inbreeding in this particular cohort.

265 Review of pedigree information from the colony at Tulane Primate Center revealed only minor
266 inbreeding. Specifically, only 3 pairs of animals shared a parent or grandparent: GI89 shares one
267 grandparent and one parent with II21 (shown in red in **Supplemental Table II**); animals IM73 and
268 HE06 both share a grandparent (shown in green in **Supplemental Table II**); IB78 and II69 both share
269 a grandparent (shown in blue in **Supplemental Table II**). Of a total of 54 peptide/macaque
270 combinations associated with the peptides, 13 were recognized by multiple animals and only 8 were
271 represented by instances in which the responding macaque was a relative of another macaque
272 responding to the same peptide (**Supplemental Table II**). Hence these epitopes are not dominant
273 because of inbreeding and common ancestry.

274

275 **Inferred MHC alleles potentially restricting detected immune responses**

276 Frequently recognized peptides may be uniquely presented by certain MHC class II allele(s)
277 commonly expressed in the general macaque population. Alternatively, the more dominant epitopes
278 might be restricted by more than one MHC class II molecule (promiscuous restriction). Indeed, in

humans it has been reported that promiscuous epitopes account for a large fraction of responses⁹⁸, and promiscuous binding predictions have been used to perform genome-wide analysis of Mtb class II responses in humans⁹⁵. To address these possibilities, we first determined which specific MHC class II allele(s) were most frequently expressed by next generation sequencing (**Supplemental Table II**). The frequency of the most common alleles is summarized in **Table IV**.

To gain more insight into potential allelic restriction for the epitopes recognized by multiple animals, we examined the relative frequency in which different alleles are expressed in animals that responded or did not respond to each epitope. Classical genetic calculations calculating Odds Ratios, Relative Frequencies (RF) and associated p values were utilized to infer MHC allelic variants potential restricting the epitope-specific responses. Four epitope/MHC allele combinations with a significant association were revealed by this analysis (**Table V**).

The Rv3874₇₁₋₈₅ER epitope was exclusively recognized by animals expressing DQB1*18:g1. In total, five of nine animals expressing this allele responded. In contrast, none of the six animals that did not express DQB1*18:g1 responded to this peptide. Thus, this epitope is likely restricted by DQB1*18:g1 (RF = 1.7; p = 0.044) (**Table V**). Similarly, the Rv0288₆₁₋₇₅AT epitope was predicted to be restricted to DPA1*02g2/B1*07:01. Three of the six animals expressing these alpha and beta DP chain alleles responded to the peptide, whereas none of remaining nine animals, which did not express this DP chain gene combination, responded to the epitope (RF = 2.5; p = 0.044) (**Table V**).

By contrast, in the case of the Rv3620_{c31-65}AR and Rv3620_{c6-20}ME epitopes, both animals expressing the haplotype DQB1*18:g2, DRB*W1:g1, DRB*W6:02, DRB*W6:g2, DRB1*03:g2 responded to these epitopes, and thus one of these alleles is likely to restrict the response (RF = 5.0; p = 0.029) (**Table V**). However, these peptides are also recognized by an additional animal not carrying this haplotype or sharing any other MHC expressed in the other two animals, and therefore are associated, by definition, with promiscuous restriction.

We selected representative macaque alleles and performed a BLAST analysis (using the HLA/MHC specific tool <http://www.ebi.ac.uk/ipd/imgt/hla/>) to test whether the macaque alleles of interest would have a human match. The following macaque/human MHC allele pairs were found with the listed percentage identity in parenthesis: Mamu-DPA1*02:01/HLA-DPA1*01:03 (93%), Mamu-DPB1*07:01/HLA-DPB1*106:01 (93%), Mamu-DQB1*18:01/HLA-DQB1*03:03 (93%), Mamu-DQB1*18:04/HLA-DQB1*03:96 (92%) and Mamu-DRB*W1:01/HLA-DRB1*14:07 (91%), with an overall mean of 92% of all the pair of Mamu/HLA alleles.

The epitopes identified allow general coverage and analysis of T cell responses in rhesus macaques

312 The results presented above identify a total of 54 different peptide epitopes, recognized in a set
313 of 15 genetically diverse animals. We hypothesized that these epitope set might allow uniform
314 coverage of Indian rhesus macaques in general. Accordingly, we generated a pool of these epitopes and
315 tested it with splenocytes derived from a set of 8 new macaques (**Table I**), in which only one animal
316 was related with one of the 15 animals used for epitope discovery and an additional 8 naïve animals
317 (**Table I; Supplemental Table II**) that had not been vaccinated or infected with Mtb.

