Immunological consequences of intragenus conservation of *Mycobacterium tuberculosis* T-cell epitopes

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A previous unbiased genome-wide analysis of CD4 *Mycobacterium tuberculosis* (MTB) recognition using peripheral blood mononuclear cells from individuals with latent MTB infection (LTBI) or nonexposed healthy controls (HCs) revealed that certain MTB sequences were unexpectedly recognized by HCs. In the present study, it was found that, based on their pattern of reactivity, epitopes could be divided into LTBI-specific, mixed reactivity, and HC-specific categories. This pattern corresponded to sequence conservation in nontuberculous mycobacteria (NTMs), suggesting environmental exposure as an underlying cause of differential reactivity. LTBI-specific epitopes were found to be hyperconserved, as previously reported, whereas the opposite was true for NTM conserved epitopes, suggesting that intragenus conservation also influences host pathogen adaptation. The biological relevance of this observation was demonstrated further by several observations. First, the T cells elicited by MTB/NTM cross-reactive epitopes in HCs were found mainly in a $CCR6^+CXCR3^+$ memory subset, similar to findings in LTBI individuals. Thus, both MTB and NTM appear to elicit a phenotypically similar T-cell response. Second, T cells reactive to MTB/NTM-conserved epitopes responded to naturally processed epitopes from MTB and NTMs, whereas T cells reactive to MTB-specific epitopes responded only to MTB. Third, cross-reactivity could be translated to antigen recognition. Several MTB candidate vaccine antigens were cross-reactive, but others were MTB-specific. Finally, NTM-specific epitopes that elicit T cells that recognize NTMs but not MTB were identified. These epitopes can be used to characterize T-cell responses to NTMs, eliminating the confounding factor of MTB cross-recognition and providing insights into vaccine design and evaluation.

tuberculosis | T-cell epitope | NTM | epitope conservation | T-cell subset

The group Actinobacteria contains the large genus *Mycobacteria* that includes not only the causative agent of tuberculosis, *Mycobacterium tuberculosis* (MTB), part of the MTB complex, but also *Mycobacterium leprae* and nontuberculous *Mycobacteria* (NTMs; also known as "environmental *Mycobacteria*," "atypical *Mycobacteria*," and "*Mycobacteria* other than tuberculosis") (1, 2).

MTB-derived epitopes recognized by human T cells have been shown to be hyperconserved relative to the rest of the genomes of the MTB complex (3). This hyperconservation suggests that these epitopes may act as decoys, diverting the immune response from recognizing more relevant MTB proteins and thereby favoring MTB persistence (4). However, the immunological consequence of the conservation of MTB sequences across other species of the *Mycobacteria* genus has received less attention at the level of the specific epitopes.

Several observations suggest reactivity to MTB in nonexposed [non–bacillus Calmette–Guérin (bCG)-vaccinated and non–MTB-infected) individuals. For example, a population in Malawi with no history or scar evidence of prior bCG vaccination or MTB infection showed responsiveness to a variety of mycobacterial antigens and purified protein derivative (PPD) from different NTMs (5, 6). In addition, non–MTB-infected and non–bCG-vaccinated individuals respond to MTB-encoded antigens, MTB lysate, and PPD (7, 8).

In particular, NTMs are a ubiquitous and heterogeneous group of environmental microorganisms found in soil, fresh water (including tap water), and seawater (9–12). Although several very rarely cause disease, most NTMs are opportunistic pathogens of humans, animals, poultry, and fish (9, 12, 13). Their prevalence in the environment results in regular human exposure to different species via different routes, and NTMs can be detected in the respiratory and gastrointestinal tract or on the skin of healthy individuals (12). This environmental exposure can influence resistance to MTB as well as interfere with or enhance the protective immune response to vaccination (14). Several lines of evidence suggest that differences in exposure to NTMs may be an important determinant of the variation in efficacy observed with bCG vaccination (15, 16).

Although there is substantial evidence for immune cross-reactivity between MTB and NTM bacteria (15–18), no studies have been performed at the level of the specific epitopes. Analyses of immune responses induced by NTM infection are limited, and the lack of NTM-specific reagents further complicates this issue. Several reports suggest that NTMs induce the

Significance

Mycobacterium tuberculosis (MTB) infection is one of the most common diseases worldwide. The *Mycobacteria* are a large bacterial family that includes MTB and nontuberculous mycobacteria commonly found in the environment (NTMs). We have shown that non–MTB-infected and non–bacillus Calmette–Guerin-vaccinated individuals nevertheless react to MTB-derived sequences. This reactivity can be explained by conservation of the epitope sequence in NTMs. Thus, the widespread exposure to various species of *Mycobacteria* influences reactivity to MTB and NTMs. We identified epitopes that are found only in NTMs, allowing dissociation of MTB- versus NTM-specific reactivity. These epitopes, in conjunction with epitopes specific to latent MTB infection, will provide a novel tool to study host–pathogen dynamics in the context of the design and evaluation of MTB vaccines and diagnostics.

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Data deposition: A complete list of epitopes is available from the Immune Epitope Database, www.iedb.org (submission ID 1000598).

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production not only of IFN-γ but also of TNF-α, IL-1β, and IL-6 (19-21). NTM-specific reagents to monitor influence of NTM exposure would be important for vaccine evaluations, development, and diagnostics alike.

Here, using data available from a genome-wide screen for MTB-derived epitopes, we characterized the response in individuals with latent MTB infection (LTBI) and nonexposed healthy controls (HCs) and investigated the role of epitope conservation. We found not only that HCs reacted to sequences derived from MTB but also that this reactivity correlated with conservation in NTMs and was mediated by the same $CD4^+CCR6^+CXCR3^+$ memory T-cell subset as in LTBI donors. Differential reactivity of the epitopes also extended to MTB and NTM epitopes generated by natural processing by antigen-presenting cells. In addition, we defined a set of NTM-specific epitopes not present in MTB. These findings have implications for the design and evaluation of vaccine candidates and diagnostic applications.

Results

Differential Reactivity of TB-Specific Epitopes in HC Individuals. We previously reported a genome-wide screen of MTB-derived predicted CD4 T-cell epitopes in LTBI donors (22). In that study MTB-uninfected/non–bCG-vaccinated donors recruited in the general San Diego area were used as HCs. For reference purposes, a summary of those already-published data are shown in Fig. 1*A*. Although the LTBI donors had a strong reactivity against selected epitopes, some degree of reactivity also was observed in the HC donors (22).

