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Incomplete functional T-cell reconstitution in immunological non-responders at one year after initiation of antiretroviral therapy possibly predisposes them to infectious diseases

Ashwini Shete[a], Sampada Dhayarkar[a], Shashikala Sangale[b], Uttam Medhe[c], Narayan Panchal[a], Girish Rahane[a], Rajendra Yelgate[a], Ashwini Dhamanage[a], Raman Gangakhedkar[a,d,*]

[a] ICMR–National AIDS Research Institute, Bhosari, Pune, India
[b] B.J. Medical College and Sassoon General Hospital, Pune, India
[c] Yashwantrao Chavan Memorial Hospital, Sant Tukaram Nagar, Pimpri, Pune, India
[d] Indian Council of Medical Research, V. Ramalingaswami Bhawan, Ansari Nagar, New Delhi, India

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ABSTRACT

Background: Immunological non-responders (INR) represent a unique category of HIV-infected patients on antiretroviral therapy. These patients have suppressed viremia but a suboptimal increase in CD4 cell count, which might have opposing effects on functional immune reconstitution. Hence, the extent of immune reconstitution in INR patients was investigated in order to determine their susceptibility to opportunistic infections.
Methods: Twenty-three INR patients (CD4 increase <50 cells/mm^3, viral load <40 copies/ml), 40 age-, sex-, and baseline CD4 count-matched responders (CD4 increase >100 cells/mm^3, viral load <40 copies/ml), and 18 treatment failures defined as per the national guidelines were enrolled at 1 year of antiretroviral therapy. The following examinations were performed: haemogram, phenotypic characterization by flow cytometry, and assessment of functional immune status by ELISPOT and intracellular cytokine assays.
Results: A higher percentage of INR patients had clinically symptomatic infections than the responders. CD8$^+$ activation and innate immune parameters, including the absolute neutrophil count and natural killer (NK) cell frequency and functionality, were restored in the INR patients. They had significantly higher non-HIV antigen-specific T-cell responses and activated CD4$^+$ cells, but significantly compromised T-cell functionality, as assessed after anti-CD3 stimulation, and lower CD31$^+$ and CD62L$^+$CD4$^+$ cells.
Conclusions: INR patients showed lower thymic output, incomplete functional T-cell reconstitution, higher responses to HIV co-pathogens, and higher symptomatic events, indicating the need for close monitoring and intervention strategies to overcome their continuing immunocompromised status.

Introduction

The initiation of antiretroviral therapy (ART) in an HIV-infected individual leads to a rapid reduction in plasma HIV-1 RNA levels and an increase in peripheral CD4$^+$ cell counts (Gulick et al., 1997; Hammer et al., 1997; Montaner et al., 1998). However discordant responses, referred to as immunological non-response (INR), whereby the CD4 count fails to rise but the plasma viral load is suppressed, may occur in approximately 20% of patients (Gaardbo et al., 2012). The main goal of ART is the suppression of HIV replication leading to an undetectable viral load. Hence it is difficult to decide whether to change antiretroviral drugs in the case of INR when there is complete suppression of the viral load.

A major concern regarding INR patients is their persistent immunosuppressed status, as indicated by low CD4 counts, leading to higher susceptibility to opportunistic infections and progression to AIDS. Since CD4 T-cells are the key cells of the immune system, it is critical to understand the extent of restoration of immune function in INR patients who have persistently low CD4 counts but suppressed HIV viremia. Viremia in HIV-infected patients is

* Corresponding author at: Epidemiology and Communicable Diseases, Indian Council of Medical Research, V. Ramalingaswami Bhawan, PO Box No. 4911, Ansari Nagar, New Delhi, India.
E-mail addresses: rgangakhedkar@nariindia.org, rgangakhedkar.hq@icmr.gov.in (R. Gangakhedkar).

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responsible for continued antigenic exposure leading to chronic immune activation. Immune activation is the hallmark of HIV infection, and its role in HIV pathogenesis is evident from the association of CD38 expression on T-cells with disease progression (Giorgi et al., 1993). Its importance in HIV pathogenesis is also stressed by studies performed in the African green monkey model, which failed to show progression to AIDS in the absence of chronic immune activation in spite of higher viral loads (Silvestri et al., 2003). Chronic immune activation is responsible for impaired immune functions because of enhanced immune exhaustion, senescence, and activation-induced apoptosis (Appay and Sauce, 2008; Sokoya et al., 2017). Immune activation has been shown to be significantly reduced in patients with virally suppressive ART for 12 months (Koblavi-Deme et al., 2003).

Continued viral suppression and a reduction in immune activation for more than 1 year has been shown to result in improvements in innate immune functions (Chehimi et al., 2007). The effect of viral suppression on T-cell functions may differ, because a reduced antigenic load as a result of viral suppression has been shown to result in a reduced frequency of HIV-specific CD8 cells (Casazza et al., 2001). However, it has been shown to restore CD8 functionality, leading to the appearance of polyfunctional cells (Rehr et al., 2008). Furthermore, although HIV-specific CD8 cells decay after ART initiation, a rapid increase in $CD8^+$ T-cell responses to other chronic viral infections has been observed in previous studies (Casazza et al., 2001), indicating the restoration of Cytotoxic T lymphocyte (CTL) functionality against these infections. Highly active antiretroviral therapy (HAART) for 6 months has also been shown to restore antigen-specific CD4 T-cell responses to several antigens (Wendland et al., 1999). However, the extent of immune restoration after viral suppression in the presence of poor CD4 cell recovery needs to be determined in order to assess the possible susceptibility of these individuals to opportunistic infections.