318 Consistent with the notion that this epitope set would allow general coverage in Indian rhesus
319 macaques, we found that 6 of the 8 new animals were associated with strong responses, with median of
320 176 SFC/10⁶ cells (interquartile range 30-523). By contrast, no or weak, responses were observed in
321 splenocytes from the 8 naïve animals tested (**Figure 3a**). These data suggest that the 54 epitopes may
322 allow general coverage and analysis of T cell responses in Indian rhesus macaques.

323 **Extensive similarity between human and NHP epitope repertoires**

324 The MHC class II molecules of NHPs bear very significant degrees of sequence homology to
325 human HLA class II molecules. A functional overlap between HLA DR and macaque DR molecules
326 has indeed been reported ⁸.

327 Based on these observations we hypothesized that at least some of the dominant epitopes
328 detected in NHPs might have been previously detected in humans. Strikingly, we found that in the
329 antigens Rv3875 (ESAT-6), Rv0288 (TB10.4), Rv1886c (85B) and Rv3874 (CFP10), every single
330 epitope we identified has also been identified in human studies (**Supplemental Table III**).

331 For the epitopes in antigen Rv3619c (EsxV), only epitope Rv3619c₆₋₂₀QQ15 was positive in
332 humans ⁹⁹(**Supplemental Table III**). The only epitope in Rv0125 (MTb32a), Rv0125₃₀₆₋₃₂₀AD15, as
333 well as the epitopes in Rv1196 (mtb39a) and Rv2608 (PPE42) have not been reported in the setting of
334 human infection. Hence, while some epitope macaque-specific epitope responses are noted, the
335 majority of epitopes in our study have also been detected in human Mtb infection.

336 **Human Mtb epitopes also allow general coverage and analysis of T cell responses in Indian** 337 **rhesus macaques**

338 The results presented above demonstrate that the majority of epitopes in our study have also
339 been detected in human Mtb infection. We, therefore, hypothesized that conversely epitopes detected in
340 humans might also allow uniform coverage of Indian macaques in general. Accordingly, we generated
341 a pool of 300 epitopes previously recognized in human LTBI ⁹⁵. This pool contained 25 of the 54
342 epitopes seen in macaque and 9 epitopes with partial overlap (10aa) to the 54 epitopes. This human
343 epitope pool was first tested in splenocytes from the original 15 animals that were immunized and/or
344 infected with tuberculosis (**Table I**). In addition, we subsequently tested this pool in splenocytes

345 derived from the set of 8 new animals that were also immunized and infected with tuberculosis (**Table**
346 **I**), and an additional 11 naïve animals (**Table I**).

347 We found that the pool of human epitopes elicited significant responses in 13 of the 15 original
348 animals (Median 142 SFC/10⁶, IQR 47-198) (**Figure 3b**). Consistent with the notion that this epitope
349 set would allow general coverage in Rhesus macaques, we found that 6 out of 8 new animals were
350 associated with vigorous responses, with median of 171 SFC/10⁶ cells (IQR 48-426; **Figure 3b**). No
351 responses were observed in splenocytes from 9 of the 11 naïve animals tested, and weak responses
352 (<70 SFC/10⁶ cells) were observed for the remaining two (**Figure 3b**). These data suggest that the
353 human epitope pool represents an alternative strategy to allow general coverage and analysis of T cell
354 responses in Indian rhesus macaques.

355 **T cell responses associated with the human and macaque Mtb epitope pools**

356 As shown above, Mtb-specific responses in Indian macaques can be investigated using either
357 macaque or human-derived epitopes. As a way to further illustrate this point, we used intracellular
358 cytokine staining (ICS) assays. Specifically, we wanted to determine the CD4/CD8 phenotype of
359 responding T cells and to compare the magnitude of responses between the two epitope pools.
360 Accordingly, we tested the two pools in 7 animals that had been exposed to TB, and 3 naïve controls.

361 As expected, the responses were almost entirely associated with CD4⁺ T cells (**Figure 4A-C**).
362 This is an important point, as until these experiments were performed the CD4/Class II phenotype was
363 inferred on the basis of the epitope size and the fact that in humans *ex vivo* responses are vastly
364 dominated by this phenotype.