In the present study, we set out to analyze in more detail the differential patterns of reactivity in LTBI and HC individuals. Specifically, epitopes recognized by two or more donors were divided into different categories based on their recognition by these donor cohorts (Fig. 1*B*). LTBI-specific epitopes (those for which no reactivity was observed in any of the 31 HC donors tested) accounted for 72% of the epitopes and 62% of the total reactivity. Mixed epitopes (for which some reactivity was observed in both LTBI and HC donors) accounted for 21% of the epitopes and 36% of the reactivity. Interestingly, a few epitopes, which accounted for 7% of the epitopes and 2% of the reactivity, were recognized exclusively by HC individuals (HC-specific).

Differentially Reactive Epitopes also Are Differentially Conserved Within MTB Sequences. Previous work suggested that MTB epitopes are hyperconserved and that this conservation results from host–pathogen coadaptation (3). Following a methodology similar to that of Comas et al. (3), we evaluated the degree of conservation in different epitope groups: 112 LTBI-specific, 33 mixed, and 10 HC-specific. Nonsynonymous (dN) and synonymous (dS) substitutions were compared to assess the relative degree of sequence conservation in the antigens' epitope and nonepitope regions.

For LTBI-specific epitopes the dN/dS values for all regions were less than 1 (Fig. 1*C*), indicating that purifying selection plays a major role in the evolution of these regions. When the dN/dS values were compared among different genomic regions, it was confirmed that these epitopes are more conserved than are antigens and nonepitopes (Fig. 1*C*), similar to the previous study of Comas et al. (3).

Strikingly, no synonymous nucleotide substitutions were present in the mixed and HC-specific epitopes. Analysis of the nonsynonymous nucleotide substitution rates indicated that these epitopes are more variable than are antigens as a whole and nonepitopes (Fig. 1 *D* and *E*). In fact, in the case of HC-specific epitopes, they are significantly less conserved than are antigens and nonepitopes (Fig. 1*E*).

Taken together, these data indicate that the LTBI-specific epitopes are hyperconserved and that the opposite is true for the epitope sequences for which reactivity is detected in HCs.

Differential Reactivity Correlates with Conservation in NTM. We further hypothesized that the pattern of reactivity presented above might result from exposure to NTMs and that the reactivity seen in HCs might be directed against epitopes conserved in these strains. To test this hypothesis, we retrieved all complete NTM genome sequences that were publicly available at the time of analysis initiation (January 2012). A total of 15 NTM species were selected: *M. abscessus*, *M. avium* 104, *M. avium* subspecies *paratuberculosis* k 10, *M. gilvum* PYR-GCK, *M. marinum* M, *M. smegmatis* strain MC2 155, *M.* sp. JDM601, *M.* sp. JLS, *M.* sp. KMS, *M.* sp. MCS, *M.* sp. Spyr1 (gilvum), *M. ulcerans* Agy99, *M. vanbaalenii* PYR-1, *M. colombiense* CECT 3035, and

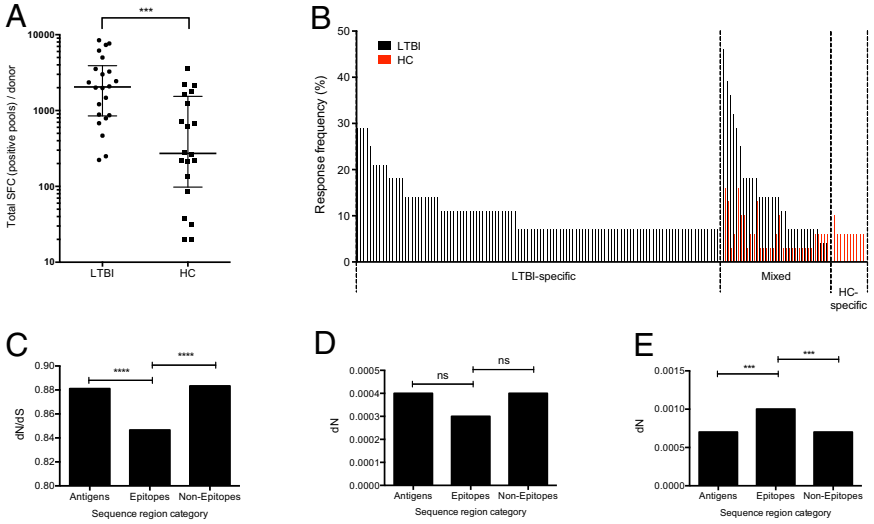

Fig. 1. Differential reactivity of epitopes in HC individuals. (*A*) Magnitude of epitope pool responses following genome-wide screen in individuals with LTBI ($n = 22$) and HC ($n = 20$). Each dot represents one donor; median ± interquartile range is indicated. ***$P < 0.001$, one-tailed Mann–Whitney test. (*B*) Epitopes recognized by two or more LTBI (black bars) and HC (red bars) donors divided into three categories of reactivity: LTBI-specific, mixed, and HC-specific. (*C*) dN/dS in LTBI-specific epitopes compared with nonepitope regions and antigens as a whole. ****$P < 0.0001$; Mann–Whitney test. (*D* and *E*) dN in mixed (*D*) and HC-specific (*E*) epitopes compared with nonepitope regions and antigens as a whole. ***$P < 0.001$; Mann–Whitney test. ns, no significant difference.

M. parascrofulaceum ATCC BAA-614. Next, the sequences of the 155 epitopes from Fig. 1*B* were compared with these full-genome sequences. The number of genomes in which each epitope was conserved was tabulated for each of the epitope classifications. To allow for likely cross-reactivity at the immunological level of closely related sequences, a single substitution was allowed per epitope.

The results of this analysis are shown in Fig. 2*A*. The LTBI-specific epitopes were clearly associated with low conservation, being found, on average, in 1.7 (11%) of the 15 NTM genomes, whereas the mixed epitopes were conserved in a significantly higher number (3.3, 22%; $P = 0.016$, unpaired t test). Even more strikingly, however, on average the HC-specific epitopes were conserved in almost 50% of the 15 genomes analyzed ($P < 0.0001$). The number of epitopes conserved in each strain appeared to correlate with how closely related the strains are in the phylogenetic tree. The highest numbers of instances of conservation were observed in *M. marinum* and *M. ulcerans*, which also are most closely related to MTB.