Patients with and without suboptimal CD4 recovery have been reported to have similar incidences of clinical events (Nakanjako et al., 2008). Conversely, the risk of developing severe clinical events has been reported for patients with INR (Lapadula et al., 2013). Although a few studies have reported an increased occurrence of CDC category B and C events (Centers for Disease Control and Prevention) (Kaufmann et al., 2005; Tan et al., 2008), this increase as well as the prognosis in these individuals did not differ significantly from those of patients with a complete response. The results of the SMART study also indicated that the risk of progression to AIDS diminishes if virological suppression is sustained irrespective of low $CD4^+$ cell counts in these patients, suggesting that people with full viral load suppression may not require prophylaxis despite lagging CD4 counts (Zoufaly et al., 2011). These findings indicate that there might be a qualitative improvement in T-cell functionality in spite of low CD4 counts.

Hence this study was conducted to determine the extent of immune reconstitution in INR patients as compared to treatment responders at 1 year following combined ART initiation. The responders were matched with INR patients to nullify the effects of age, sex, and nadir CD4 counts on immune functionality.

Materials and methods

Study population

Patients taking ART for 1 year, who were being treated at Yashwantrao Chavan Memorial Hospital (YCM) and B.J. Medical College (BJMC) ART centres under the National AIDS Control Organization (NACO) programme, and who had >95% adherence at all visits, were enrolled and assigned to one of three categories: (1) INR patients, who had an increase of <50 CD4 cells/mm^3 at the end of 1 year and a viral load of <40 copies/ml; (2) patients with an increase of >100 CD4 cells/mm^3 at the end of 1 year and who were matched for age (±5 years), sex, and baseline CD4 count with the INR patients (control group of matched responders); (3) patients with treatment failure, identified as per the national guidelines.

The study was approved by the institutional ethics committees. Blood samples were collected after obtaining written informed consent from the patients. The samples were used for haemogram, CD4 count estimation, and viral load testing. Peripheral blood mononuclear cells (PBMCs) separated from the samples were cryopreserved. Thawed PBMCs were rested for 2 h prior to performing the various immunological assays.

ELISPOT assay

The interferon gamma (IFN-γ) secretory ELISPOT assay was performed as described elsewhere (Lichterfeld et al., 2004). Briefly, the procedure included incubation of 1×10^5 PBMCs per well from the study participants with 5 μg/ml of Gag (NIH AIDS Research and Reference Reagent Program) and FEC (a pool of peptides from influenza virus, Epstein–Barr virus, and cytomegalovirus; Mab-Tech) peptide pools. Unstimulated cells with only media served as a negative control and phytohaemagglutinin (PHA)-stimulated cells served as a positive control. PBMCs were also incubated with K562 cells (a target cell line for natural killer (NK) cells) at an effector-to-target ratio of 10:1 for the assessment of NK cell functionality. The incubation was done in a pre-wetted anti-human IFN-γ antibody-coated plate at 37 °C in a 5% CO_2 incubator for 16–20 h. Detection antibody conjugated with enzyme and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate was added after the overnight incubation with intermittent washing steps. The reaction was stopped by washing the plate thoroughly with distilled water and the plate was read on an automated ELISPOT reader (AID, Germany) after complete drying of the plate.

Intracellular cytokine assay

The intracellular cytokine assay was performed using the revived PBMCs after 2 h of resting. The cells were stimulated with FEC peptide pool (5 μg/ml) and anti-CD3/CD28 antibodies. For anti-CD3/CD28 stimulation, round bottom tubes (BD Biosciences) (fluorescence-activated cell sorting) were coated with 1 μg/ml of each of the antibodies overnight at 4 °C. PBMCs were added to the tubes after washing them three times with sterile phosphate-buffered saline (PBS). PBMCs (1×10^6) were incubated with FEC or coated anti-CD3/CD28 at 37 °C in a 5% CO_2 atmosphere for 6 h in the presence of brefeldin A (5 μg/ml; Sigma-Aldrich) and anti-CD107a–APC-H7 (BD Biosciences, USA), a degranulation marker. Ethylenediaminetetraacetic acid (EDTA) 10 mM was added to the cells and the cells were washed with PBS containing 0.5% bovine serum albumin (BSA) (wash buffer). The cells were stained for surface markers using anti-CD4 PE/Dazzle 594 (Biolegend, USA) and anti-CD8 BUV737 (BD Biosciences) antibodies for 30 min at room temperature in the dark. The cells were washed and fixed with lysing solution (BD Biosciences) for 10 min at room temperature. The cells were then washed once with wash buffer and permeabilized using permeabilizing solution 2 (BD Biosciences) for 10 min at room temperature. The cells were subsequently washed with wash buffer and stained with anti-TNF-α PECy7, anti-IL-2 BV605, and TNF-α BUV395 (BD Biosciences) for 30 min at room temperature. The cells were then washed and resuspended in the fixative (3% formaldehyde). Fifty thousand gated lymphocytes per sample were acquired using a FACSAria Fusion flow cytometer (BD Biosciences) and analyzed using FACSDiva software. PBMCs without any antigen stimulation were used as negative control.

