Indian Long-term Non-Progressors Show Broad ADCC Responses with Preferential Recognition of V3 Region of Envelope and a Region from Tat Protein

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HIV-specific antibody-dependent cell cytotoxicity (ADCC) is likely to be important in governing protection from human immunodeficiency virus (HIV) and slowing disease progression. Little is known about the ADCC responses to HIV-1 subtype C. We characterized ADCC responses in HIV-1 subtype C-infected Indian subjects with slow disease progression and identified the dominant antigenic regions recognized by these antibodies. ADCC responses were measured in plasma from 34 long-term non-progressors (LTNPs), who were asymptomatic and maintained CD4 count above 500 cells/mm³ for the last 7 years in the absence of antiretroviral therapy (ART), and 58 ART naïve progressors with CD4 count <500 cells/mm³ against overlapping HIV-1 peptides using a flow cytometry-based antibody-dependent natural killer (NK) cell activation assay. The assay measured CD107a expression on NK cells as a marker of antibody-dependent NK cell activation and IFN-γ secretion by NK cells upon activation. The ADCC epitopes were mapped using the matrix of overlapping peptides. Indian LTNPs showed higher and broader ADCC responses compared to the progressors. The Env-C and Tat-specific ADCC responses were associated with lower plasma viral load, whereas the Env-C responses were also associated with higher CD4 counts. Five of 10 LTNP responders targeted epitopes in the V3 region (amino acids 288–330) of Env-C. Additionally, three Tat regions were targeted by ADCC antibodies from LTNPs. ADCC responses were associated with slow HIV progression in Indian subtype C-infected cohort. The frequently recognized peptides from the V3 loop of Env and the novel epitopes from Tat by the LTNPs warrants further study to understand the role of ADCC responses to these regions in control and prevention of HIV-1 infection.

Keywords: LTNP, NK cells, ADCC, envelope, Tat
INTRODUCTION

A vaccine is urgently needed for ultimate success in controlling human immunodeficiency virus (HIV) epidemic. However, one of the major obstacles in this endeavor is that the correlates of immunity to HIV are poorly understood. Fc-mediated functions of Env-specific antibodies, such as antibody-dependent cell cytotoxicity (ADCC), correlated with protection against HIV infection in an RV 144 phase III HIV vaccine trial (1). Further, ADCC responses have been associated with protection against infection in newborns (2). However, data are scarce on the ADCC responses in the individuals infected with different HIV subtypes especially infected with Indian strains of HIV-1.

Human immunodeficiency virus-infected individuals who are able to maintain CD4 counts above 500 cells/mm³ for extended time period, usually more than 7 years of infection in the absence of antiretroviral treatment (ART) are commonly termed as long-term non-progressors (LTNPs) (3). Although a subset of LTNPs generates broadly neutralizing antibodies or efficient T cell immunity to HIV, such immune responses do not fully explain their slower disease progression (4–6). Fc-mediated antibody responses can mediate clearance of infected cells and may contribute to slower disease progression (7). However, these responses have not been characterized well in subtype C-infected Indian subjects. Further, the information on HIV epitopes recognized by ADCC antibodies across different HIV clades is limited.

We have previously shown that the natural killer (NK) cells from Indian LTNPs are functionally competent in lysing HIV-infected cells (8). However, the information about ADCC-mediating antibodies in these patients was not known. Recent advances in assays to measure the ability of ADCC antibodies to activate NK cells in response to pools of overlapping HIV-1 peptides (9) allowed us to characterize linear peptide ADCC epitopes recognized by HIV subtype C-infected Indian subjects. This study was undertaken to estimate and characterize the ADCC responses for their magnitude and breadth in Indian LTNPs and to identify the epitopes recognized by them.

MATERIALS AND METHODS

Study Participants

Study participants were identified among the attendees of the outpatient clinics of the National AIDS Research Institute who visited the clinic regularly for care and support. LTNPs were identified as individuals with asymptomatic HIV infection for more than 7 years who had stable CD4 counts above 500 cells/µl and who had not taken ART (3). Progressors were identified as ART-naïve HIV-infected patients with CD4 counts <500 cells/µl. Thirty-four LTNPs and 58 progressors were enrolled in the study along with 14 HIV-uninfected healthy individuals. Demographic, immunological, and virological characteristics of 92 HIV+ subjects are shown in Table 1.