In conclusion, these results suggest that HCs cross-recognize MTB sequences and that this recognition correlates with the conservation of those sequences in NTMs.

Differential Recognition of Epitope Pools Based on Their Genus-Wide Conservation Features. Next, we undertook the converse experiment to test whether conservation across different mycobacteria species directed the specificity of recognition in different donor cohorts. Epitopes from Fig. 1*B* were classified into those found only in MTB (55 epitopes) and those also conserved in NTMs (69 epitopes) (Dataset S1). Epitope conservation in 44 classes of bacteria other than *Mycobacterium* was determined also, and these 51 broadly conserved epitopes were analyzed separately (Dataset S1). To maximize signal strength, pools containing broadly conserved epitopes included all epitopes identified, not only epitopes recognized by two or more donors (22).

Pools of ∼20 epitopes each were prepared and then tested for reactivity in 20 LTBI donors and 20 HCs. The total response per donor observed for the various epitope categories is shown in Fig. 2*B*.

As expected, significantly higher reactivity was detected in LTBI donors than in HCs, for both the MTB-only and the MTB/NTM-conserved pools. Significantly more reactivity was directed toward MTB/NTM-conserved sequences, suggesting that NTM exposure may boost the reactivity to conserved sequences. Conversely, for HC individuals, a trend toward higher responses was noted for the pools of epitopes conserved in NTMs.

Interestingly, for both LTBI and HC individuals the detected reactivity against broadly conserved epitopes was significantly less than that to MTB/NTM-conserved sequences. Very broad conservation of a peptide from MTB in other species could mean that it is conserved in the human proteome as well, so that lack of reactivity to the peptide in humans could be a consequence of self-tolerance. To examine this possibility, we compared the sequences of the peptides broadly conserved in other bacterial genomes with the human proteome. Less than 0.1% of these peptides were found to be conserved in humans, suggesting that mechanisms other than self-tolerance must underlie this effect.

The Antigenic Basis of Differential Reactivity. The data presented above imply that in a population with low exposure to the MTB complex, such as our donor cohort from the general San Diego area, preexisting immunity and occasional boosting might be provided by exposure to NTMs. In terms of vaccine design or evaluation, preexisting immunity might be viewed as desirable. Alternatively, in the context of the evaluation of vaccination strategies, preexisting immunity may contribute an undesired confounding factor. In this respect, the fact that the different epitope classes appear to be differentially conserved raises the possibility that they might be used differentially in pathogen-evasion strategies.

To examine this issue, epitope reactivity data next were mapped back to the specific antigens of provenance (22). Antigens were categorized further as LTBI-specific or cross-reactive as described in *Materials and Methods*.

Fig. 3 *A* and *B* shows the response frequency and magnitude of antigens currently included in tuberculosis (TB) vaccines in clinical development. As can be seen, vaccine antigens Rv0288, Rv1886c, Rv2608, and Rv3804c are widely cross-reactive, whereas antigens Rv1196, Rv3619c, Rv3620c, and Rv3875 appear to be exclusively LTBI-specific. Thus, a dichotomy exists in candidate vaccine antigens, with approximately half being classified as LTBI-specific and half being classified as cross-reactive.

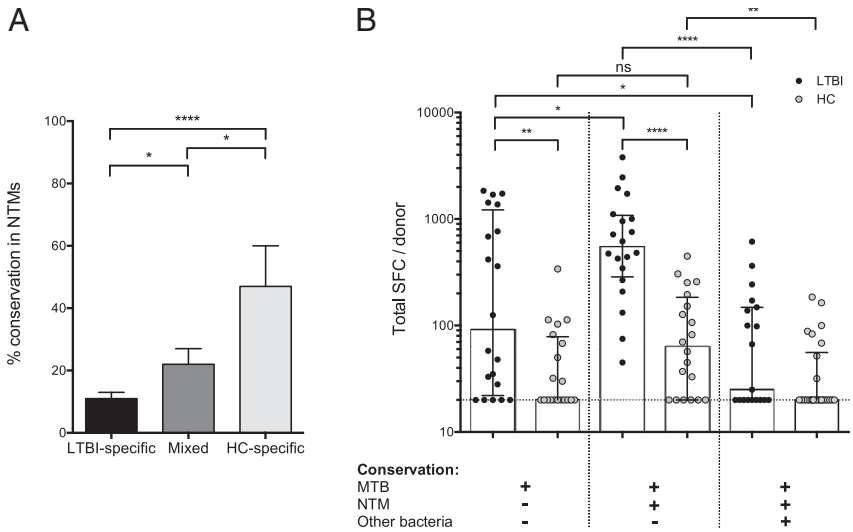

Fig. 2. Differential reactivity correlates with conservation. (*A*) Conservation of LTBI-specific, mixed, and HC-specific epitopes in 15 NTM. *$P < 0.05$, ****$P < 0.0001$, unpaired t test. (*B*) Magnitude of epitope pool responses following division based on conservation in MTB or MTB/NTM or broadly conserved across bacteria in LTBI ($n = 20$) and HC ($n = 20$) donors. Each dot represents one donor; the median ± interquartile range is indicated. The dashed line at 20 SFC indicates the threshold of positivity. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; one-tailed Mann–Whitney test. ns, not significant.

