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Study of KIR Expression and HLA Ligands in CD56⁺ Lymphocytes of Drug Resistant Tuberculosis Patients

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ABSTRACT

Analysis of receptor–ligand interactions in the context of diseases necessitates to understand how HLA–KIR genotypes function in diseases. Although CD56⁺ lymphocytes are derived from multiple lineages, they share a functional association with immunosurveillance and antimicrobial responses.

The present study aimed to determine whether KIR phenotype in CD56 lymphocytes and corresponding HLA-class 1 ligands are associated with multidrug resistance tuberculosis (MDR-TB). We compared the frequencies of HLA-C and HLA-BW4 genes, the expression of KIRs 2DL1/2DS1, 2DL2/2DL3, 3DL1, and 2DS4 and the combinations of HLA/KIR in 32 Nifamycin and Isoniazid-resistant TB with those in 68 drug non resistant (NR) sputum smear positive pulmonary TB patients. PCR-SSP and flow cytometry were performed for HLA and KIRs typing, respectively.

We showed no significant differences between inhibitory or activating KIRs as well as HLA ligands in MDR TB patients compared with NR-TB . The combinations of inhibitory KIR-HLA ligands in MDR-TB were much more prevalent, but not statistically significant than in NR patients ($p=0.07$). The frequency of MDR patients with all HLA-C and HLA-BW4 ligands was higher than NR-TB ($p<0.009$). Conversely, the percentage of MDR patients having only one kind of HLA gene was significantly lower than NR-TB ($p<0.01$). We conclude that the expression of inhibitory KIRs with corresponding HLA ligands genes, and/or co-existence of three HLA class 1 ligands for inhibitory KIRs may be associated with drug resistance in pulmonary tuberculosis.

Keywords: Human Leukocyte Antigen; Killer Cell Immunoglobulin Like Receptor; MDR-TB; Tuberculosis

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INTRODUCTION

Tuberculosis (TB) is one of the major infectious diseases causing morbidity and mortality in the world.¹ Treatment of patients with drug resistant strains of tuberculosis is expensive, difficult, and less effective.² Understanding of the immune response generated against tuberculosis has been improved due to advances in immunological tools. The inflammatory environment upon M.tb infection recruits innate effector cells such as macrophages, polymorphonuclear neutrophils (PMN) and NK cells to the infectious area. This eventually leads to the establishment of acquired T cell immunity which appears to be protective in more than 90% of infected individuals.³

Host susceptibility factors and immunological differences between TB patients and healthy individuals were studied in many investigations. Identification of risk factors could help to understand the causal associations especially in multi-drug resistant (MDR) TB. Several evidences indicated the involvement of the major histocompatibility complex (MHC) in susceptibility to TB. Association of HLA, with development of pulmonary TB has been established in various studies in different ethnic groups.⁴⁻⁶ In our country a report on the frequencies of HLA-B17 and -DR14 in TB patients suggests that these alleles could be either positively or negatively associated with pulmonary TB.⁷ A study on leukocytes and CD56/CD16 lymphocytes of TB patients was previously reported from our laboratory.⁸ However, genetic host susceptibility to MDR-TB is not clearly defined in most of reported studies.^{9,10}

Killer cell immunoglobulin-like receptors (KIRs) are polymorphic glycoproteins expressed on the cell surface of natural killer (NK) and subsets of T cells. KIRs interact with specific motifs on HLA class I molecules and modulate NK cytolytic activity.¹¹⁻¹³ KIRs have a multifunctional role in disease. Many studies have been performed to determine the role of these receptors and also their HLA class I ligands in human diseases. Salim I et al. have summarized the findings from a number of disease association studies and discussed on the activating and inhibitory roles of this genes.¹⁴

Up to 15 activation and inhibitory KIR genes are expressed on NK cells. All mature NK cells express at least one receptor specific for self MHC. Besides NK cell connecting innate and adaptive immunity, NK is an

effector lymphocyte in the early immune response by killing the abnormal cells and by releasing immunomodulatory cytokines.^{15,16,17}

Human NK cells share granulysin with cytotoxic T cells to kill M. tuberculosis. NK cells are able to directly lyse M. tb-infected monocytes and macrophages in vitro¹⁸ they mediate early protection against M.tb and a variety of intracellular pathogens.¹⁹ However, although the recent reports indicate the role of NK cells to mounting a strong response to M. tuberculosis, their exact function in vivo remains unclear.