365 When the average magnitude of *ex vivo* responses to the two pools was compared, 0.1% (IQR
366 0.04-0.8%) of the total CD4⁺ T cells secreted IFN- γ in response to the pool of macaque epitopes, while
367 0.2% (IQR 0.04-0.3%) of the total CD4 cells responded in response to the pool of human epitopes.
368 Weak or no IFN- γ responses were detected from CD8⁺ T cells and naïve animals (**Figure 4A**). The
369 difference in response between the 54 epitope and the 300 epitope pools was not significant, but
370 nevertheless the 300 epitope pool tended to have a stronger response and lower background in naïve
371 animals. These data strengthens the feasibility of utilizing the human 300 epitope pool as an alternative
372 to analyze T cell responses against Mtb in Indian rhesus macaques.

373 **Rhesus and human Mtb epitopes also allow general coverage and analysis of T cell responses in** 374 **cynomolgus macaques**

375 The previous data demonstrated a high cross-reactivity between human and rhesus class II
376 molecules. Based on this observation, we reasoned that both the rhesus and human epitopes might also
377 be crossreactively recognized by cynomolgus macaques, the other widely used NHP species. To test

378 this hypothesis, we tested a set of cynomolgus macaques with latent TB infection (LTBI; n=8) and
379 active TB infection (n=8) with the pool of 54 rhesus epitopes and the 300 human epitope pool.

380 All of the cynomolgus macaques, irrespective of latent TB infection or active TB, tested
381 responded strongly to the pool of rhesus epitopes (Median 743 SFC/ 10^6 , IQR 236-1076 for LTBI and
382 Median 535 SFC/ 10^6 , IQR 471-689 for active TB) (**Figure 5a**). As shown in **Fig 5b**, responses of
383 LTBI and active TB individuals against the 300 human epitopes pool were also high, with median 761
384 SFC/ 10^6 (372-1075) and 743 SFC/ 10^6 (623-925), respectively. For both peptide pools tested, there
385 was no significant difference between the two groups (Mann Whitney test). In conclusion, these data
386 suggest that these epitope sets allow for broad coverage across multiple NHP species.

387 **Discussion:**

388 While NHP class I responses have been rather extensively characterized, much less is known at
389 level of MHC class II restricted responses. To our knowledge, this is the first comprehensive epitope
390 identification effort for NHPs in general, and in the context of Mtb in particular. We identified 54 new
391 epitopes, significantly expanding the number of specific epitopes that can be used to monitor immune
392 responses after vaccination and experimental infection. In fact, before this study, only one CD4⁺ T cell
393 epitope had been extensively characterized in NHP ⁸⁷. Of general importance, in the present study we
394 report that epitope repertoire of human and NHP class II overlaps significantly, to the point of allowing
395 use of human epitopes to detect responses in both rhesus and cynomolgus macaques, and the use of
396 rhesus epitopes to detect responses in cynomolgus macaques.

397 It is important to consider the molecular basis of the general epitope overlap described herein.
398 The most likely explanation resides in the extreme degree of sequence similarity between different
399 class II alleles in various primates. In many cases, different human and macaque alleles are more
400 similar to each other than the same human or macaque allele is to other alleles of the same species. For
401 example, the macaque class II alleles Mamu DRB*W1:01 and DRB*21:04 share 89.5% identity. Yet,
402 DRB*W1:01 shares 91.4% identity to HLA DRB1*14:07, while DRB*21:04 is 91.7% identical to
403 HLA DRB1*04:01. In fact, more similarity exist between different HLA DR and macaque DR alleles,
404 than between HLA DR and HLA DQ molecules ^{9, 100-102}. Furthermore, previous studies highlighted a
405 significant degree of overlap in the repertoire of peptides bound by different alleles of the same human
406 HLA class II locus, and even across HLA class II loci ⁴⁶. Accordingly promiscuous epitope recognition
407 across different alleles of a given locus or even across different HLA class II loci is not uncommon ^{46,}
408 ^{98, 103}. In addition, a functional overlap between HLA DR and macaque DR molecules has also been
409 reported ⁸.

410 It is also possible that peptides that are cross-presented and recognized by CD4⁺ T cells are
411 associated with special unique features. A recent study from our laboratory specifically investigated
412 prediction of epitopes promiscuously cross-recognized in humans (Paul et al., J. Imm. Methods, *in*
413 *press*). Future studies will address whether these features also extend to human-primate cross-reactive
414 epitopes.