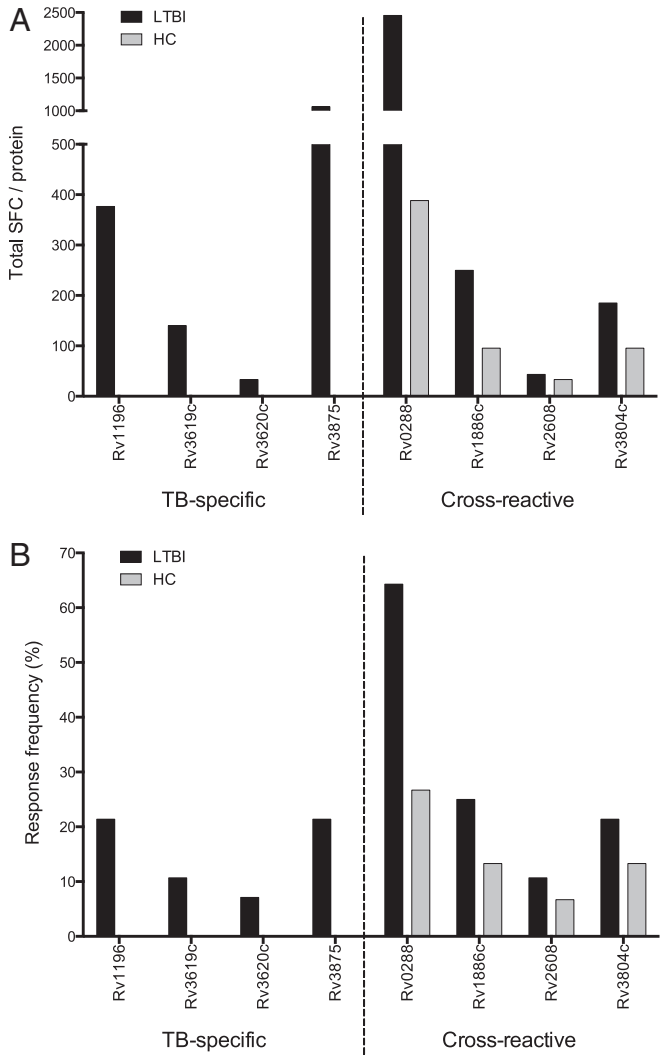

Fig. 3. Antigenic basis of differential reactivity. T-cell reactivity in LTBI (black bars) and HC (gay bars) donors to antigens currently included in TB vaccine trials divided in two categories of reactivity: LTBI-specific and cross-reactive. (*A*) Magnitude of response as total SFC per protein. (*B*) Percentage response frequency per protein.

CD4$^+$ T-Cell Responses to MTB/NTM-Conserved Epitopes in HC Donors Are Restricted to the CCR6$^+$CXCR3$^+$ T-Cell Subset. The results presented above demonstrate that TB epitopes whose sequences are conserved in NTMs also can be recognized by HCs. We next used the T-cell library approach (23) to investigate whether the T cells recognizing these sequences in HC donors are associated with a similar or dissimilar phenotype, compared with those recognizing the same sequences in LTBIs. For these experiments, different CD4 T-cell subsets were sorted (Fig. 4 *A* and *B*), corresponding to CXCR3$^+$CCR6$^+$ cells [previously described as identifying the majority of LTBI-derived MTB-reactive T cells (22)], CXCR3$^-$CCR6$^+$ cells (enriched for Th17), and CCR6$^-$ cells (the rest of memory T cells, enriched for Th1 and Th2). HC-derived T cells responding to an MTB/NTM epitope pool were highly enriched in cultures derived from the CXCR3$^+$CCR6$^+$ T-cell subset (Fig. 4*C*). This pattern of distribution was consistent in the three different HC donors analyzed, with 80% of the MTB/NTM-reactive memory CD4 T-cell response residing in the CXCR3$^+$CCR6$^+$ subset (Fig. 4*D*).

In conclusion, these data demonstrate that the MTB/NTM-specific T cells responding in HC donors are largely confined to the same CXCR3$^+$CCR6$^+$ subset previously described in LTBI donors.

Definition of NTM-Specific Epitopes. Because T-cell reactivity can be detected in HC individuals, presumably induced by exposure to NTMs, it should be possible to identify NTM-specific epitopes that are not conserved in MTB. To address this possibility, a total of 1,583 predicted HLA promiscuous binders, selected as described in *Materials and Methods*, were tested for ex vivo production of IFN-γ by peripheral blood mononuclear cells (PBMCs) from 30 HC individuals (Fig. 5*A*). A total of 106 individual NTM-epitopes, not present in MTB, were identified (Fig. 5*B* and Dataset S2). The top 20 epitopes account for 46% of the total response and are conserved, on average, in 40% of the 15 NTM strains. Individual donors recognized an average of seven epitopes (median of 1, range 0–79), underlining the exposure rate of NTMs in these individuals.

Next, the top 20 epitopes were pooled and tested further in 20 LTBI and 20 HC donors (Fig. 5*C*). As expected, the overall frequency and magnitude of response to the NTM-specific epitope pool was comparable in the two donor cohorts, presumably because both cohorts are exposed to NTMs.

Conserved NTM/MTB and NTM-Specific Epitopes Are Generated by Natural Processing of NTM Antigens. To demonstrate that the patterns of sequence conservation reflect the patterns of specificity observed in antigens derived from living bacteria and epitopes generated by natural processing, we again used the T-cell library approach. CCR6$^+$CXCR3$^+$ T-cell libraries reactive to *M. tuberculosis*, *M. avium* subspecies *paratuberculosis*, *M. marinum*, *M. avium*, and *M. abscessus* were established by expansion with heat-killed bacteria from these five representative mycobacterial strains (Fig. 6*A*) in three representative HC donors. Significant baseline reactivity against both MTB and NTMs exists in HCs and was detected by this approach.

Next, we investigated whether these mycobacteria-specific cultures recognized the MTB/NTM-conserved or NTM-specific epitope pools. For this purpose, T-cell libraries first were expanded using MTB or pooled representative NTM whole-cell bacteria (i.e., *M. abscessus*, *M. avium*, and *M. avium* subspecies *paratuberculosis*). Then the cultures were challenged with the MTB-specific, MTB/NTM-conserved, or NTM-specific peptide pools. The results clearly showed that (*i*) MTB-specific epitope pools are recognized exclusively by LTBI donors after MTB expansion, (*ii*) MTB/NTM-conserved pools are recognized by both LTBIs and HCs after MTB or NTM expansion, and (*iii*) the NTM-specific pool is recognized exclusively following NTM expansion (Fig. 6*B*).

Conversely, we also tested CCR6$^+$CXCR3$^+$ T cells expanded with the MTB/NTM-conserved (Fig. 6 *C* and *D*) or NTM-specific epitope pools (Fig. 6*D*) for recognition of the five mycobacterial strains. The MTB/NTM-specific cells proliferated in response to all strains, whereas NTM-specific cells proliferated only upon challenge with NTMs and not in response to MTB.

Collectively, these results demonstrate that MTB/NTM epitope pool-specific T cells respond, with varying degrees of cross-reactivity, to a variety of *Mycobacteria* strains and confirm that the T-cell reactivity elicited by the NTM-pool is NTM-specific. The results also demonstrate that the epitopes conserved in NTMs and MTB are generated by natural processing in a variety of different NTM bacteria.