Surface phenotyping

PBMCs were assessed for CD4 and CD8 cell phenotypic characterization, activation marker expression, and frequency of different populations of NK and B cells. PBMCs were stained with anti-CD3 PE/Dazzle 594 (Biolegend), anti-CD62L PE, and anti-CD31 PE/Cy7 for phenotypic characterization; anti-CD3 PE/Dazzle 594 (Biolegend), anti-CD8 APC/Cy7, anti-HLA-DR Percp5.5, anti-CD38 FITC, anti-CD25 APC, and anti-CD127 PE/Cy7 for activation marker analysis; and anti-CD3 PE/Cy7, anti-CD56 APC/Cy7, anti-CD16 FITC, anti-CD19 APC, and CD27 PE for NK and B cell phenotyping (all from BD Biosciences) in the second tube for 30 min at room temperature in the dark. CD4 cells in the first two tubes were identified as $CD8^{-}CD3^{+}$ cells. The cells were washed and fixed by adding 3% formaldehyde and were acquired within 24 h to obtain 50 000 gated lymphocyte events by FACSAria Fusion (BD Biosciences). The data were analyzed using FACSDiva software.

Human total IgG ELISA

Plasma samples from the study participants were used to measure total immunoglobulin G (IgG) levels using a commercially available ELISA kit according to the manufacturer's protocol (Invitrogen, USA). Plasma samples were diluted 1:500 000 as per the manufacturer's instructions and an appropriate dilution factor was applied to calculate the levels.

Statistical analysis

The statistical analysis was performed and graphs plotted using GraphPad Prism software version 5. Continuous variables and frequencies were compared between the two groups using the Mann–Whitney test and Fisher's exact test in 2×2 contingency tables, respectively. p-Values of <0.05 were considered significant.

Results

Study population

The characteristics of the study participants are shown in Table 1. The participants in the INR group and responder group were matched for age, sex, and baseline CD4 count. Hence these parameters did not differ between the groups. The absolute CD4 count ($p < 0.0001$) and CD4% ($p = 0.024$) at 1 year were significantly lower in the INR group as compared to the responder group. The treatment failure group had significantly lower CD4 counts than the INR group at baseline as well as at 1 year ($p = 0.004$). The number of participants with a CD4 count <200 cells/mm^3 did not differ among the groups at baseline, but there were significantly more at 1 year in the INR group as compared to the responder group. The proportion of symptomatic participants with World Health Organization (WHO) clinical stage 2–4 disease was higher in the INR group than in the other groups at baseline, although the difference was not statistically significant. However, the proportion was significantly higher in the INR group than in the responder group at the first year of follow-up ($p = 0.024$). Haemogram reports showed a significantly higher leukocyte count in the responders than in the other two groups. Differential counts indicated significantly higher absolute lymphocyte and eosinophil counts in the responder group as compared to the other two groups. However, the INR group showed a significantly higher absolute neutrophil count than the treatment failure group, which was comparable to that of the responders.

Table 1
Characteristics of the study participants.[a]

Characteristic	INR ($n = 23$)	Matched responders ($n = 40$)	Treatment failures ($n = 18$)	p-Value compared with responders	p-Value compared with treatment failures
Age (years)	42 (23–60)	39.5 (25–62)	37.5 (24–52)	NS	NS
Sex, M:F	11:12	20:20	9:9	NS	NS
Baseline CD4 (cells/mm^3)	270 (14–380)	283.5 (25–352)	142.5 (8–358)	NS	0.0046
Patients with baseline CD4 count of <200 cells/mm^3 (%)	30.4%	42.5%	66.6%	NS	NS
Enrolment CD4 (cells/mm^3)	227 (13–360)	479 (246–1387)	89 (19–331)	<0.0001	0.004
Patients with CD4 count of <200 cells/mm^3 at enrolment (%)	30.4%	0%	72.2%	0.0017	NS
CD4 increase (cells/mm^3)	3 (−295 to 48)	242 (104 to 1035)	−7 (−162 to 96)	<0.0001	NS
Enrolment CD4%	20 (4–32)	23.5 (11–41)	8 (2–21)	0.024	0.003
WHO clinical stage 2–4 at baseline (%)	29%	10%	9%	NS	NS
WHO clinical stage 2–4 during first year (%)	41%	13%	36%	0.0249	NS
Viral load (copies/ml)	<40	<40	63 165 (1891–526 175)	NS	<0.0001
WBC count ($\times 10^9$/l)	4.9 (1.2–12.9)	5.95 (3.3–11)	3.65 (2–7.7)	0.03	0.04
Absolute neutrophil count ($\times 10^9$/l)	3.27 (1.3–11.04)	3.3 (1.6–6.19)	2.13 (1.33–4.66)	NS	0.007
Absolute lymphocyte count ($\times 10^9$/l)	1.22 (0.36–2.3)	2.0 (1.13–4.47)	1.17 (0.36–2.2)	<0.0001	NS
Absolute monocyte count ($\times 10^9$/l)	0.24 (0.08–0.57)	0.28 (0.12–0.56)	0.25 (0.17–0.39)	NS	NS
Absolute eosinophil count ($\times 10^9$/l)	0.09 (0.03–0.68)	0.19 (0.03–0.76)	0.1 (0.03–0.62)	0.0009	NS
Absolute basophil count ($\times 10^9$/l)	0.03 (0–0.09)	0.03 (0.01–0.7)	0.03 (0.01–0.3)	NS	NS

F, female; INR, immunological non-responders; M, male; NS, non-significant; WBC, white blood cell.
[a] Results are presented as the median (range), unless stated otherwise.

IFN-γ secretory ELISPOT assay

The IFN-γ secretory response was assessed against HIV, non-HIV antigens, and PHA (Figure 1). The INR and treatment failure groups had lower responses to PHA than the responder group, although the response did not differ significantly. The HIV-specific response was assessed using the HIV Gag peptide pool. The Gag-specific response did not differ between the INR and responder groups, but was significantly lower ($p = 0.04$) in the treatment failure group as compared to the responder group. Similarly, the response was also determined against common viral infections using FEC peptides. The INR group showed a higher response against FEC peptides than the other groups, and this was significantly higher as compared to the response of the failure group ($p = 0.03$). All samples showed fewer than 5 spots in unstimulated wells. NK cell functionality was also assessed in the ELISPOT assay by using K562 cells, which serve as the targets for NK cells. The treatment failure group had significantly suppressed NK cell functionality as compared to the responders ($p = 0.046$) and INR patients ($p = 0.044$).