415 Here, we found that the majority of TB epitopes we identified in NHPs has also been identified
416 in human studies ^{80, 86, 87, 104-110}. Indeed, we further show that a pool of epitopes previously identified in
417 humans could be used to measure immune responses after vaccination and experimental infection in
418 NHP. These results demonstrate that these reagents can be used to broadly characterize TB-specific

419 NHP responses. They further suggest that this approach might be generally used to characterize NHP
420 responses in other pathogen systems.

421 The observation that the repertoire of peptides recognized by CD4⁺ T cells restricted by specific
422 alleles is similar in macaques and humans could have suggest potential approaches to rapidly defining
423 epitope specificity and restriction of macaque responses. Though it is beyond the scope of this paper,
424 we predict that it should be feasible to find macaque class II molecules with the same binding motif as
425 a human class II molecule and show that the two consistently elicit the same T cell responses. Indeed,
426 we previously pointed out extensive similarities between Mamu-DRB*w201 and HLA-DRB1*0101
427 peptide binding repertoires ⁹.

428 In our study, we evaluated the mechanism underlying the observation that certain epitopes were
429 frequently recognized in the cohort of rhesus macaques analyzed. We concluded that the shared
430 responses were not due specific MHC class II allele(s), unexpectedly common due to common
431 pedigrees and inbreeding. Rather, the analysis demonstrates that in some cases these shared responses
432 are likely restricted by alleles commonly expressed in the general macaque population. In other
433 instances, based on the fact that responding macaques shared no MHC class II molecules, it is most
434 likely that promiscuous binding and restriction is associated with epitope recognition. While
435 promiscuous epitopes account for a large fraction of responses in humans ⁹⁸, this is the first account of
436 this phenomenon in the NHP in general, and in the rhesus macaque Mtb infection model in particular.

437 The large overlap in repertoire observed between macaques and humans is particularly
438 striking. While some precedence for this has been noted for MHC class I molecules ¹⁴, this has not
439 been generally noted for MHC class II because macaques epitopes have been most studied in the
440 context of SIV/HIV and HIV is used in humans and SIV in monkeys. This can also be interpreted in
441 the context of research addressing the coevolution of humans and Mtb. The results suggest that human
442 T cell epitopes within MTB are highly conserved, suggesting purifying selection acting on T cell
443 epitopes in MTB ¹¹¹. This has led to the hypothesis that MTB is benefiting from being recognized by T
444 cells, possibly because this response may drive lung inflammation and transmission of MTB. A
445 possible explanation for this resides in the fact that HLA and macaque MHC class II molecules dictate
446 a similar repertoire of epitope presentation. Indeed, the motifs and repertoires of human and macaque
447 DR are very similar ⁹.

448 The specific system we have chosen to perform our studies is TB infection and vaccination. The
449 current, commercially available, BCG vaccine is associated with variable effectiveness against Mtb
450 infection and disease, and is not indicated for immunocompromised individuals ¹¹²⁻¹¹⁴. Testing new TB
451 vaccines in NHP models is of considerable interest, to optimize schedule and administration regimens,

as well as comparison of different adjuvant and delivery strategies. Furthermore, accurate quantification of immune responses in the context of vaccinations associated with varied degrees of protection would allow identification of immunological correlates of protection. NHP models with the specific use of aerosolized Mtb have yielded the best parallel infection by modeling human outcomes^{72, 75}. However, most studies in have focused on the molecular biology of the pathogen after infection and whether vaccination had a direct impact on subsequent infection, with lesser emphasis on immunological characterizations^{75, 115-119}.

Several Mtb antigens immunodominant in humans^{84, 85, 103, 107, 114, 120-124} are included in the design of new TB vaccines. Herein, we tested the immunogenicity in rhesus macaques of a set of antigens that have been used in human clinical trials and diagnostics. Of these antigens, a subset has been shown to be protective in animal models (Rv3619c, Rv3620c, Rv2608), highly immunogenic in humans (Rv3875, Rv3874, Rv0288, Rv1196) and strongly recognized in the initial stages of infection in humans (Rv0288 and Rv3875)^{125, 126}. We show that several of these antigens, including Rv3875 (ESAT-6) and Rv3874 (CFP10), which are immunodominant in humans¹²⁷⁻¹³⁴, are also immunodominant in rhesus macaques. Our results therefore support the feasibility of evaluation in NHPs of vaccines based on these antigens. By the same token, we detected weak and inconsistent responses in the case of other vaccine antigen candidates, such as Rv0125, Rv1886c, Rv1813c, Rv3619c and Rv3620c. Even though the present study has expanded our understanding of the cellular immune response to selected antigens, the analysis is limited to Mtb antigens that are either immunodominant or that were considered potentially protective⁹⁵. This is a limitation of the study especially given that protection is not defined in the context of these studies. Therefore, caution should be exercised in interpreting results of vaccines based on these antigens in NHP, as the low immunogenicity in macaques might not allow extrapolating the results to humans.