CD56 expressing NK cells comprise 5–10% of the PBMCs. CD56 or neural cell adhesion molecules are expressed on the surface of neurons, glia, skeletal muscle, activated T cells and natural killer cells. They have a role in cell-cell adhesion and are involved in the mechanisms by which these cells home into inflammation areas. In this study we investigated. CD56/KIR bearing NK and activated NK-T cells in PBMCs from MDR and DR tuberculosis patients using antibodies against CD56 and KIRs. As the KIR genes are not always expressed on the cell surface, the purpose of this study was to determine whether KIR expressions and their HLA ligands either alone or in combinations are associated with MDR- TB. For this, we compared the frequencies of each KIR phenotype as well as HLA-C and HLA-Bw4 alleles in MDR- TB patients with those in drug non resistant (NR) TB patients.²⁰

PATIENTS AND METHODS

The study consisted of 100 patients referred to Masih Daneshvari hospital during the period between 2007-2009. All patients had symptoms of pulmonary tuberculosis and were classified into two groups; MDR-TB ($n = 32$) and drug sensitive group ($n = 68$) based on the resistance to both rifampicin and isoniazid, determined by drug sensitivity testing.²¹ Resistance was indicated by the growth of more than 1% of the colonies in the drug-containing medium. This research project had been approved by the university. More over an informed consent was taken from all participants.

Monoclonal antibodies (mAbs) -The mAbs used in this study were FITC-conjugated anti-CD56, PE-conjugated anti-CD 158a/h (KIR2DL1/2DS1), PE-conjugated anti-CD 158b (KIR2DL2/2DL3),

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PE-conjugated anti-CD 158 e (KIR3DL1), PE-conjugated anti-CD158i (KIR2DS4) and PE or FITC conjugated mouse isotype controls. All antibodies were obtained from CALTAG laboratories, CA.

Flow cytometry Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Lymphoprep (Axis-shield AS, Oslo, Norway) gradient centrifugation. In order to exclude adherent cells, Each PBMC was incubated in a culture dish in a humidified 5% CO₂, 95% air atmosphere at 37 °C for 60 minutes. After incubation, non adherent cells were collected and washed twice in phosphate-buffered saline (PBS). Two-color immunofluorescence staining was carried out for surface phenotyping using mAbs to CD56 and one anti- KIR. The stained cells were then suspended in 0.5 ML of PBS and analyzed by flow cytometry. Lymphocyte subsets were identified by gating analysis, and fluorescence profiles were obtained for 10000 cells from each sample. Negative controls for each experiment were performed with FITC- and PE-labeled mouse immunoglobulin-G and unlabeled samples. The frequency of each KIR-expressing CD56⁺ lymphocytes was calculated using dot plots.

HLA typing- DNA was extracted from each peripheral blood. PCR-SSP method was performed for HLA- typing (KIR-TYPE/EPITOP-TYPE kit, BAG health care, Germany) according to recommended instruction.

Statistical analysis - Data obtained from flow cytometry are expressed as percentages. Data were analyzed using SPSS program. The Pearson chi-square or Fishers exact tests was performed to assess the association of KIR surface antigens and HLA genes (alone or in- combination) with MDR-TB. All statistical tests were considered as significant if $P < .05$.

RESULTS

We investigated the expression of KIRs on the surface of lymphocytes with and without their corresponding HLA class 1 ligands. We stained PBMCs using mAbs to CD56 and KIRs and gated lymphocyte area to detect positive cells. Therefore, the majority of NK and NK-T, the important effector cells against M.tb infection existed in gated region. All results obtained from flow cytometry in two groups of TB patients are represented in figures 1 to 3.