The study was approved by the institutional ethical committee. Participants were enrolled after obtaining written informed consent.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic, immunological, and virological characteristics of human immunodeficiency virus-infected study participants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>Long-term non-progressor</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>34</td>
</tr>
<tr>
<td>IQR (35–45)</td>
<td>38.5</td>
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<tr>
<td>Male/female</td>
<td>11/23</td>
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<tr>
<td>Years of seropositivity (mean and range)</td>
<td>10.8 years (7–19 years)</td>
</tr>
<tr>
<td>CD4 count at the testing visit (cells/µl) [median and IQR]</td>
<td>699 (632–942)</td>
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<tr>
<td>Mann–Whitney U-test p &lt; 0.0001</td>
<td></td>
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<tr>
<td>Plasma viral load at testing visit (RNA copies/ml) [median and IQR]</td>
<td>2,174 (422–9,799)</td>
</tr>
<tr>
<td>Mann–Whitney U-test &lt; 0.0003</td>
<td></td>
</tr>
<tr>
<td>Number of viremic controllers (viral load &lt;2,000 RNA copies/ml)</td>
<td>12</td>
</tr>
<tr>
<td>Number of non-viremic individuals (viral load &gt;2,000 RNA copies/ml)</td>
<td>22</td>
</tr>
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ADCC Intracellular Cytokine Staining Assay (NK Cell Activation Assay)

The ADCC response in the serum/plasma samples of the study participants was determined using NK cell activation assay as described previously (10). The assay uses CD107a and IFN-γ expression by NK cells as a final readout. We preferred this assay, although it does not measure the killing of infected target cells, as this assay was useful in helping us to map the linear epitopes recognized by ADCC-mediating antibodies and also captured cytokine secretion (IFN-γ), an another important aspect of antibody-mediated NK cell activation. Also, it has been recently shown that the ADCC activity observed in NK cell activation assay correlates significantly with functional killing assays such as the rapid fluorometric ADCC assay (11) and other assays (12–17). The anti-HIV ADCC responses were assessed against consensus envelope [both HIV-1 B and C], Gag (HIV-1C consensus), Pol (HIV-1 B consensus), and accessory proteins: Nef, Vpu, Rev, and Tat (HIV-1 B consensus) using pools of 15mer peptides overlapping by 11aa (National Institutes of Health AIDS reagent program). The detailed description of the peptides and the procedure for preparation of peptide pools is given in Supplementary Material. Briefly, 150 µl whole blood from healthy donor (as a source of NK cells) and 50 µl of heat-inactivated heparin-anticoagulated plasma or serum [final concentration as1:4] from the study participants was incubated at 37°C with HIV peptide pools (at a final concentration of 1 µg/ml/peptide) for 5 h in the presence of brefeldin A (10 µg/ml) (Sigma) and monensin (0.68 µl/ml) (BD Biosciences). After incubation, CD3–CD56dim NK cells were gated and analyzed for the expression of intracellular IFN-γ and CD107a using fluorescent tagged antibodies [CD3 Per Cp: (clone-SK7), CD56 PE-Cy7: (clone-HCD56), and CD107a APC H7: (clone-H4A3) (all from Biolegend), and IFN-γ APC: (clone-B27) (BD Biosciences)]. The gating strategy is shown in Figures 1A–C. Stimulation by purified anti-CD16 monoclonal
antibody (clone-3g8, at 2.5 μg/ml concentration) was used as a positive control (Figure 1D). Plasma samples of all study groups incubated with HIV-negative donor blood in the absence of antigen was considered as a negative control for the respective sample. Mock control containing plasma, HIV-negative donor blood, and DMSO equivalent solution was used to negate the effect of DMSO that was used to dissolve peptides on NK cell activation (Figure 1E). The percentage of activated NK cells as a marker of ADCC activity was represented as the sum of the percentage of any activated NK cells, expressing only CD107a, both CD107a and IFN-γ and only IFN-γ (Figures 1F,G). The percent NK cell activation seen in mock stimulation (donor blood (source of NK cells) and test plasma incubated along with DMSO equivalent solution) for each sample was subtracted from this sum. The NK cell activation in mock control (plasma+cells without stimulation) ranged from 0.124 to 4.41% with the mean of 1.48%. The response was considered to be positive if the following two criteria were fulfilled: (1) the total percentage of CD107a and IFN-γ expressing (activated) NK cells was more than three times the percentage of activated NK cells from the negative control of the respective sample and (2) the total percentage of activated NK cells was greater than the mean plus two SD of the percent activated NK cells from 14 HIV-negative donors tested for each of the HIV antigens. The interassay variation was found to be <10% as assessed by determining the % CV for positive control [Anti-CD16 stimulation] was 7.18% and negative control [donor blood (source of NK cells) without any stimulant] was 9.8%.