Discussion

The *Mycobacteria* genus encompasses not only the well-known MTB and related species referred to as the "MTB complex" but also a series of other *Mycobacteria*, NTMs, which are closely related and seldom are pathogenic for humans in normal conditions, despite their ubiquitous presence. That these NTMs

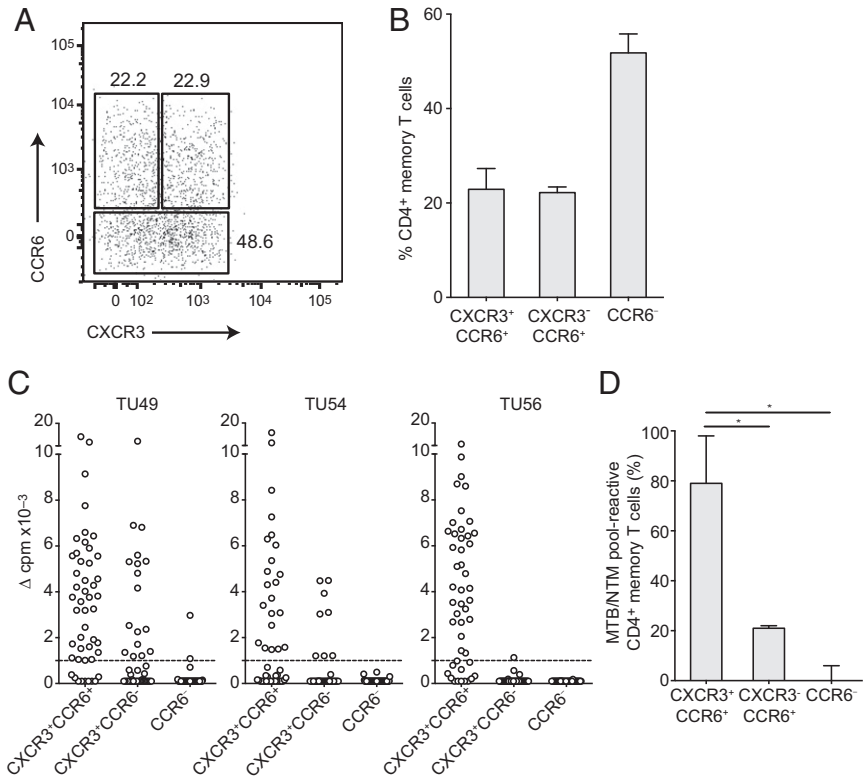

Fig. 4. MTB/NTM-specific T-cell responses in HC donors are restricted to a $CXCR3^+CCR6^+$ memory subset. (*A* and *B*) Three $CD45RA^-CD25^-CD4^+$ memory T-cell subsets from three HC donors were sorted: (*i*) $CCR6^+CXCR3^+$; (*ii*) $CCR6^+CXCR3^-$; and (*iii*) $CCR6^-$. (*A*) Representative dot plot from one donor. (*B*) Median percentages of the T-cell subsets on total $CD4^+$ memory T cells. Error bars indicate interquartile range. $n = 3$. (*C*) T-cell libraries were set up from the sorted subsets by polyclonal stimulation and were expanded for 3–4 wk. Libraries were analyzed for the presence of antigen-specific T cells by stimulation with the MTB/NTM epitope pool and measurement of ^{3}H-thymidine incorporation. Shown is proliferation (cpm) of individual cultures from three different donors. Dotted lines represent the cutoff value. (*D*) Distribution of epitope pool-specific T cells in the three memory T-cell subsets. Data represent median ± interquartile range from three donors. $*P < 0.05$; Mann–Whitney test.

might be cross-reactive with MTB-complex bacteria has been appreciated for several years (15–18), but until now the molecular mechanisms involved in this cross-reactivity were not addressed in detail. Our original interest in this field was sparked by the finding that HC (non–MTB-exposed, non–bCG- vaccinated) individuals reacted to MTB-derived epitope sequences, albeit with lower frequency and magnitude than did LTBI individuals. Our analysis revealed that this background reactivity is directed against epitopes conserved among MTB-complex bacteria and NTMs.

Our study provides an alternative, but not conflicting, dimension to the analysis of sequence conservation within the MTB species by analyzing conservation within the mycobacterial genus as a whole, and beyond as well. Indeed, we confirm the previous observation made by Comas et al. (3) that MTB epitopes are hyperconserved, suggesting that these epitopes might be used by MTB as a mechanism to generate a decoy immune response ultimately benefitting the bacteria. Totally unexpected was the observation that the epitopes with reactivity in both LTBI and HC

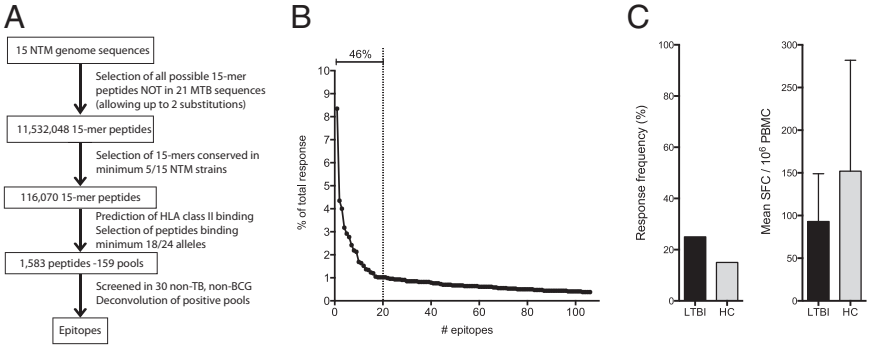

Fig. 5. Definition of NTM-specific epitopes. (*A*) Summary of the steps involved in the epitope identification pipeline showing number of genomes, 15-mer peptides, and selected peptides. (*B*) Identified epitopes plotted as a function of the percentage of the total response. Black dashed lines indicate the top 20 epitopes. The capped line indicates the percentage of total response captured by the top 20 epitopes. (*C*) T-cell reactivity in 20 LTBI and 20 HC donors to the top 20 NTM-specific epitopes. Shown are the response frequency (*Left*) and the average magnitude of response ± SD for responding donors (*Right*).