Considering any distinct KIR or HLA marker, neither KIRs molecules (2DL1, 2DL2/ 2DL3, 2DL1/2DS1, 3DL1 and KIR2DS4), nor HLA genes (C1group, C2group and Bw4 motifs) showed significant differences ($P \geq 0.1$) between MDR and NR TB patients (Figures 1 and 2). Moreover, we compared MDR and DR TB patients based on the number of HLA-class 1 alleles in each patient. According Data represented in figure 2, the majority of MDR patients had all three ligands (HLA-C1 and 2 groups, HLA-BW4) in their genome ($p < 0.007$). Conversely, the percentage of MDR patients having only one kind of HLA ligand gene for inhibitory KIRs was significantly lower than those in NR-TB ($p < 0.009$). Regarding KIR/HLA combinations, as shown in figure 3, although the majority of MDR-TB patients with inhibitory KIRs were positive for corresponding HLA ligands. The differences between MDR and NR TB patients were not found to be statistically significant ($p \geq 0.08$).

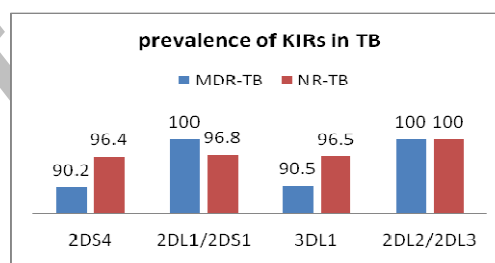


Figure 1. Distribution of KIRs molecules in drug sensitive (NR-TB) and drug resistant (MDR) tuberculosis patients. PBMCs were double-stained with anti-CD56 and anti-CD158i for KIR2DS4 or anti-CD158a/h for KIR2DL1/2DS1 or anti-CD158e for KIR3DL1 or anti-CD158b for KIR2DL2/2DL3. No significant differences are seen between two groups ($p \geq 0.1$).

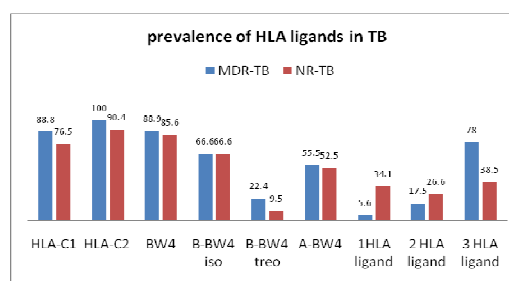


Figure 2. Distribution of HLA class-1 ligands for inhibitory KIRs in MDR and NR tuberculosis. The significant decrease for having only 1 HLA ($P \leq 0.009$) and increase for having the three HLA ligand ($P \leq 0.007$) are seen in MDR-TB compared with ND-TB patients.

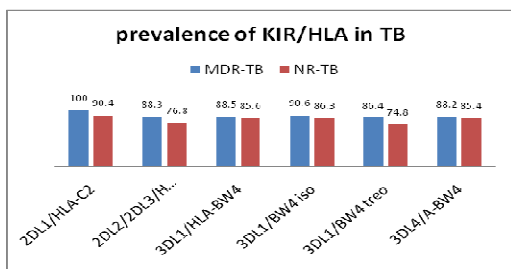


Figure 3. Distribution of different KIR molecules and their corresponding ligands in drug sensitive and MDR tuberculosis patients. PBMCs were double-stained with anti-CD56 and anti- CD158i for KIR2DS4 or anti-CD158a/h for KIR2DL1/2DS1 or anti-CD158e for KIR3DL1 or anti-CD158b for KIR2DL2/2DL3. HLA alleles were typed using PCR- SSP. No significant difference is detected between two patients groups ($p \geq 0.01$).

DISCUSSION

MDR- TB is a growing problem worldwide. It also increases the mortality rate by co-infection with human immunodeficiency virus. Only a small percentage of patients infected with *Mycobacterium tuberculosis* develop clinical TB. Reports on the clinical and immunogenetic association for the development of multi-drug resistant tuberculosis suggest that HLA complex acts as an independent predictor for the development of MDR-TB.^{10,22} However, association of KIR/HLA with multidrug resistance TB has not been studied yet.