**Mapping of Epitopes Recognized by ADCC Antibodies**

The samples of ADCC responders were further used to identify linear peptides recognized by ADCC antibodies using the peptide matrix pools in NK cell activation assay. The matrix pools were designed in such a way that each peptide is present in two different pools as described previously (18). The peptide that was common in two pools showing that ADCC response was identified as the peptide recognized by ADCC antibodies and the recognition of the particular peptide was further confirmed by repeating the NK cell activation assay using the single peptide. For mapping the epitopes, 30 pools for HIV-1 subtype C Env peptides and 10 pools for HIV-1 subtype B Tat peptides were used. Details of the pools are given in Figures S1A,B in Supplementary Material.
Statistical Analysis
Statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA). Data were analyzed by Mann–Whitney U test to compare the magnitude of ADCC responses between LTNP and progressor groups. Correlation analyses were performed using Spearman correlation between CD4 count, viral load, and magnitude of ADCC responses. Fisher's exact test and chi square tests were used to analyze the significance of ADCC responses in different categories.

RESULTS
LTNP and Progressor Cohorts
Thirty-four HIV-infected individuals who fulfill criteria for LTNP were enrolled in the study. As a comparator, we selected 58 progressor subjects with CD4 T cell counts of <500/μl. The median age of LTNPs (median 39 years [IQR 35–45]) and progressors (median 35 [IQR 31–39]) was not significantly different. Of the 34 LTNPs, 23 were female and 11 were male, versus 24 and 34 among the 58 progressors, respectively. The mean seropositivity in LTNP cohort was 10.8 years with a range from 7 to 19 years. The CD4 counts of LTNPs at the study visit (median 699 cells/μl [IQR 632–942]) were significantly higher than in the progressors (median 409 cells/μl [IQR 315–456]) (p < 0.001). As expected, the plasma viral load values at the study visits were significantly lower in LTNP (median 2,174 HIV RNA copies/ml [IQR 422–9,799]) compared to the values from progressors (median 17,148 [IQR 5,543–87,100]) (p = 0.0002) (Table 1).

Indian LTNP have Significantly Higher Levels of ADCC Responses
A total of 20 of 34 LTNPs (58.8%) and 20 of 58 progressors (34.4%) showed antibody-dependent NK cell activation against at least one of the HIV overlapping peptide pools. The trend of higher magnitude in LTNP was observed in the case of Env-C [LTNP mean 18.2%; range 8.4–35%, progressors mean 13.72%; range 5.6–24.5%], Tat B [LTNP mean 10.8%; range 5.8–16.4%, progressors mean 4.3%], Nef B [LTNP mean 7.6%; range 4.6–15.87%, progressors mean 5.6%; range 3.5–10.6%], and Vpu [LTNP mean 13%; range 5.1–20.8%, progressors mean 11%; range 3.6–21%]. Whereas a trend of higher magnitude in progressors was observed in the case of Env B (LTNP mean 9.36%; range 2.8–21%, progressors mean 13.01%; range 2.7–43%) and Rev B [LTNP mean 6.2%; range 4–9.2%, progressors mean 9%; range 3.7–16.6%]. In the case of Gag C, ADCC responses were seen only in the LTNP cohort and only one from progressor group showed the ADCC response against Pol B peptide pool (Figures 2A,B). The number of LTNPs showing Env-C and Gag-specific ADCC responses was also significantly higher than among progressors (p < 0.007 and p < 0.016, respectively). The number of responders against Tat antigen was also higher in LTNPs, but the difference did not reach significance (p = 0.06, Fisher's 2 × 2 exact test), whereas the number of responders against HIV-1 subtype B Env peptides, Pol, Nef, Rev, and Vpu antigens was similar in both groups (Figure 2C). Although the magnitude of ADCC response is shown as % total NK cell activation (sum of % NK cells expressing CD107a and/or IFN-γ), overall the distribution of CD107a and IFN-γ expression of activated NK cells by the NK cell-activating antibodies within the plasma of the LTNPs and progressors was found to be skewed toward higher IFN-γ expression in both groups (Table S2 in Supplementary Material).