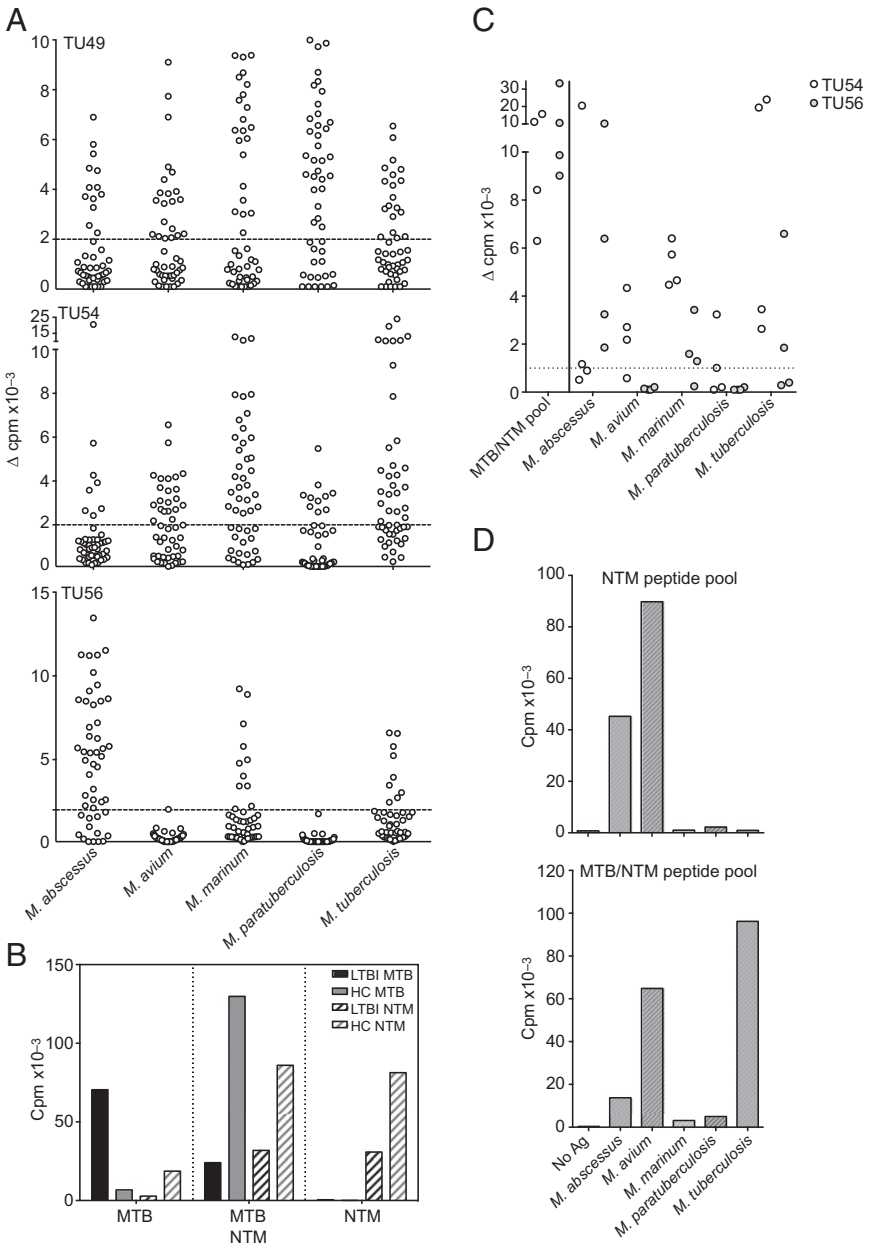

Fig. 6. Conserved and NTM-specific epitopes are generated by natural processing of NTM antigens. (*A*) Sorted CCR6^{+}CXCR3^{+} T cells from three representative HC donors were polyclonally expanded and analyzed for the presence of *Mycobacteria*-specific T cells by stimulation with irradiated autologous monocytes with or without heat-killed bacteria from the indicated mycobacterial strains. Shown is proliferation (cpm) of individual cultures from three different donors. Dotted lines represent the cutoff value. (*B*) Cells from one HC and one LTBI donor were stimulated with MTB lysate (solid bars) or a with pool of *M. abscessus*, *M. marinum,* and *M. avium* subspecies *paratuberculosis* lysates (hatched bars) for 5 d. CFSE^{lo} cells were sorted and restimulated with conserved MTB-, MTB/NTM, or NTM-specific epitope pools. Shown is proliferation (cpm). (*C*) Cells from two HCs (filled and open circles, respectively) were stimulated with the MTB/NTM epitope pool for 5 d. CFSE^{lo} cells were sorted and restimulated with the MTB/NTM epitope pool as a control and the indicated whole-cell lysates from *Mycobacteria*. Shown is the proliferation of individual cultures. The dotted line represents the cutoff value. (*D*) Sorted CCR6^{+}CXCR3^{+} T cells from one representative HC were stimulated with the MTB/NTM (*Lower*) or NTM-specific (*Upper*) epitope pool for 5 d. CFSE^{lo} cells were sorted and polyclonally expanded and were analyzed for the presence of *Mycobacteria*-specific T cells by stimulation with irradiated autologous monocytes with or without indicated mycobacterial strains. Shown is proliferation (cpm).

donors and conserved in NTMs are not hyperconserved. This finding is interesting, because it implies that NTM do not use the same T-cell recognition decoy strategy that has been hypothesized for MTB. Indeed, it is possible that, rather than acting as a decoy, the hyperconserved T-cell reactivity seen in MTB may reflect the need to stimulate the formation of the caseating granulomas needed for aerosol transmission of the pathogen (24). NTMs, on the other hand, do not require caseation (or infected hosts) for transmission, and therefore the capacity to induce hyperconserved T-cell reactivity may not have evolved in association with these mycobacterial species. This observation raises the speculation that, in the setting of MTB vaccination, aiming immune responses to MTB/NTM-conserved epitopes and antigens that apparently are not used or are used less often as a decoy by MTB might be more advantageous than aiming at the hyperconserved ones.