NK cell activity in TB infection is controlled by cytokines and a complex repertoire of activating and inhibitory receptors. Due to the involvement of KIR-HLA combinations mentioned in some infectious and non-infectious diseases²³⁻²⁵ we investigated a number of HLA class 1 genes (HLA-C and HLA-BW4) and expression of their corresponding inhibitory receptors (KIR2DL1, KIR2DL2 / KIR2DL3, KIR3DL1) as well as activating KIR2DS4 expression in peripheral blood lymphocytes from TB patients. For this, we selected CD56 positive lymphocytes to investigate the expression of KIRs on the surfaces of NK and activated T cells.

It has been reported that diverse human peripheral blood lymphocytes (PBL) populations express CD56 molecule on their surfaces. Among these lymphocytes, NK and NK-T cells are identified.²⁰ The killing activity of CD56 lymphocytes is balanced by contra-regulatory

signals derived from inhibitory and activating KIR receptors.²⁶⁻²⁹ In this study, as the CD56 bearing adherent cells had been removed from PBMCs, all CD56/KIR positive NK and activated T lymphocytes were included in the experiments.

Although, the number of TB patients with KIR2DL1 or KIR2DL3 genes have been previously shown to be different compared with controls,^{26,30} we found no significant differences between expression of either inhibitory or activating KIRs in MDR-TB compared to DR-TB patients. We suggest that different results (phenotype versus genotype studies) could be due to the failure of some KIR gene expression or differences in case/control populations.

Until now, there is no documented report on the HLA associations with MDR-TB from Iran. In this study no changes of HLA-C and BW4 allele frequencies were detected in MDR compared to NR – TB patients. We also calculated the percentages of different combinations of HLA ligands to determine the frequencies of MDR and NR TB-patients bearing one, two or three HLA alleles in their genomes. After analysis of HLA alleles, we noted that the percentage of patients bearing only one ligand for inhibitory KIRs was significantly lower in MDR in comparison to those in NR-TB. Conversely, higher proportion of MDR-TB patients had all three kinds of HLA-class 1 genes. Thus, coexistence of HLA-class 1 ligands may lead to more inhibition of cytotoxic activities in TB patients resulting drug resistance status.

As the allelic diversity of KIRs and their respective MHC class 1 ligands creates a further level of complexity to interpretation, both KIRs and their HLA class 1 ligands must be considered together in disease association studies.^{28,30} However, all KIR alleles do not express on the cell surface and/or are not functional. Therefore, to understand any relationship between these markers and MDR-TB, we studied on the expressed KIR molecules and HLA ligand genes together. Analysis of KIRs phenotypes together with related HLA ligands indicated that the percentages of any combination of KIR/HLA was higher, but not statistically significant in patients with MDR-TB compared to NR-TB. Thus, we suggest that expansion of inhibitory KIRs plus their HLA ligands may associate with drug resistance and reducing NK cell activation in TB patients. However, as we found no statistically significant differences between MDR and NR-TB, this could be attributed to limited sample sizes.

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Taken together, some different findings are noted between this study and other reports investigated on KIRs and HLA ligands associations.^{10,22-26,31-33} We suggest that inconsistency between our and others results may be attributed to: 1) different study subjects, in many reports subjects were TB patients and normal controls while in this study MDR patients were compared with NR-TB. 2) Different methods for KIR assay; in the majority of studies KIRs were detected genetically while in this study phenotypes of gene expressions were studied. In this study the importance of KIR expressions was shown besides the genetic studies. Although our results suggest greater inhibition in MDR patients relative to drug sensitive TB controls, further studies on other KIRs and also HLA and non HLA ligands are needed to confirm these findings. We suggest that phenotypic studies and genetic link for disease development could help to improve treatment strategies in MDR-TB. However, in order to confirm the exact influence of KIR/HLA combinations in MDR-TB, studies on extended number of patients are required. We Hope that a molecular basis for MDR-TB associations with HLA– KIR combinations will be provided in near future.

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