In conclusion, this study highlights a previously unappreciated extensive overlap in epitope recognition at the level of class II responses in humans, rhesus macaques and cynomolgus macaques. This is particularly unique given the species differences and the varying routes of antigen exposure. The observation provides the basis for the development of epitope-based tools to study the immunodominance patterns in the context of novel vaccines as well as characterizing different outcomes of infection in NHP in any pathogen system, and potentially also in the context of immune intervention in cancer, autoimmunity and allergies. In particular, our findings have immediate applicability for the evaluation of new vaccines and diagnostics in the setting of non-human primate model of TB.

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992
993

994 **Table Ia. Immunization and infection regimens for Indian rhesus macaques that were initially**
995 **tested with all pools of peptides from antigens²**

996

<i>Animal #</i>	<i>Vaccination</i>	<i>Booster</i>	<i>Infection</i>	<i>2nd</i> <i>Infection</i>	<i>3rd</i> <i>Infection</i>
IB78	3D-BCG	MVA-Ag85B	Mtb CDC1551	-	-
GP21, II69	3D-BCG	MVA control	Mtb CDC1551	-	-
HN69, IJ42, HE06, II21	BCG-Aero	-	Mtb	-	-
IJ23, IN85, IM73, CK53, II07	SigH-Aero	-	Mtb	-	-
GI89, II43	-	-	Mtb-Mutant Pools	Mtb-Mutant Pools	SIV-Mac239
HB95	-	-	Mtb-Pool 1,2,3	-	-

997

² Each of the animals was infected with Mtb CDC1551. The animals were either vaccinated and/or immunized or considered a negative control. Immunizations consisted of the recombinant 3D-BCG strain provided by Dr. Doug Kernodle. The animals vaccinated with the 3D-BCG strain were boosted six weeks later with either a control MVA or MVA:Ag85B and then challenged six weeks after the boost with Mtb CDC1551 to deliver 1000 cfu into the lungs of macaques (86). Aerosol vaccination with a single, one time dose of BCG (Danish) (130, 131) as well as Mtb:Δ-sigH (CDC1551 background) (Kaushal et al, Nat Med in review) were performed in order to deliver ~5000 cfu of bacilli into the lungs of macaques. The animals were challenged 8 weeks after the single pulmonary vaccination with the identical dose of Mtb CDC1551 (1000 cfu delivered into the lungs of macaques). SIV co-infection was performed 9 weeks after initial low-dose (25-50 cfu delivered to the lungs of macaques) Mtb CDC1551 infection.

998 **Table Ib. Immunization and infection regimens for newly tested Indian rhesus macaques**
999

<i>Animal #</i>	<i>Vaccination</i>	<i>Booster</i>	<i>Infection</i>	<i>Infection</i>	<i>Infection</i>
GK87, CL10	-	-	<i>Mtb</i> <i>CDC1551</i>	-	-
CR57, EA97	BCG-Aero	-	<i>Mtb</i> <i>CDC1551</i>	-	-
FM29, DF44	SigH-Aero	-	<i>Mtb</i> <i>CDC1551</i>	-	-
HK78	3D-BCG	MVA- AG858	<i>Mtb</i> <i>CDC1551</i>	-	-
IL33	BCG-Aero	-	<i>Mtb</i>	-	-

1000

1001

1002 **Table Ic. Negative control Indian rhesus macaques.**

1003

<i>Animal #</i>	<i>Peptide pool tested</i>	<i>Vaccination</i>	<i>Booster</i>	<i>Infection</i>
EA43, FD79, EP16	Pool of 300	None	None	None
IB62, DR27, HE68, FT88, IM14, HT41, CT52, BK01	Pool of 54 and 300	None	None	None