In addition, we have shown that NTM exposure is an important factor in shaping the immune reactivity against MTB and that intragenus as well as intraspecies epitope conservation should be taken into account when investigating species-specific responses. Recent studies have shown that this conservation is important in diverse settings, such as human herpesviruses and grass pollen allergy (25, 26). Chiu et al. (26) showed that CD8 T cells responding to a varicella zoster virus epitope also respond to homologous epitopes derived from viruses as divergent as α- and γ-herpesviruses, independent of previous infection (26). Similarly, Archila et al. (25) showed that pollen-derived cross-reactive epitopes can be found in a wide variety of grass species. Cross-reactivity of T cells rather than subclinical disease thus might be an explanation for detectable pathogen-specific reactivity in individuals that are negative for the pathogen.

The partial overlap with donors previously used is a limitation of the analysis. This limitation is important to note because, although it does not impact the analytic approach and extensive *in silico* analysis, it most likely does affect the final list of peptides/proteins identified as NTM-driven responses in HCs. One third of the donor cohort are new donors not previously investigated. Thus, the results have been at validated least partially in a different cohort. The final definitive list of peptides that reflect true pathogen versus environmental responses undoubtedly will be winnowed down in the years to come with the expansion of the mixed category. Furthermore, the list of cross-reactive epitopes identified herein is not exhaustive, and many more cross-reactive epitopes are likely to exist, because the degree of sequence identity required to activate cross-reactive T-cell responses is not easily predictable, and highly divergent epitopes can elicit cross-reactive responses (27). Indeed, epitope conservation can exist between structurally unrelated antigens with little sequence homology (28–30). We found here that epitopes conserved beyond the *Mycobacteria* genus are recognized less frequently than those conserved in MTB and NTMs. This less frequent recognition was not caused by scarcity of these broadly conserved epitopes in the peptide library studied. An alternative explanation for this phenomenon is that broad expression and conservation among different bacterial classes might lead to tolerization or exhaustion of T cells specific for those sequences or to the induction of T cells with regulatory activity. Ongoing studies are investigating this possibility in more detail.

Our data further imply that preexisting immunity and occasional boosting might be provided by exposure to NTMs, at least in a population with low exposure to the MTB complex, such as the one from the general San Diego area. In this context, it is noteworthy that other studies also have reported reactivity to MTB-encoded antigens in HCs and NTM-infected/exposed individuals that is hypothesized to be as a result of exposure to NTMs (7, 8, 31). Furthermore, NTM exposure may explain the higher baseline reactivity to PPD that was found in MTB-naive young adults as compared with children (32). Other studies highlighted that the majority of small MTB-PPD reactions could be attributed to NTM exposure or disease (33). Although the likelihood is strongly suggested by T-cell reactivity, we acknowledge there is no formal evidence that the HCs with positive responses actually were exposed to NTMs. Further studies are being planned to test individuals who have recovered from NTM infections to confirm that they have similar reactivity. In this respect, it can be pointed out that the difference in frequency of recognition of NTM-specific versus other nonpathogenic bacterial epitopes recognized also can be used to support this hypothesis.

Several lines of evidence suggest that differences in exposure to environmental mycobacteria may be an important determinant of the variation in efficacy observed with bCG vaccination (15, 17, 18, 34–36). NTM exposure may influence bCG protection by providing partial protection that bCG vaccination might improve only marginally. Exposure to NTMs might inhibit bCG replication and thereby prevent the induction of an efficient bCG-mediated immune response and corresponding protection against TB (15, 37, 38). Alternatively, it is possible that natural exposure to certain NTMs might induce an inappropriate immune response that is boosted by the bCG vaccine (39). If certain antigens were dominant in bCG vaccination but were recognized only weakly or not at all in TB infection, a bCG vaccine lacking those antigens might induce a more potent and cross-reactive response.

NTM-based preexisting immunity and boosting might be viewed as desirable in the context of vaccination strategies, especially if the response could be directed against less hyperconserved epitopes. Alternatively, it might contribute an undesired confounding factor in the evaluation of vaccination strategies. One of the cross-reactive antigens with clear reactivity detected in HCs is the Rv3804c antigen (Ag 85A). This antigen was selected on the basis of conservation among all mycobacterial species as a vaccine candidate, MVA85A (40). MVA85A recently was evaluated in phase II trials in which it was used to boost bCG vaccinees, but it failed to protect against MTB infection (41). Interestingly, response to this antigen has not yet been shown to be dominant following bCG vaccination in humans. The data presented here indicate that the Rv3804c antigen may be a rather dominant antigen in the context of NTM exposure and thus raise the possibility that boosting bCG responses with different antigens that are more dominant in bCG than in NTM might lead to a different outcome.

Interestingly, the memory T cells responsible for the reactivity in HCs have the $CCR6^{+}CXCR3^{+}$ phenotype previously described for LTBI and PPD-reactive individuals (22, 23, 42). The transcriptional signature of these cells shows that they express molecular features, such as *ABCB1* (MDR1), *KIT* (CD117), *CCR2*, *BAFF*, and *IL12RB2,* thought to be relevant for controlling chronic/latent infections (43). This finding predicts that NTM-based vaccines should elicit CD4 T-cell responses phenotypically similar to those elicited by MTB infection.

The data presented here demonstrate that it is possible to develop well-defined reagents to measure NTM exposure, thereby addressing fundamental questions about how NTM exposure influences the protective effect of existing and new vaccine candidates. Previous studies have investigated NTM exposure using PPD derived from NTM species (33, 44) and identified certain NTM-specific antigens (45). However, because of the degree of sequence homology between members of the MTB complex, the definition of entire antigens that are NTM-specific is, in general, problematic. Instead, the approach of defining specific epitopes allows one to dissect specific responses more easily.

In conclusion, this study describes significant reactivity to MTB-derived epitopes in the general population that is directed against epitopes conserved in MTB and NTMs and shows that intragenus epitope conservation is an important issue to be considered in addition to intraspecies epitope conservation. The study of intragenus epitope conservation promises to provide important insights into host–pathogen interactions, is relevant for diagnostic applications, and also might offer insights in the design and evaluation of MTB vaccine candidates.

Materials and Methods

Ethics Statement. Research conducted for this study was performed in accordance with approvals from the Institutional Review Board at the La Jolla Institute for Allergy and Immunology (FWA#00000032). All donors provided written informed consent before participation.