Intracellular cytokine assay

Intracellular cytokine assays were performed to assess different cytokines expressed in response to the FEC peptide pool as well as anti-CD3 stimulation. Individuals who responded to FEC peptides in the ELISPOT assay were assessed for their response in the intracellular cytokine assay. Figure 2 shows the intracellular cytokines expressed by the CD8 and CD4 cells of these individuals. The INR group showed higher IFN-γ- and tumour necrosis factor alpha (TNF-α)-expressing CD8 cells at baseline than the responder group. Interestingly, the FEC-specific response was higher in the INR group and was significantly higher in terms of TNF-α ($p = 0.04$) and interleukin (IL)-2 (one-tailed $p = 0.02$) expression than that of the responder group. However, the INR group showed compromised IFN-γ ($p = 0.004$ and one-tailed $p = 0.02$, respectively) and TNF-α ($p = 0.0007$ and $p = 0.01$, respectively) expressing CD8 and CD4 responses in response to anti-CD3 stimulation as compared to the responder group. CD107a-expressing CD8 cells were also significantly lower in the INR group in response to anti-CD3 stimulation. Polyfunctional responses were not observed much in these groups. Bifunctional responses against FEC as well as anti-CD3 stimulation did not differ between these two groups.

T-cell activation and CD4 cell phenotyping

T-cell activation was determined by flow cytometry. Activated CD4 cells (Figure 3a), as determined by CD38, HLA-DR, and CD25 expression, were significantly higher in treatment failure patients as compared to responders, as well as INR patients ($p = 0.0002$, $p < 0.0001$, $p < 0.0001$ and $p = 0.02$, $p = 0.002$, $p = 0.0001$, respectively). INR patients also had higher CD38 ($p = 0.005$), HLA-DR ($p = 0.035$), and CD25 ($p = 0.018$) expressing CD4 cells than the responders. CD8 cell activation (Figure 3b) was assessed by determining the expression of CD38, HLA-DR, and both together. Treatment failure patients had higher expression of these markers as compared to the responders, as well as INR patients ($p < 0.0001$, $p = 0.039$, $p < 0.0001$ and $p = 0.0007$, $p > 0.05$ (not significant), $p = 0.0018$, respectively). However, INR patients did not have higher activated CD8 cells as compared to the responders. CD4 cells were also analysed for their maturation phenotypes by determining the expression of CD45RA and CD62L markers on their cell surface. Naïve ($CD45RA^+CD62L^+$) and central memory ($CD45RA^-CD62L^+$) CD4 cells were less, while effector memory ($CD45RA^-CD62L^-$) and effectors were comparatively more in INR patients as compared with responders (Figure 3c). CD62L-expressing CD4 cells were significantly less in INR patients than in responders ($p = 0.046$). CD31-expressing CD4 cells were also significantly ($p = 0.047$) less in INR and treatment failure patients than in responders, indicating lower thymic output in these patients (Figure 3c).

NK and B cell frequency

Percentages of NK cells and their different types were determined by the expression of CD56 and CD16 markers on CD3-negative cell populations (Figure 4a). Total ($CD56^+$ and/or $CD16^+$), regulatory ($CD56^+CD16^-$), cytotoxic ($CD56^+CD16^+$), and defective NK cells ($CD56^-CD16^+$) also did not differ significantly among responders and the INR group. The responders had significantly lower defective NK cells ($p = 0.02$) as compared to the treatment failure group. However, regulatory NK cells were significantly higher in the INR group than in the treatment failure group ($p = 0.001$). B ($CD19^+CD3^-$) cells were differentiated as naïve and memory cells by the expression of CD27 (Figure 4b). The percentages of total and naïve B cells ($CD19^+CD27^-$), although higher in responders, did not differ significantly among the three groups. However, the percentage of memory B cells ($CD19^+CD27^+$) was significantly higher in the responder group than in the treatment failure group ($p = 0.002$).

Total IgG levels

Total IgG levels in plasma samples of the study participants were measured by ELISA (Figure 4c). Plasma IgG levels in treatment failure patients were significantly higher than those in responders

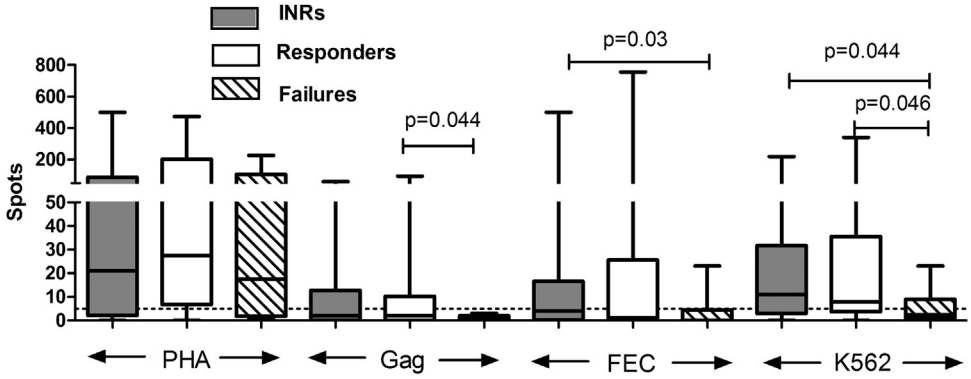

Figure 1. Interferon gamma (IFN-γ) secretory response by ELISPOT assay. The figure shows the number of spots (*y*-axis) in the IFN-γ secretory ELISPOT assay by peripheral blood mononuclear cells (PBMCs) of the different group participants against different stimuli (*x*-axis). The bars represent the medians and the error bars indicate the interquartile ranges for the values. *p*-Values showing significant differences ($p < 0.05$) between the groups as calculated by Mann–Whitney test are shown in the figure.