We then analyzed whether the magnitude of the Env subtype C, Tat, and Gag-specific ADCC responses in the HIV-infected individuals correlated with CD4 T cell count and viral load; standard markers of HIV disease progression. Anti-Env ADCC responses were modestly correlated with CD4 T cell counts (r = 0.235, p = 0.02, Figure 3A) and Tat (r = 0.263, p = 0.016, Figure 3D) antigens. Also, a significant association between Env-C ADCC responses and lower plasma viral load was observed when only LTNP group was separately analyzed (Spearman r = −0.377; p = 0.04). Besides this, the median plasma viral load values (calculated based on annual viral load values obtained during the follow-up period) among LTNPs were also negatively correlated with ADCC responses against Env-C specific ADCC (r = −0.367, p = 0.0006, Figure 3C) and Tat (r = −0.263, p = 0.016, Figure 3D) antigens.

The ADCC Responses in LTNPs Have Higher Breadth
The ability of ADCC responses to recognize diverse HIV strains is likely to improve the effectiveness of ADCC antibodies as a prevention strategy. Hence, to study the breadth of the ADCC antibody specificities, we analyzed whether LTNPs and progressors recognized both subtype C and B Env peptide pools. We found that of the 34 LTNPs, 20.6% (N = 7) had ADCC responses against both HIV-1 C and B Env peptides, whereas only 3.4% (2/58) of the progressors showed response against both B and C Env peptides (p < 0.01 by Fisher’s 2 × 2 exact test, Figure 4A).

Combinations of broad ADCC responses might also contribute to slow HIV progression given our findings on Env- and Tat-specific ADCC responses as mentioned above. To study this, we analyzed both the cohorts that had positive ADCC responses to 0, 1, 2, 3 or >3 of the HIV antigens represented by peptide
A Magnitude of ADCC response to structural proteins

B Magnitude of ADCC response to accessory proteins

FIGURE 2 | Continued
pools. We found that the ADCC responses in the LTNPs showed significantly greater breadth compared to the progressors. A total of 21% of LTNPs showed responses against more than three antigens, compared to 7% in progressors (chi square test, \( p < 0.0001 \), Figure 4B).

**Indian LTNPs Showed Preferential Recognition of Env-C V3 and Tat Epitopes**

ADCC responses targeting specific epitopes may be beneficial in HIV control, similar to that observed with specific CD8+ CTL responses. To identify epitopes recognized by the ADCC antibodies from the Indian LTNPs, we used a matrix approach using subpools of peptides. The peptide pools in the matrix were designed in such a way that a single peptide was common in both the pools. ADCC epitopes identified in the matrix approach were then confirmed by NK cell activation assay using the single peptide present in both the pools to which the response was observed. We studied epitopes from Env-C and Tat antigens, as the responses to these antigens were associated with lower plasma viral load and also were higher in frequencies and magnitudes in the LTNP cohort. Due to the scarcity of the samples from the progressor group, we mapped the potential ADCC epitopes only in LTNPs and only the frequently recognized peptides (when recognized by 3 or more than 3 LTNPs) were tested in progressors.

