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A model of phenotypic susceptibility to Tuberculosis: Deficient in silico selection of Mycobacterium tuberculosis epitopes by HLA alleles

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Abstract. HLA-DR allelic variants have been associated with tuberculosis (TB) susceptibility in different populations with risk ratios of 3.7 to 7.2. We hypothesized that the genetic susceptibility to TB depends upon the reduced capability of HLA-class II alleles of TB patients to bind and select peptide antigen from the Mycobacterium tuberculosis (MTB) expressed genome. To test this hypothesis, we developed a software that can predict HLA-DR restricted epitopes within the whole MTB genome based on quantitative peptide binding matrices. We analyzed the number of MTB epitopes recognized in two previously described populations of TB patients and matched controls and in a control population comprised of individuals affected by a sarcoid-like granuloma induced by beryllium and by healthy exposed controls. The number of putative epitopes within the whole MTB genome which could be bound by any HLA-DR allele (HLA-DR immunome of MTB) was 405,422 out of 1,304,277 possible 9-mers i.e., 31.08% of the global capability, instead of the expected 35%. When tested at an affinity level equivalent of the 1% of the best binder peptides, the HLA-DR alleles (HLA-DRB1*0801, *0802, *1401, *1501 and *1502) associated with TB susceptibility recognized a significantly lower mean number of MTB-epitopes (7,862±4,258) than the MTB-epitopes recognized by HLA-DR alleles (HLA-DRB1*0301, *0701, *1101, *1102, *1301 and *1302) negatively associated with TB (11,376±1,984, $p<0.032$). The number of epitopes bound at high affinity out of the whole MTB genome by the combination of the two HLA-DR alleles carried by each individual was lower in TB patients [TB-population 1: 11,341±908 (mean+SEM); TB-population 2: 15,303±657] than in matched healthy controls (CTR-population 1: 13,587±605, $p<0.03$ vs TB-population 1; CTR-population 2: 1,6841±555, $p<0.04$ vs TB-population 2). No difference was seen in individuals with the sarcoid-like granuloma induced by beryllium compared to the exposed healthy (beryllium-hypersensitivity: 17,593+447; controls 18,014±421; $p=0.57$). The data suggest that HLA-DR alleles associated with susceptibility to tuberculosis may be endowed with a reduced capability to bind at high affinity T-cell epitopes and select them for antigen presentation. The same alleles may contribute to determine the reaction to mycobacteria in non tuberculous granulomatous disorders. *(Sarcoidosis Vasc Diffuse Lung Dis 2008; 25: 21-28)*

Key words: tuberculosis, susceptibility, HLA, epitope prediction, T-cell response, sarcoid-like granulomas

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Introduction

Classic tuberculosis (TB) epidemiologic studies indicate that the genetic background may play an important role in susceptibility to TB (1). With the expansion of molecular genetics studies, a large number of genes have been associated with TB, leading to think that susceptibility might be the result of an imbalance between the effects of susceptibility genes and of protective genes such as the natural resistance-associated macrophage protein Nramp1, the IFN-α receptor 1, the IL-12 receptor β1 genes and the HLA genes. In this regard, a number of allelic variants of the HLA locus which have been associated with TB risk in population studies indicating DQB1*05, *06 and DRB1*08, *14, *15 and *16 as susceptibility genes with risk ratios ranging from 3.7 to 7.2, and HLA-DRB1*03, *07, *11 and *13 as "protective" genes (2-10).

HLA genes code for surface receptors that are known to play a pivotal role in the generation of antimicrobial immunity (11). HLA class II proteins (HLA-DP, -DQ and -DR) bind peptides derived from the digestion of microbes in the phago-lysosome of antigen presenting cells, carry them to the cell surface and present them to cytokine-producing CD4 T-cells. Structure-function studies of HLA class II molecules have indicated that the selection of antigenic peptides by the HLA receptors is dictated by the chemico-physical interaction between the amino acid side chains lining receptor-like pockets on the floor of the HLA antigen binding groove and the the agretopes i.e., the aminoacid side chains of the antigenic peptides (12, 13).

As most of the polymorphisms generating allelic variability of the HLA molecules code for aminoacid changes in the peptide binding groove's pockets, each HLA allele will bind a unique set of aminoacid side chains hence selecting a discrete set of antigenic peptides for antigen presentation (14, 15). As a consequence, the ability of HLA alleles to select a peptide antigen repertoire from a given microbe for antigen presentation, hence to induce a protective immune response, may vary widely, leading to greater susceptibility to infection of the subjects carrying the HLA alleles less efficiently binding and presenting antigens (16).