1004

1005

1006 **Table II. List of Antigens tested in the present study³**

Antigen	Rv#	Vaccine trial	Reference
Mtb32A	Rv0125	Mtb72F	135
TB10.4	Rv0288	HyVac4, AERAS-402	136
Mtb39A	Rv1196	Mtb72F	135
	Rv1813c	ID93	137, 138
Ag85B	Rv1886c	H1, HyVac4, H56, AERAS-402	139, 140
	Rv2608	ID93	137, 138
EsxV	Rv3619c	ID93	137, 138
EsxW	Rv3620c	ID93	137, 138
CFP10	Rv3874		
ESAT-6	Rv3875	H1, H56	141-143

1007

1008

³ List of Tuberculosis antigens tested in this study. The antigens studied correspond to antigens currently considered as vaccine candidates, or included in the QuantiFERON-TB Gold In-tube test.

1009 **Table III. Epitopes detected in 2 or more animals⁴**

Antigen	Position	Sequence	Magnitude	Frequency	# Animals
Rv0125	306-320	AVDGAPINSATAMAD	83.3	14%	2
Rv3619c	76-90	QAAGNNMAQTDSAVG	183.3	14%	2
Rv3620c	16-30	AGRFEVHAQTVEDEA	103.3	14%	2
Rv3620c	61-75	NQAFRNIVNMLHGVR	80.0	14%	2
Rv3620c	76-90	DGLVRDANNYEQQEQ	75.0	14%	2
Rv3874	31-45	QVESTAGSLQGQWRG	83.3	14%	2
Rv3874	51-65	AQAAVVRFQEAANKQ	1146.6	21%	3
Rv3874	71-85	EISTNIRQAGVQYSR	745.0	36%	5
Rv0288	61-75	AMEDLVRAYHAMSSST	265.0	21%	3
Rv3620c	6-20	MTDPHAMRDMAGRFE	81.7	21%	3
Rv3620c	51-65	ATSLDTMTQMNQAFR	106.7	21%	3
Rv3875	1-15	MTEQQWNFAGIEAAA	2836.7	57%	8
Rv3875	51-65	YQGVQQKWDATATEL	375.0	29%	4

⁴ Epitope responses detected in at least two animals are listed, with specific information including antigen source, position, as well as specific information about the response elicited, including total magnitude as measured by SFCs in ELISPOT, frequency of positive animals and specific numbers of animals responding to epitope.

1012 **Table IV. MHC class II alleles present in more than two animals⁵**

Allele or cluster of alleles	Frequency
DRB1*03:03	33%
DRB1*03:09	20%
DRB1*04:06	20%
DRB*W21:04	20%
DQA1*26:g1 ¹	80%
DQA1*01:g2	40%
DQA1*01:g3	20%
DQA1*01:04	33%
DQB1*18:g1	60%
DQB1*06:01	40%
DQB1*06:g1	20%
DPA1*02:g2	40%
DPA1*02:g1	67%
DPA1*06	40%
DPB1*07:01	40%

1013

1014

⁵ Allele nomenclature including a :g designation reflects that a unique allele was not identified, that this includes a cluster of alleles.

1015 **Table V: Predicted Restrictions of TB Epitopes in Macaques⁶**

Antigen	Sequence	Allele	A+R+	A- R+	A+R-	A- R-	RF	P- value
Rv0288	AMEDLVRAYHA MSST	DPA1*02:						
		g2	3	0	3	9	2.5	0.044
		DPB1*07:						
		01	3	0	3	9	2.5	0.044
Rv3620c	ATSLDTMTQMN QAFR	DQB1*18:						
		g2	2	1	0	12	5.0	0.029
		DRB*W1:						
		g1	2	1	0	12	5.0	0.029
		DRB*W6:						
		02	2	1	0	12	5.0	0.029
		DRB*W6:						
		g2	2	1	0	12	5.0	0.029
Rv3874	EISTNIRQAGVQ YSR	DRB1*03:						
		g2	2	1	0	12	5.0	0.029
Rv3620c	MTDPHAMRDM AGRFE	DQB1*18:						
		g2	2	1	0	12	5.0	0.029
		DRB*W1:						
		g1	2	1	0	12	5.0	0.029
		DRB*W6:						
		02	2	1	0	12	5.0	0.029
		DRB*W6:						
		g2	2	1	0	12	5.0	0.029
		DRB1*03:						
		g2	2	1	0	12	5.0	0.029

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⁶ A+: genotyped HLA allele positive; A-: genotyped HLA allele negative; R+: epitope response positive; R-: epitope response negative. P value is calculated from Fisher exact test from A+R+, A-R+, A+R-, and A-R-.