The consensus Env-C overlapping peptides were 212 in number and the number of consensus Tat B peptides were 23. Out of 212 Env-C peptides, 153 peptides were recognized by ADCC antibodies from 10 LTNPs and 16 out of 23 Tat B peptides were recognized by 3 LTNPs (Table 2). One LTNP showed response to Env and Tat peptides. It was observed that the conserved C1, C2, C4, and C5 region and the variable V3, V4, and V5 and a few CD4 binding sites were recognized by the ADCC antibodies from Indian LTNPs (Table 2). In
**DISCUSSION**

Understanding the immune responses controlling HIV replication and disease progression will be critical in the development of improved preventive and curative strategies. LTNPs and elite controllers can be considered as a model for slow disease progression with better virus control. In addition to cellular immunity, there is emerging literature on the role of functional antibodies in virus control in these populations (23, 24). Ackerman and colleagues recently showed that subtype B-infected elite controllers with spontaneous virus control have a combination of various antibody effector mechanisms, including ADCC (25). Our study showed that the anti-Env ADCC responses were significantly higher in LTNPs with low plasma viral load (<2,000 copies/ml), suggesting a probable role for ADCC in controlling viral replication in subtype C HIV infections in India. We also found that ADCC antibodies from LTNPs were significantly more likely to identify more than one antigen simultaneously compared to progressors. Broad CTL responses were associated with higher CD4 count and lower plasma viral load. CD4 counts at the testing visit (N = 92) (X axis) with envelope C peptide pool (Y axis) (p = 0.02) (A) and Tat-specific ADCC responses (Y axis) (p = 0.07) (B). (C,D) Association of plasma viral load at the testing visit (N = 92) with ADCC response against Env-C peptide pool and Tat B peptide pool, respectively. (E) Magnitude of envelope C-specific ADCC response in viremic controllers and non-controllers within long-term non-progressors (LTNPs). The magnitude of ADCC responses [% natural killer cell activation] was comparable in between the groups, but the number of responders to envelope C was high in viremic controllers (8 out of 12) (plasma viral load <2,000 copies/ml) as against 4 out of 22 LTNPs with plasma viral load >2,000 copies/ml. Additionally, the preferential recognition of V3 region (aa 288–330) as indicated by blue dot was seen in four out of eight responders from viremic controller LTNPs as against only one out of four responders from non-controllers.

**FIGURE 3** ADCC responses were associated with higher CD4 count and lower plasma viral load. CD4 counts at the testing visit (N = 92) (X axis) with envelope C peptide pool (Y axis) (p = 0.02) (A) and Tat-specific ADCC responses (Y axis) (p = 0.07) (B). (C,D) Association of plasma viral load at the testing visit (N = 92) with ADCC response against Env-C peptide pool and Tat B peptide pool, respectively. (E) Magnitude of envelope C-specific ADCC response in viremic controllers and non-controllers within long-term non-progressors (LTNPs). The magnitude of ADCC responses [% natural killer cell activation] was comparable in between the groups, but the number of responders to envelope C was high in viremic controllers (8 out of 12) (plasma viral load <2,000 copies/ml) as against 4 out of 22 LTNPs with plasma viral load >2,000 copies/ml. Additionally, the preferential recognition of V3 region (aa 288–330) as indicated by blue dot was seen in four out of eight responders from viremic controller LTNPs as against only one out of four responders from non-controllers.
Figure 4 | LTNPs showed cross clade as well as broad ADCC responses. (A) Percentage of the responders (Y axis) to both Env-C and -B peptides is significantly higher in LTNPs as compared to the progressors (p = 0.01 in a 2 × 2 Fisher's exact test). (B) The breadth of the ADCC response. The number of human immunodeficiency virus antigens (Y axis) recognized by ADCC antibodies from LTNPs were significantly higher than the number of antigens recognized by progressors (chi square test, p < 0.0001).

responses have been shown to be associated with slow or no progression (26–28). It might be possible that broad ADCC responses limit opportunities for immune escape from ADCC reducing the disease progression (9). It will be interesting to estimate ADCC responses to various epitopes over time in this cohort to confirm this hypothesis.

Further characterization of anti-Env and anti-Tat ADCC responses in our Indian LTNP cohort found that they were associated with higher CD4 counts and lower plasma viral loads at the time of study visit and also with lower median plasma viral load calculated for the entire follow-up period. Similar findings have been reported by other groups studying subjects infected with HIV-1 subtypes B and A (5, 29–31), suggesting that the role of ADCC responses in controlling viral multiplication holds true across multiple clades.