In this context though, since each individual co-expresses at least 2 HLA-DR molecules on the cell surface of the APC, it is reasonable to think that individual susceptibility to infection shall be determined by the capability of each subject's two HLA-DR molecules combined to recognize microbial antigen epitopes, rather than the carriage of a single susceptibility allele. Moreover, among the HLA-DR alleles, there is a predominant expression of HLA-DRB1 alleles, being expressed at a level five times higher than its paralogues DRB3, DRB4 and DRB5 (17-19).

The assessment of HLA-associated susceptibility to TB, as model for susceptibility to granulomatous disorder mediated by mycobacteria, might thus require an analysis of disease associated phenotypes instead of disease associated alleles.

To assess this hypothesis, we took advantage of bioinformatics tools allowing the identification of antigenic peptides in whole microbial genomes by quantitative peptide binding motifs analysis for the HLA alleles (20). We developed software that can predict HLA-DR restricted epitopes in the whole MTB genome based on quantitative implemented peptide binding matrices and used this tool to determine the number of epitopes potentially recognized in the MTB genome in two already described populations of TB patients and matched healthy controls (21, 22). In addition, a population of patients affected by the sarcoid-like granulomatous reaction induced by beryllium and matched beryllium-exposed subjects (23).

Methods

Patients' characteristics:

The study populations was composed by the TB patients and matched controls of two already described separate reports on the genetic susceptibility to TB in which HLA-DR high resolution typing was available for all study subjects (21,22). A population of patients with beryllium hypersensitivity and matched beryllium-exposed unaffected subjects were used as disease control population (23).

They were 160 patients with tuberculosis (TB-population 1), and 200 controls (CTR-population 1) (see table 1) included in the Kim HS et al. study (22) and the 127 patients with tuberculosis (TB-population 2) and 120 matched controls (CTR-population 2) included in the Vejbaesya S et al. study (21) (see

table 1). The disease control population included 74 subjects with beryllium hypersensitivity (BeH) and 86 beryllium exposed matched controls (Be-CTR) from the Amicosante et al. study (23).

The quantitative implemented peptide binding motifs are available only for 52 over more than 300 HLA-DRB1 alleles (24, 25). All together they may cover, with at least one allele, about 90% of the HLA-DR variability of different human populations (26). For the purpose of this study, only the subjects carrying both HLA-DR alleles with an available HLA-DR binding motif were used (see table 1). Specifically, 106 out of 320 (33%) control subjects and 85 out of 287 (30%) TB patients could be subjected to the analysis, as 168 (52%) controls and 151 (53%) TB patients had only one HLA-DR allele with an available binding motif, while 48 (15%) controls and 51 (18%) TB patients had both HLA-DR alleles without an available binding motif. Consequently, the alleles which were analyzed in the study populations were: HLA-DRB1* 0101, 0102, 0301, 0401, 0402, 0404, 0405, 0408, 0410, 0701, 0802, 0806, 1101, 1102, 1104, 1106, 1301, 1302, 1307, 1401, 1501, 1502.

The selected subgroups did not differ for demographical characteristics from the subgroups of subjects excluded from the study for having one or both HLA-DR alleles without a known binding motif (data not shown).

Genomes

The genome of *Mycobacterium tuberculosis* H37Rv strain (NC 000962.2) composed of 4048 genes transcribed into 3,989 proteins and the genome of *Escherichia coli* K12 (NC 000913.2) were used in this study for the immuno-informatic analysis.

Software for the identification and enumeration of epitopes in whole genomes

To enumerate the T-cell epitopes present in data sets of proteins as large as a microbial genome, we developed a software for the identification and enumeration of peptide binding epitopes to HLA-DR molecules. This software was developed on LabView platform (National Instruments, US) using a graphic language. Basically, it is an open system in which two different databases are uploaded and crossed.

Briefly, the first input database is represented by the protein sequences that can be uploaded from a file in FASTA format. The second database is represented by matrice(s) describing the peptide binding capabilities of the HLA-DR alleles under analysis. The present version of the software is equipped with a set of 52 additional matrices for HLA-DR peptide binding profiles (15, 27).