1019 **Figure legends (captions to illustrations)**

1020 **Figure 1. T Cell Responses Detected for TB Antigens in Macaques.** Splenocytes from 15 individual
1021 macaques (Table I), were tested in ELISPOT assays to determine immunological responses to pools of
1022 overlapping peptides spanning ten common TB antigens. Splenocytes were incubated with 10 µg/ml
1023 per peptide, after which the number of IFN- γ^+ producing cells were enumerated in an ELISPOT assay.
1024 The total magnitude of response, as measured by total spot forming cells (SFC) is shown on the left y-
1025 axis, depicted in blue. Student's T-Test, $p < 0.05$. The frequency of animals (right y-axis) that responded
1026 to each specific antigen is depicted in red. Each individual peptide was tested in triplicate.

1027

1028 **Figure 2. Epitope profiles for each of the antigens.** Splenocytes from macaques (Table I) were
1029 incubated with 20 µg/ml per peptide, after which the number of IFN- γ^+ cells were enumerated in an
1030 ELISPOT assay. Each panel represents the peptides from the peptides from a unique antigen: a)
1031 Rv3875, b) Rv3874, c) Rv2608, d) Rv1886c, e) Rv1196, f) Rv3619c, g) Rv0125 and h) Rv0288. The total
1032 magnitude of response, as measured by total spot forming cells (SFC) is shown on the left y-axis,
1033 depicted in blue. Student's T-Test, $p < 0.05$. The x-axis represents the overlapping peptides tested
1034 (except for 2c, which depicts every other peptide). The frequency of animals (right y-axis) that
1035 responded to each specific antigen is depicted in red. Each individual peptide was tested in triplicate.

1036

1037 **Figure 3. Human epitopes allow general coverage and analysis of T cell responses in Indian**
1038 **rhesus macaques.** Splenocytes from Indian rhesus macaques (black dots, positive responses; white
1039 dots, negative responses as per criteria described in the materials and methods section, Table I) were
1040 incubated with 2µg/ml per peptide in pool, after which the number of IFN- γ^+ cells were enumerated in
1041 an ELISPOT assay. Each dot represents mean SFC/ 10^6 cells in one animal. Each individual pool was
1042 tested in triplicate. One-tailed Mann Whitney test, ****, $p < 0.0001$, **, $p < 0.001$, two-tailed Mann
1043 Whitney test, ns, no significant difference. A) Pool of 54 epitopes, defined in macaques, tested in TB
1044 animals (n=8) and naïve animals (n=8). B) Pool of 300 epitopes, defined in humans, tested in TB
1045 animals (n=15 + 8) and naïve animals (n=11).

1046

1047 **Figure 4. Mtb-specific CD4⁺ T cell responses can be analyzed using epitopes derived from human**
1048 **studies.** Epitope-specific IFN- γ production by splenocytes from Indian rhesus macaques (Table I)
1049 measured after 6 h peptide pool stimulation. A) % IFN- γ -producing CD4⁺ (black dots) or CD8⁺ (grey
1050 dots) T cells in response to the pool of 54 and 300 epitopes. TB animals (IB78, II69, GP21, IJ42, IM73,
1051 II43, GI89, n=7, left) and naïve animals (IB62, DR27, HE68, n=3, right). B and C) Representative dot

1052 plots from one TB animal (B) and one naïve animal (C). Plots are gated on total CD4⁺ (left panel) or
1053 CD8⁺ (right panel) T cells. Numbers indicate percentage of IFN- γ -producing cells.

1054

1055 **Figure 5. Human epitopes allow general coverage and analysis of T cell responses in cynomolgus**
1056 **macaques.** PBMC from cynomolgus macaques (Materials and Methods) were incubated with 2 μ g/ml
1057 per peptide in pool, after which the number of IFN- γ -producing cells were enumerated in an ELISPOT
1058 assay. Each dot represents mean SFC/10⁶ cells in one animal. Each individual pool was tested in
1059 triplicate. Two-tailed Mann Whitney test, ns, no significant difference. Peptide pools tested in LTBI
1060 animals (n=8) and animals with active TB (n=8). A) Pool of 54 epitopes B) Pool of 300 epitopes.

1061

1062

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

B