Study Subjects. Leukapheresis or whole-unit blood samples from 75 adults were obtained from the La Jolla Institute for Allergy and Immunology and the University of California, San Diego Antiviral Research Center Clinic. Donors were classified into LTBI and HC groups based on T-spot.TB reactivity. LTBI was confirmed by positive T-spot.TB as well as a physical examination and/or chest X-ray that was not consistent with active tuberculosis. None of the donors had been vaccinated with bCG, and all HCs had a negative T-spot.TB.

For the analysis of whether differential reactivity correlates with conservation as demonstrated in Fig. 2*B*, 18 of the 20 LTBI individuals and 9 of 20 HC donors were included in our previous study (22).

Peptides. The 15-mer peptides were synthesized as crude material on a small (1-mg) scale by Mimotopes and/or A and A. The Immune Epitope Database submission number for the NTM peptides is 1000598.

PBMC Isolation. PBMCs were purified by density-gradient centrifugation (Ficoll-Hypaque; Amersham Biosciences) from 100 mL of leukapheresis sample or 450 mL of whole blood, according to the manufacturer's instructions. Cells were cryo-preserved in liquid nitrogen suspended in FBS (Gemini Bio-Products) containing 10% (vol/vol) DMSO.

Ex Vivo IFN-γ ELISpot Assay. ELISpot assays were performed as previously described (22). Briefly, PBMCs were plated at a density of 2×10^5 cells per well in 96-well plates (Immobilon-P; Millipore) coated with anti–IFN-γ (AN18; Mabtech). PBMCs were stimulated with peptide pools (5 μg/mL), individual peptides (10 μg/mL), phytohemagglutinin (PHA; 10 μg/mL), or medium containing DMSO corresponding to the percent of DMSO in the peptide pool dilutions. Each condition was tested in triplicate wells. Plates were incubated at 37 °C for 20 h, after which wells were washed with PBS/0.05% (vol/vol) Tween 20 and incubated with 2 μg/mL biotinylated anti–IFN-γ (R4-6A2; Mabtech) for 2 h at 37 °C. Spots were developed using Vectastain ABC peroxidase (Vector Laboratories) followed by 3-amino-9ethylcarbazole (Sigma-Aldrich) and Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). Spots were counted using computer-assisted image analysis (KS-ELISPOT reader; Zeiss). For a response to be considered positive, three criteria had to be fulfilled: net spot-forming cells (SFC) per $10^6 \geq 20$, stimulation index ≥ 2 (sample divided by negative control), and $P < 0.05$ (mean of triplicate values of the sample vs. the negative control; Student's *t* test).

T-Cell Library. T-cell library assays were performed as previously described (23). CD4 T cells were isolated from PBMCs by positive selection with microbeads (Miltenyi Biotec). Memory $CD4^+$ T-cell subsets were sorted with a FACSAria (BD Biosciences) to over 98% purity excluding $CD45RA^+$, $CD25^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. Antibodies used for positive selection were anti–CCR6-PE (11A9; BD Pharmingen), anti–CCR4-PE-Cy7 (1G1; BD Pharmingen) and anti–CXCR3-APC (G025H7; BioLegend). Cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate, penicillin (50 U/mL), streptomycin (50 μg/mL) (all from Invitrogen), and 5% (vol/vol) heat-inactivated human serum (Swiss Red Cross). T cells (1,000 cells per well) were stimulated polyclonally with 1 μg/mL PHA (Remel) in the presence of irradiated (45 Gy) allogeneic feeder cells (1.0×10^5 cells per well) and IL-2 (500 IU/mL) in a 96-well plate format, and T-cell lines were expanded as previously described (23). Library screening was performed at day 14–21 by culturing extensively washed T cells ($\sim 2.5 \times 10^5$ cells per well) with irradiated autologous monocytes (2.5×10^4), either unpulsed or pulsed for 3 h with *Mycobacteria* whole particles (ratio 1:3). Microbes were killed by heating at 95 °C for 10 min according to standard methods. In some experiments, T cells were cultured with peptide pools (2 μg/mL). Proliferation was measured on day 2 or 3 after 16-h incubation with 1 μCi/mL [methyl-^{3}H]-thymidine (Perkin-Elmer). Precursor frequencies were calculated based on numbers of negative wells according to the Poisson distribution and are expressed per million cells.

In some experiments, carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells were cultured with peptide pools (2 μg/mL) or with heat-killed mycobacteria. On day 5 $CFSE^{lo}$ cells were sorted, and cells were stimulated polyclonally with mycobacterial strains or with peptide pools, and libraries were screened as described above.

Categorization of Antigens. Antigens recognized by two or more donors (i.e., epitope reactivity mapping to specific antigens) were classified based on frequency and magnitude (total SFC) of response. If both the frequency and magnitude of response were at least fourfold greater in LTBI individuals than in HCs, the antigen was determined to be LTBI-specific. All other antigens were categorized as cross-reactive.

NTM-Specific Epitope Identification. A summary of the epitope identification pipeline is shown in Fig. 5*A*. All possible 15-mers were selected from 15 strains of NTM [*M. abscessus*, *M. avium* 104, *M. avium* subspecies *paratuberculosis* k 10, *M. gilvum* PYR-GCK, *M. marinum* M, *M. smegmatis* strain MC2 155, *M.* sp. JDM601, *M.* sp. JLS, *M.* sp. KMS, *M.* sp. MCS, *M.* sp. Spyr1 (gilvum), *M. ulcerans* Agy99, *M. vanbaalenii* PYR-1, *M. colombiense* CECT 3035, and *M. parascrofulaceum* ATCC BAA-614]. Next, all peptides absent from 21 MTB strains (22) were selected, allowing up to two substitutions. This selection resulted in a total of 11,532,048 peptides. Of these, all 15-mers conserved in 5 or more of the 15 strains were selected for a total of 116,070 peptides. The binding affinity of each peptide then was predicted for a panel of 24 HLA class II alleles (46). Finally, the 1,583 15-mer peptides with the highest predicted binding promiscuity were synthesized and screened for recognition in donor samples using an IFN-γ ELISpot assay.

Calculation of dN/dS. Nucleotide sequence concatenates were made for epitope and nonepitope regions from 21 MTB strains (22). The number of nonsynonymous nucleotide substitutions (resulting in amino acid sequence change) per nonsynonymous site (dN) and the number of synonymous nucleotide substitutions (no amino acid change) per synonymous site (dS) were calculated by the Nei–Gojobori method (47) as implemented in the MEGA5 program (48).

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