The matrices database includes also the threshold values for different affinity levels as reported in the original packages. The distribution of the matrix results of all the 20^9 possible peptides that can theoretically bound HLA-DR molecule is automatically generated as well as theoretical affinity thresholds. The analysis can be customized by selecting single/multiple proteins and HLA alleles among the set of data loaded, threshold and other parameters. The software generates all the possible nonamer peptides in a protein sequence and analyse them on the HLA-peptide binding matrices in analysis. For each protein and each HLA allele, all the peptides presenting a permissive

Table 1. Study populations.

Population	Group	Cases (N)	Subjects with both HLA-DR alleles with known peptide binding motif	Reference
TB population 1	TB	160	48	Kim HS, Hum Immunol 2005 (15)
	CTR	200	65	
TB population 2	TB	127	37	Vejbaesya S Eur J Immunogenet 2002 (14)
	CTR	120	39	
Control population	Be-hypersensitive	74	65	Amicosante M, Respir Res 2005 (16)
	Be-exposed controls	86	70	

Numbers of subjects for each study population, used for the epitopes prediction analysis; the reference of the study of origin is reported on the left column. Be: beryllium.

aminoacid (AA) in relative position P1 are stored in memory together with its matrix score and relative position in the protein.

For the purpose of this work, two analysis were implemented in the software. A first analysis is represented by the identification of the epitopes recognised, in each single protein of the data set at the affinity threshold applied for each HLA-DR allele in analysis. This allows the enumeration of the epitopes in the whole data set and the identification and enumeration of proteins that present a defined number of epitopes, such as the proteins that are putatively not recognised by the HLA-DR allele at the affinity threshold used, as they present zero epitopes. A second specific analysis for the identification of the epitopes recognised in a set of proteins by 2 HLA-alleles together has been developed to mime the situation of the HLA-DR recognition in a single subject. In this analysis, the software identify and enumerate the common and the different peptide epitopes recognised by the two HLA-DR alleles of the subject under evaluation by the position in the test set of proteins at the threshold of affinity applied.

The whole MTB and *E. coli* genomes were analysed for the HLA-DR alleles negatively and positively associated to TB and for the enumeration of epitopes recognized by single subjects in analysis at the different thresholds of affinities equivalent to the 1%, 2%, 3%, 4% and 5% of the best binding natural peptides for HLA-DR alleles (27). The Threshold of affinity is a preselected numerical value used to differentiate between binders and non binders, any peptide frame scoring higher than this value is predicted as binder or vice versa; it correlates with the peptide score (15) and therefore with HLA-ligand interaction, therefore it is an indicator for the likelihood that predicted peptide is capable of binding to a given HLA-molecule. To express the results, we have chosen, the percentage of 1% in order to lower the false positive rate.

The number of epitopes recognized by each study subject has been evaluated both as absolute number and as a relative number respect the amount of MTB epitopes in the whole genome.

Statistical analysis

All the data are expressed as mean ± standard deviation of the mean (SD). Comparisons between groups are made by Student's t test.

Results

1. *The immunome of MTB H37Rv*

HLA-DR molecules bind a core of nine aminoacids long protein fragments, when they carry in the relative position 1 (hereafter named P1) non-polar residues (I, L, M, F, W, Y, V) i.e., 7 out of 20 aminoacids or 35% of all the aminoacids. Analysis of the genome of *M. tuberculosis* H37Rv strain (NC_000962), which comprises 4,048 genes that can be transcribed into 3,989 proteins, allowed to estimate that, independently of the relative affinity of the epitopes for the HLA-DR molecules, the number of putative epitopes capable of binding any HLA-DR allele in the whole MTB genome was 405,422 out of 1,304,277 possible 9-mers, that we define as the HLA-DR MTB-immunome. This indicates that the HLA-DR MTB-immunome encompasses 31.08% of the whole MTB nonamer population. This number is equivalent to the 88% of the expected theoretical recognition that is equivalent to 35% of the all the peptides, thus suggesting that MTB encompasses a lower number of epitopes than expected if its genome presented a normal aminoacid distribution.

Differently from MTB, the number of putative epitopes capable of binding any HLA-DR allele in the *E.coli* genome was 537,294 out of 1,566,080 nonamers (34.30%). This is equivalent to 98% of the expected theoretical recognition, a fraction that is significantly higher than that of the MTB HLA-DR immunome ($p<0.0001$).