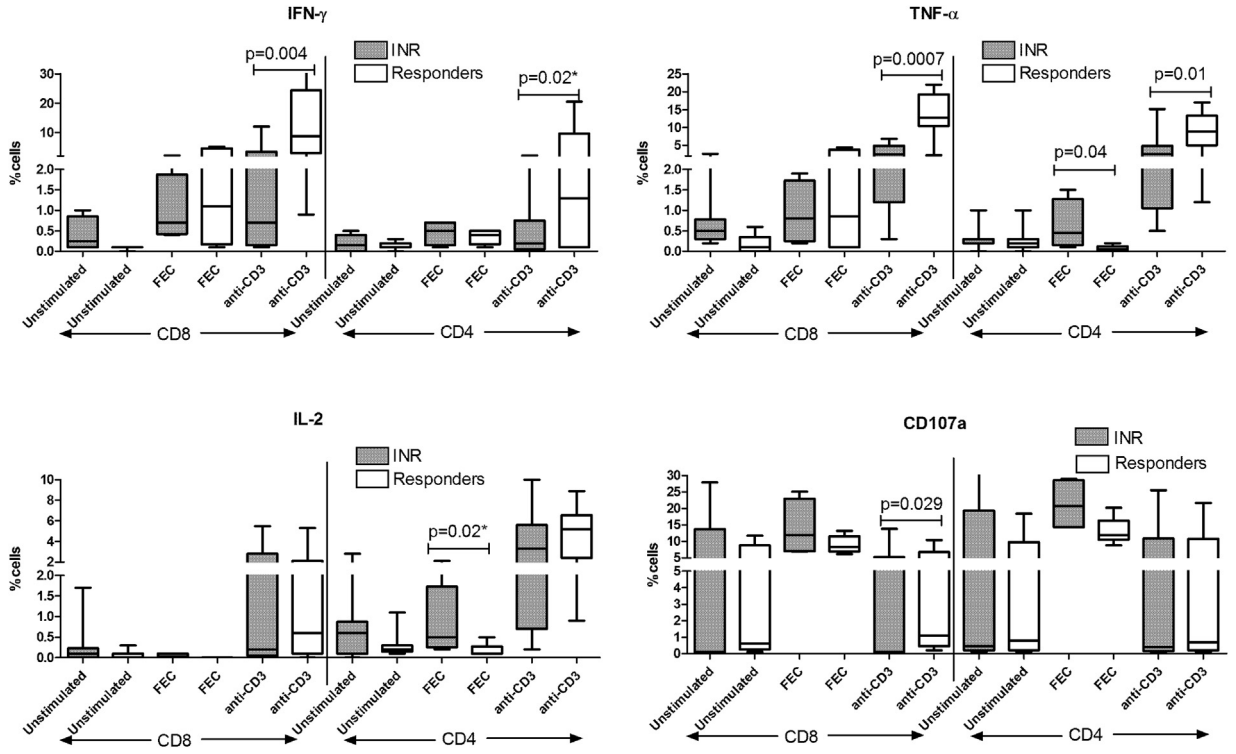

Figure 2. Cytokine response by intracellular cytokine assay. The figure shows the percentages of CD8 and CD4 cells (*y*-axis) of the immunological non-responders (INRs) and responders expressing different cytokines or degranulation marker CD107a after stimulation with FEC or anti-CD3 antibodies (*x*-axis) by flow cytometry. The bars represent the medians and the error bars indicate the interquartile ranges for the values. *p*-Values showing significant differences ($p < 0.05$) between the groups as calculated by Mann–Whitney test are shown in the figure.

($p = 0.002$) and INR patients ($p = 0.041$). The levels in the INR patients and responders did not differ significantly, although INR patients had higher levels than the responders. The levels in the study participants correlated negatively with CD4 counts ($r = -0.37$, $p = 0.003$) and positively with viral loads ($r = 0.33$, $p = 0.007$).

Discussion

Immunological non-responders are HIV-infected patients with discordant responses in terms of suppressed viral load but suboptimal increase in CD4 count after the initiation of HAART. It is important to evaluate the recovery of these immune responses in these patients, as compromised immune functions owing to poor CD4 recovery might increase their susceptibility to different opportunistic infections. Although participants could not be analysed separately according to WHO clinical stage because of the small sample size, those with symptomatic infections classified as WHO clinical stage 2 and above were analysed together. In this study, the proportion of such symptomatic INR patients was significantly higher than that in the responder group. The proportion of INR patients with a CD4 count <200 cell/mm^3 at 1 year was also significantly higher in the INR group than in the responder category, indicating a severe immunosuppressed status in these patients. However, they were not likely to be AIDS presenters at ART initiation, as the percentages of study participants with a baseline CD4 count <200 cell/mm^3 did not differ significantly among the groups. Immune responses were assessed at 1 year of ART in this study, as many patients show a significant, although not complete, reconstitution by that time (Chehimi et al., 2007; Serpa et al., 2010; Wendland et al., 1999). Early responses within the first year have also been shown to influence life-expectancy in the long run (May et al., 2014). It is also important to identify defects in immune responses, as well as possible mechanisms of immune non-reconstitution as early as possible in order to develop intervention strategies to prevent further morbidity.