The envelope protein is frequently targeted by ADCC antibodies from subtype B-infected individuals and non-human primates (22, 32–37). We have shown that ADCC responses in Indian LTNPs also preferentially target Env-C region, confirming a key role for Env-specific ADCC responses in virus control. The use of overlapping peptide pools allowed us to map Env-specific ADCC responses to individual linear peptide epitopes. We found ADCC antibodies from Indian LTNPs recognized both variable and conserved regions of Env-C antigens. Different ADCC monoclonal antibodies generated from HIV-1 subtype B infected population were shown to be specific to CD4-induced epitopes (19, 38). In our LTNP cohort, we found that the peptides spanning the V3 region of Env were commonly targeted (5/10 of Env-C responders), indicating that the antibodies recognizing HIV-1 Env region independent of CD4 binding region might also be important in viral control. Indeed, in our study, four of the five LTNP who recognized this epitope have undetectable plasma viral load (Figure 3E). The main caveat of the anti-variable loop ADCC responses is the variability in this region across viruses. However, detection of these responses in HIV-1 LTNPs emphasizes a potential role of anti-V3 ADCC responses in viral control in HIV-1 C-infected patients. Effective V3-specific ADCC antibodies could also have neutralizing capacity, providing an additional level of immune control (39). Additional research is required to know whether these antibodies also have a role in clearing of latent reservoirs where the virus can downregulate CD4, making it inaccessible for CD4-dependent ADCC antibodies. It was also observed that a few previously identified regions such as V2 region of gp120 and immunodominant region of gp41 were not recognized by Indian LTNP (22). This differential recognition of ADCC epitopes by individuals infected with different subtypes has implications for vaccine designs and immunotherapeutic strategies. The importance of recognition of these epitopes could be assessed in longitudinal samples to determine whether the sustained recognition of these regions influences disease progression.

Our finding that Indian LTNPs develop Tat-specific ADCC responses was novel and unexpected although only 4 of 34 LTNP showed Tat-specific response. A number of researchers have reported ADCC responses against Nef, Vpu, and Pol (7, 40, 41), but in our cohort, we found significantly higher and more common recognition of Tat peptides in LTNPs. Tat is one of the earliest expressed proteins in HIV-1 infection and is responsible for efficient transcription (42–45). It has been shown that the presence of Tat-specific CTLs and/or anti-Tat antibody is associated with control of viral replication and slow progression to AIDS (46–48). In non-human primate models, greater protection was correlated with Tat- and Env-binding antibodies along with higher ADCC and ADCVI activities (49). The ADCC antibodies generated against the antigen expressed early in the life cycle might be able to control viral replication early. It will be interesting to study Tat-specific monoclonal and polyclonal antibodies
to determine whether antibodies generated against individual Tat epitopes can lyse HIV-infected cells and control HIV infection in vitro. We have used subtype B Tat peptides in the study as subtype C Tat peptides were not available. This is the limitation of the study. However, we carried out a blast analysis of Tat sequences of consensus subtype C and consensus subtype B, which showed that the three Tat epitope regions identified by Indian LTNPs had 69, 93, and 82% similarity (Figure S2 in Supplementary Material). Given our findings, future studies using consensus subtype C Tat peptides or autologous peptide sets based on individual viruses are now warranted.

Since the ADCC activity observed in the NK cell activation assays significantly correlate with a functional killing assay (11), we can speculate that NK cell activating antibodies from Indian LTNPs would have an ability to kill HIV-infected cells in vivo; future studies are needed to study the efficiency of target cell killing by these antibodies.

In conclusion, ADCC responses have been characterized in Indian LTNPs for the first time. The findings added to the global data and confirm the association of broad ADCC responses in viral containment across different subtypes. The frequently recognized peptides from the V3 loop of Env-C and Tat antigen by LTNPs with controlled viral replication warrants further studies to understand their probable role in clearing the latent infection and also in HIV protection.

**ETHICS STATEMENT**

The study design was approved by an institutional ethics review board of National AIDS Research Institute. All study participants provided informed consent before the collection of blood samples. The study did not involve any vulnerable population.

**AUTHOR CONTRIBUTIONS**

AK performed the research, data analyses, and wrote the manuscript. MT and SJK designed the study and commented on manuscript. RP commented on manuscript. AS, SK, and VM performed parts of the research and helped in manuscript writing. MG and SG contributed clinical information and samples for the study.

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REFERENCES


SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00005/full#supplementary-material.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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