2. Impaired MTB proteins recognition by HLA-DR alleles associated with TB susceptibility.

HLA-DR alleles HLA-DRB1*0801, *0802, *1401, *1501, *1502, which have been associated with TB susceptibility in previous studies, recognized significantly lower number of MTB-epitopes (7,862±4,258) than the HLA-DR alleles HLA-DRB1*0301, *0701, *1101, *1102, *1301 and *1302, associated with TB resistance [11,376±1,984 ($P<0.032$)], at the affinity level of 1%. Consequently, there was a significantly higher number of MTB proteins (1,268±686) which could not be recognized by the HLA-DR alleles associated with TB susceptibility compared to HLA-DR alleles negatively associated with TB (776±232; $p<0.001$).

3. *Phenotypic analysis of the ability of HLA-DR alleles of TB patients to bind whole MTB genome peptides.*

When this analysis was applied to those individual subjects carrying a pair of HLA-DR alleles with known peptide-binding motifs, the number of MTB epitopes recognized by TB patients in both populations was significantly lower than that recognized by controls [TB-population 1: 15,303±657, CTR-population 1: 16,841±555; p=0.038 compared to TB population 1; TB-population 2: 11,341±908, CTR-population 2: 13,587±605, p=0.035 compared to TB-population 2 (Figure 1 panel A)].

Interestingly, when a population of individuals affected by the sarcoid-like granulomatous reaction to beryllium was analyzed as a control, no differences where observed between granuloma-affected and unaffected subjects in their recognition ability of MTB genome peptides (beryllium-hypersensitivity: 17,593±447; beryllium-exposed controls 18,014±421; p=0.608).

Finally, when the the three population groups were tested for their ability to recognize *E. coli* genome peptides, no differences were seen between TB and their matched controls nor between Be-hypersensitive subjects and their matched controls (figure 1 panel B), suggesting that the epitope binding defect seen in TB patients was restricted to, or more pronounced for, the MTB genome.

Discussion

The binding of antigenic peptides by the host HLA proteins expressed by antigen presenting cells is thought to represents a limiting step in the development of an antimicrobial immune response. In the context of the importance of HLA-DR genes in the immune response to MTB (28, 29) and of the observations positively or negatively linking different alleles of the HLA-DR, HLA-DQ, HLA-DP, and HLA class I genes to TB susceptibility in HLA association studies (10), it is reasonable to think that altered peptide binding by susceptible HLA molecules may be the cause of susceptibility to disease (11).

With this as a background and in the context that TB susceptibility is increased in homozygous, compared to heterozygous twins (30), it is reasonable to hypothesize that a deficient antigen recognition capability of the immune system might be at the basis of the inefficacious response to MTB in susceptible individuals, and that the determinant of susceptibility to MTB infection and disease may be the combination of the recognition abilities of both HLA-DR alleles expressed by each subject, i.e., the HLA-DR phenotype. The finding of this *in silico* model that the number of MTB epitopes recognized by the combination of the two HLA-DR alleles by TB subject was significantly lower than the matched