Patients with INR had significantly lower absolute and percentage CD4 counts than the matched responders at 1 year. However, their CD4 counts were significantly higher than those of the treatment failure patients at baseline as well as at 1 year. Patients with lower CD4 counts at ART initiation have been shown to be at high risk of developing subsequent virological failure (Deeks, 2000). Since leukocytes are the cells of the immune system that are involved in protecting the body against infectious disease, differential counts were studied in the three study groups. The absolute lymphocyte count has been shown to correlate with CD4 counts in HIV-infected patients (Kakar et al., 2011). The absolute lymphocyte count in the INR patients was found to be similar to that in treatment failure patients and significantly lower than the count in responders. Neutropenia has been shown to occur in 10–50% patients and to be associated with HIV disease progression (Levine et al., 2006). HAART has been shown to cause resolution of neutropenia (Levine et al., 2006). Although treatment failure patients had lower numbers of neutrophils, virally suppressive ART had resulted in the restoration of the neutrophil count in the INR patients. Interestingly, eosinophil counts in INR patients and treatment failure patients were significantly lower than those in the responders. Impairment in IL-5 production during HIV infection is reported to be a factor associated with the paradoxical eosinopenia observed in tropical areas (Diagbouga et al., 1999). Human eosinophils have also been shown to express CD4 receptors (Lucey et al., 1989) and hence the presence of any common mechanism for non-reconstitution of these cells needs to be investigated.

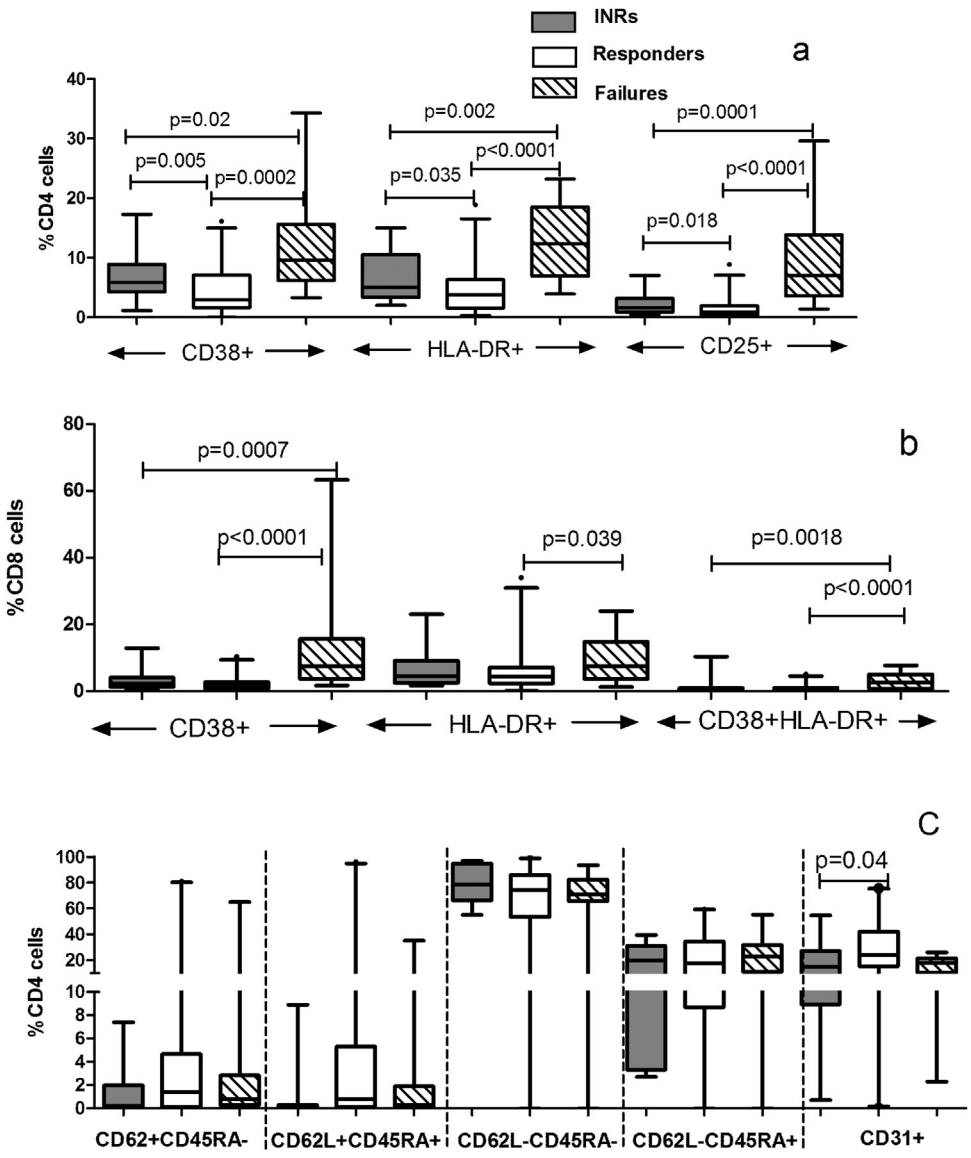

Figure 3. CD4 and CD8 cell phenotyping by flow cytometry: (a) frequency of CD4 cells of different study participants (*y*-axis) expressing activation markers CD38, HLA-DR, and CD25 as shown on the *x*-axis; (b) frequency of CD8 cells of different study participants (*y*-axis) expressing activation markers CD38, HLA-DR, and both together $CD38^{+}HLA^{-}DR^{+}$ as shown on the *x*-axis; (c) frequency (*y*-axis) of maturation phenotypes of CD4 cells of different study participants determined by expression of CD45RA and CD62L, as well as CD31-expressing CD4 cells, as shown on the *x*-axis. The bars represent the medians and the error bars indicate the interquartile ranges for the values. *p*-Values showing significant differences ($p < 0.05$) between the groups as calculated by Mann–Whitney test are shown in the figure.