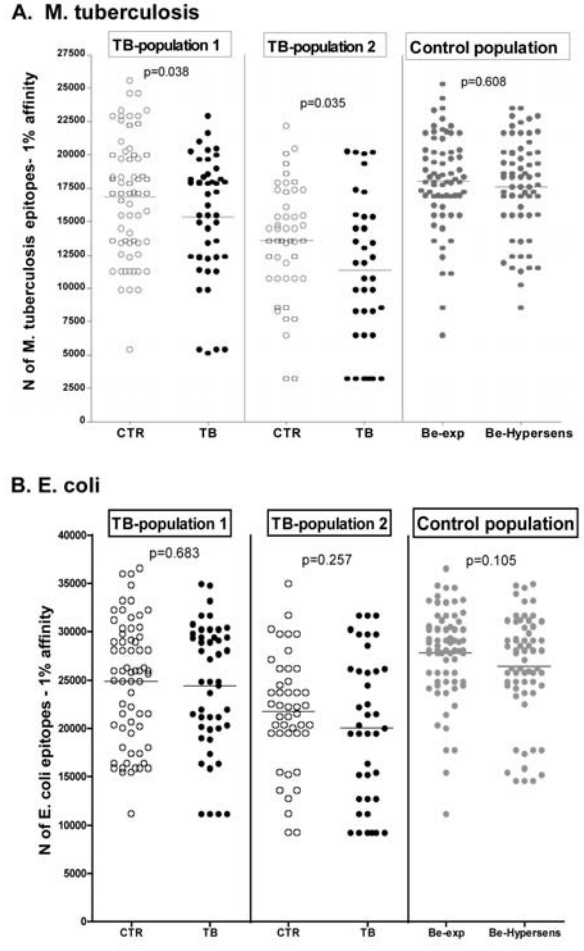

Fig. 1. Total number of epitopes recognized by *M. tuberculosis* and *E. coli*.
The number of *M. tuberculosis* (panel A) and *E. coli* (panel B) epitopes recognised in silico by each study subject with the combination of the two carried HLA-DR alleles at the threshold affinity of 1% in the two genomes. The three populations evaluated (TB-population 1, TB-population 2, and Be-exposed control population) are presented separately. Open circles, control subjects; closed circles, TB patients; grey circles Be-exposed control population. p value has been determined by Student's t-test.

controls in both study populations suggest that TB patients present may be affected by a deficient capability of recognition of the MTB proteome compared to the non affected subjects in the population.

Interestingly, Delgado et al. have recently reported a highly significant association between progressive pulmonary TB and homozygosity for HLA-DQ beta57-Asp alleles where a single polymorphism in the HLA-DQ beta chain played a critical role in the binding of ESAT-6, a highly immunogenic MTB protein and in the ensuing CD4+ T-cells immune response. Although they do not explain the mechanism of susceptibility to the development active TB, these data provided a functional link between an HLA polymorphism and susceptibility to progressive tuberculosis infection (31).

In contrast to infection, current concepts are that in hypersensitivity and autoimmune diseases susceptibility is associated with excessive HLA binding or the binding of specific (neo)-antigens by the HLA allele associated to the diseases (32).

In this context, it has been shown that the immune reaction to non-tuberculous mycobacteria can be characterized by an exaggerated reaction leading to the hypersensitivity pneumonias of the hot tub lung (33), to the metalworking fluid-associated hypersensitivity pneumonitis (34), or to the formation of granulomas within the bronchial walls leading to the formation of bronchiectasis (35), a condition that's been associated with HLA-DR 6, i.e., the alleles 13 and 14 (36). Mycobacteria have also been implicated in sarcoidosis, where acid fast rods have been seen in affected tissues (37), wall-deficient form (l-form) of mycobacteria have been isolated (38), and mycobacterial DNA has been detected (39). Interestingly, a reaction to mycobacterial antigens has been described in sarcoidosis as T-cell and antibody responses to MTB ESAT-6 and KatG protein (40), HSP70 (41), and superoxide dismutase (SodA) (42) have been observed in patients with sarcoidosis and some antigens such as the Heat Shock Protein (HSP) and Catalase-Peroxidase (KatG) have been detected by immunohistochemistry in sarcoid tissues (43, 44).

It is worth noticing in this regard, that the same HLA allelic variants which have been associated with susceptibility or resistance to tuberculosis have also been implicated in susceptibility to sarcoidosis. The HLA-DRB1*03 alleles, which have been negatively associated with TB, being therefore dubbed as "resistance" genes (45), have been associated with acute, self limiting, sarcoidosis (46). In contrast, the HLA-DRB1*15 alleles, which have been associated with the susceptibility to develop active TB (45), have been associated with stage III, or chronic sarcoidosis (47). Thus, one might hypothesize that the lack of HLA binding and presentation of mycobacterial antigens could determine disease progression in tuberculosis as well as in sarcoidosis, the type of reaction –extensively necrotizing versus non-necrotizing, being possibly driven by the expression of allelic variants of the host's genes of the innate immune response (48) or by variants expressed in the infected organism of the parasite virulence genes (49).

The virtual approximation to truth made by *in silico* models, together with the limitation imposed by the reduced number of HLA-DR phenotypes that can be analyzed in the different populations, require that *in silico* results be confirmed by studies using conventional biological techniques. With this caveats, is counceivable that the use of immuno-informatic tools for the prediction of T-cells epitopes also on other HLA class I and II alleles on data sets as large as an the entire MTB genome, will help with generating mechanistic hypothesis on the determinants of HLA-associated genetic susceptibility to TB, as well as to other granulomatous disorders caused, or induced, by mycobacteria.

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