ART has been shown to restore many effector functions in HIV-specific CTL, but it reduces the number of circulating HIV antigens cells. CTL responses as assessed by the IFN-γ secretory response against HIV as well as non-HIV antigens in the present study were found to be comparable in INR patients and responders. The treatment failure group showed significantly lower responses against Gag than the responder group. A previous study found Gag-specific $CD8^{+}$ T-cells to be inversely correlated with viral load (Alatrakchi et al., 2005), indicating the importance of this response in controlling viral replication during the initial period after ART initiation. Interestingly, INR patients showed significantly higher cytokine responses against FEC than the responder patients in the intracellular cytokine assay. Since there was a significantly higher percentage of symptomatic INR patients than symptomatic responders, ongoing active infections with these pathogens leading to higher immune responses need to be ruled out. The presence of co-pathogens has been associated with discordant immune responses to ART (Tincati et al., 2018). Contrary to FEC-specific responses, anti-CD3-induced CD4 and CD8 cell responses were found to be severely compromised in INR patients, indicating incomplete recovery of immune functions in these patients. Polyfunctional responses were rare even in responders in the present study, as it may take longer for these responses to recover; this has been reported in a previous study (Rehr et al., 2008).

Since immune activation plays an important role in contributing to immunocompromised conditions in HIV infection, restoration of the activation status of T-cells was determined. Although INR patients had significantly lower activated CD4 and CD8 T-cells as compared to treatment failure patients, they still had higher activated CD4 T-cells than the responder group. Poor CD4 recovery despite suppressive ART has been reported to be related to hyper-activation of CD4 cells (Lederman et al., 2011; Massanella et al., 2010). Contrary to CD4 cells, the frequency of activated CD8 T-cells was similar in the INR and responder groups. Activated CD8 cells, especially $CD38^{+}HLA^{-}DR^{+}$ CD8 cells, have been shown to correlate directly with HIV viral load (Sachdeva et al., 2010). Since both of

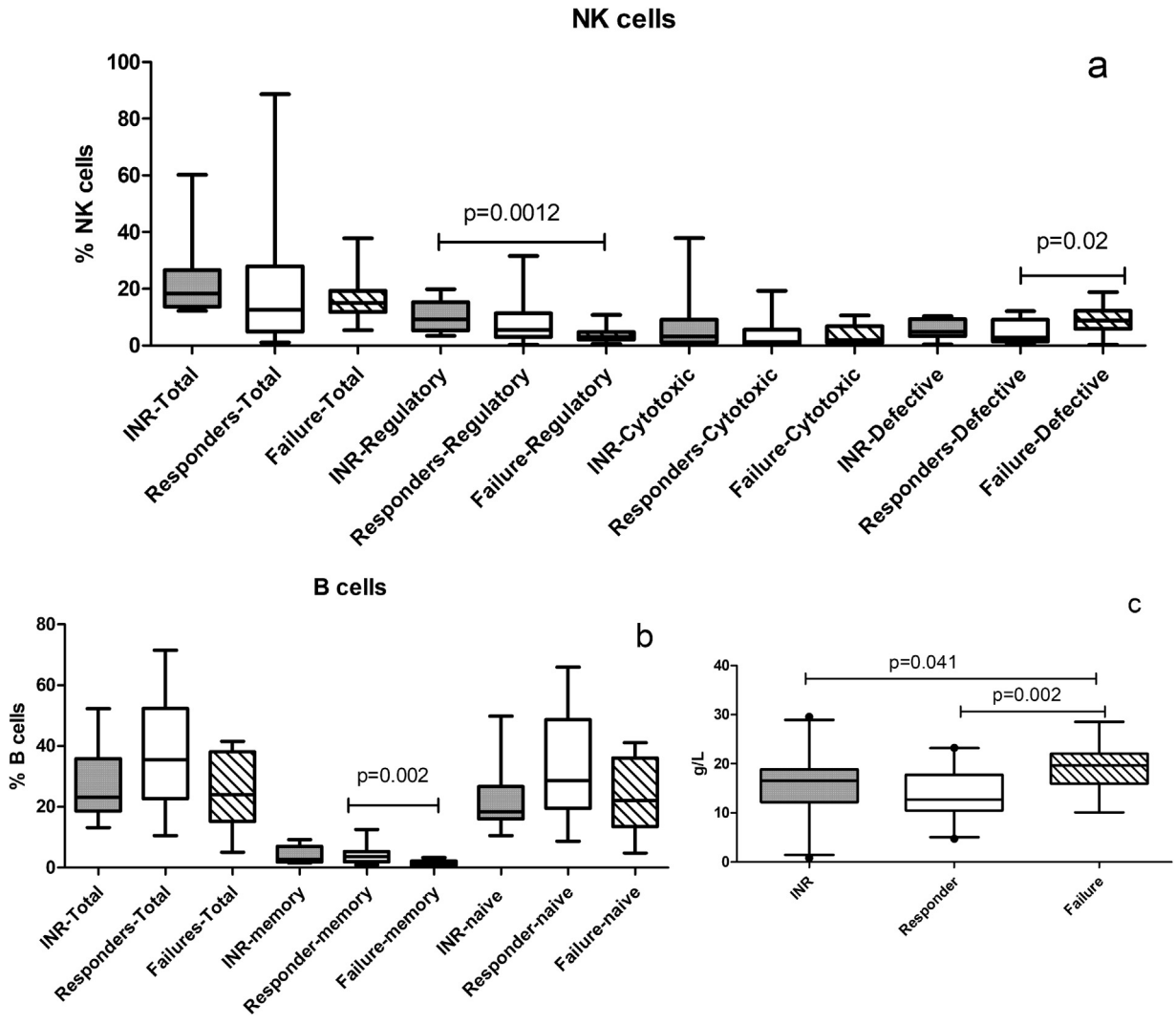

Figure 4. NK and B cell frequency by flow cytometry: (a) frequency (*y*-axis) of NK cells and their types in different study participants (*x*-axis) as determined by CD56 and CD16 expression; (b) frequency (*y*-axis) of total, memory, and naïve B cells in different study participants (*x*-axis) as determined by CD19 and CD27 expression; (c) total IgG levels (g/l) plotted on the *y*-axis for the different study groups (*x*-axis). The bars represent the medians and the error bars indicate the interquartile ranges for the values. *p*-Values showing significant differences ($p < 0.05$) between the groups as calculated by Mann–Whitney test are shown in the figure.

these groups had undetectable viral loads, they might have had lower activated CD8 cells as compared to the treatment failure group. The INR group had significantly lower CD62L-expressing CD4 cells, which comprise naïve and central memory cells. They also had significantly lower CD31-expressing cells, indicating lower thymic output, as CD31 is a marker of recent thymic emigrants among $CD4^+$ T-cells (Tanaskovic et al., 2010). Although thymic function is known to decline with age, a substantial output is still maintained into late adulthood, playing a prominent role in the supply of naïve T-cells (Poulin et al., 1999). Many studies have shown that the adult thymus can contribute to immune reconstitution following HAART (Douek et al., 1998; Harris et al., 2005), and reduced thymic output has been shown to be a major mechanism of immune reconstitution failure in HIV-infected patients after long-term ART (Poulin et al., 1999). Lower naïve cells and thymic output indicate a lower ability to respond to new antigens, limiting their immune responses to newer pathogens and thereby enhancing their susceptibility.

B cells also form an important arm of the adaptive immune response. Circulating memory B cells have been shown to be severely reduced in HIV-infected patients (Titanji et al., 2006). Treatment failure patients also had lower memory B cells as compared to responders, indicating continued memory B cell deficiency in these patients; this was restored in the INR patients who had similar frequencies to the responders. HIV infection has been shown to cause polyclonal B cell activation and hypergammaglobulinemia, affecting humoral immunity (Serpa et al., 2010). The initiation of ART has been shown to cause a significant reduction in gamma-globulin levels, indicating possible improvements in humoral immune responses (Chong et al., 2001; Serpa et al., 2010). A significant reduction in gamma-globulin levels in responders and INR patients compared to treatment failure patients was also found in the present study. The levels correlated negatively with CD4 counts, and INR patients had higher levels of IgG than responders, although this was not statistically significant, indicating suboptimal recovery of B cell function in these patients. NK cell frequency and functionality was assessed to determine the effect of viral suppression on their restoration in INR patients. Although total NK cell frequency did not differ significantly between the three groups, INR patients had relatively higher NK cells, even as compared to the responders. This could be because of lower numbers of CD4 cells in the lymphocyte population affecting relative NK cell percentages in these patients. The frequency of regulatory NK cells was significantly higher in INR patients as

compared to the treatment failure group. This could have been the reason for the significantly lower IFN-γ secretory response of NK cells in response to K562 target cells in the treatment failure patients as compared to the INR patients. High levels of chronic HIV-1 viremia have been shown to result in the appearance of functionally impaired CD56-negative NK cells (Mavilio et al., 2005; Mikulak et al., 2017). Such defective NK cells were also found to be higher in the treatment failure patients in the present study.

In conclusion, this study indicated restored CD8 activation in the presence of suppressed viremia in INR patients. Innate immune parameters such as the absolute neutrophil count and NK cell frequency and functionality were also restored in these patients. However, they had lower thymic output and CD4 activation status, and furthermore, T-cell functionality assessed after anti-CD3 stimulation was not restored in them. Interestingly, INR patients showed higher T-cell responses against HIV co-pathogens, indicating the need for the diagnosis and treatment of other active infections in these patients. It is possible that some of the differences determined at 1 year in this study did not reach the level of statistical significance because the immune responses might take longer to recover; hence there is a need to perform further studies to examine these factors after long-term ART. Since INR patients presented a higher frequency of symptomatic infections at 1 year, they need close monitoring in order to diagnose and manage these clinical events in a timely manner. Intervention strategies to improve thymic output also need to be evaluated in these patients to overcome their continuing immunocompromised status.

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Ethics

The study was approved by the institutional ethics committees and was conducted in accordance with the Declaration of Helsinki. Patients were enrolled after obtaining written informed consent.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Author contributions

Data acquisition, revising and approving the final manuscript: Ashwini Shete, Sampada Dhayarkar, Shashikala Sangale, Uttam Medhe, Narayan Panchal, Girish Rahane, Rajendra Yelgate, Ashwini Dhamanage, Raman Gangakhedkar. Design of the study: Ashwini Shete, Sampada Dhayarkar, Raman Gangakhedkar. Data analysis and writing of the paper: Ashwini Shete, Sampada Dhayarkar, Raman Gangakhedkar, Ashwini Dhamanage.

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