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A decrease in PPD specific CD4 T cell CD38 and HLA-DR expression in pulmonary tuberculosis patients after 8 weeks of therapy correlates with successful anti-tuberculosis treatment

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ABSTRACT

Tuberculosis (TB) is a major health problem in Indonesia with a million new cases each year. The CD4 T cell adaptive immune response against *Mycobacterium tuberculosis* (MTB) is central to the control of this disease. We investigated whether standard therapy of TB causes changes to these cells in the early stages of treatment. To do this we took blood samples from 2 groups of TB patients in Banda Aceh, Indonesia; one from a group of patients before treatment, and the other from a group who become smear negative after 8 weeks treatment. MTB specific CD4 T cells were identified by *ex vivo* stimulation with PPD and flow cytometric measurement of intracellular cytokines and surface markers. We found no difference in total PPD specific CD4 T cells between the groups, but that the proportion of these cells CD38 + HLA-DR+ was significantly lower in the treatment group. This decrease was not specific to Interferon gamma (IFNg), Interleukin-2 (IL-2) or Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) producing cells. Our findings show that anti-MTB treatment affects the adaptive immune response, and that measuring the decrease of the PPD specific CD4 T cell CD38+HLA-DR+ phenotype could be a useful parameter for determination of treatment success.

1. Introduction

Tuberculosis (TB) is a major health concern worldwide, with widespread infection and morbidity. In Indonesia annual TB incidence is reaching 1,020,000 cases [1]. Currently standard treatment for TB consists of a combination of the antibiotics Isoniazid, Rifampicin, Pyrazinamide and Ethambutol in an intensive phase for 2 months, followed by Isoniazid and Rifampicin for at least another 6 months. While there is a growing problem of drug resistant forms of MTB that do not respond to this treatment regime, overall it is quite successful with cured rates of greater than 85% [1]. TB is also known to exist in a state of latent infection, where the organism may still reside in a person but does not cause any outward symptoms [2]. This may be the case for successfully treated patients as it is still unclear if treatment fully removes the organism from an individual [3].

The CD4 T cell response is vital to the immune system's control of MTB infection, and measurement of this response is the basis of the tuberculin skin test and interferon gamma release assays that are used to determine if a person has been exposed/infected with MTB [4]. We and others have demonstrated that measurement and characterisation of this response can also be done with flow cytometry, particularly using intracellular cytokine staining (ICS) [5,6]. Using this technique particular phenotypes of MTB specific CD4 T cells have been found that can distinguish active from latent TB infection, such as higher expression of activation markers CD38 and HLA-DR and also combinations of cytokine staining [7,8].

We wanted to investigate whether we could detect any changes in the phenotype and frequency of circulating CD4 T cells specific for MTB in the early stages of treatment. To test for this we obtained blood samples from two groups of pulmonary TB patients who visited the

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Zainoel Abidin Hospital, Banda Aceh, Indonesia. One group consisted of patients prior to treatment, while the second group had their blood taken after 8 weeks of their anti-MTB medications and who had become smear negative by that time. To measure MTB specific CD4 T cells we used a whole blood *ex vivo* assay to stimulate the CD4 T cells with PPD. Following the stimulation these blood samples were stored in preservative for no more than 10 days and then transported to General Hospital Dr Soetomo in Surabaya, Indonesia for antibody staining and flow cytometric analysis. We used a comprehensive 15 colour antibody panel against cytokines and surface markers to identify and phenotype the PPD specific CD4 T cells in both groups. From this we could determine if there were any differences in these cells between the before and during treatment groups.

2. Material and methods

2.1. Study subjects

Subjects were recruited from Dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia. This took place from 01 November to 30 November 2019 under Ethics Approval Number 113/EA/FK-RSUDZA/2019, Fakultas Kedokteran Universitas Syiah Kuala Rumah Sakit Umum Daerah Dr Zainoel Abdin. All subjects were adults and gave written informed consent for study participation. These subjects all had active TB as defined by positive Ziehl-Neelsen staining of sputum and PCR test using GenXpert prior to treatment.

Two groups of subjects were studied: Active TB before treatment (n = 18), and a second group of previously Active TB subjects that were 8 weeks into treatment, and who had become negative on their Ziehl-Neelsen sputum staining (n = 18).

The treatment used on all subjects for anti-MTB therapy was rifampicin, isoniazide, ethambutol and pyrazinamide. Later follow up on the individuals of both groups found that they were cured after 24 weeks treatment, as defined by subjects showing clinical improvement and negative Ziehl-Neelsen staining.

2.2. PPD specific T-cell measurement

We used an adapted version of the whole blood antigen stimulation method [9]. Briefly, 4 ml of Li Heparin blood was collected from each patient at Dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia. Previous testing from the subjects' referring centre revealed no abnormalities in the lymphocyte count for each of the subjects tested. The blood was split into two 2 ml aliquots. Both had 1 μg/ml of anti-CD28 and -CD49d (eBioscience, USA) added, and to one of them Tuberculin PPD (10 μg/ml final, Statens Serum Institute, Denmark) was added. The other aliquot served as the no antigen control. After two hours incubation (37C, 5% CO_2) brefeldin A was added at a final concentration of 1 μg/ml to both aliquots and then incubated overnight for another 14 hours. Following this 2 ml of Streck Cell preservative (Streck, USA) was added to each sample and inverted 3 times to mix. The preserved samples were stored at room temperature for up to 10 days after which they were air freighted to the Department of Clinical Pathology, General Hospital Dr Soetomo, Surabaya, Indonesia for processing and flow cytometric analysis.

For immunofluorescent labelling the following was done. The preserved samples were spun down, and the supernatant removed. The pelleted sample was then resuspended in ×1 NH_4Cl solution (×10 Stock Solution: NH_4Cl 80.2 g, $NaHCO_3$ 8.4 g, EDTA 3.7 g in 1 L H_20) to lyse the red blood cells and spun down again. Next the leukocytes were stained with surface markers anti-CD3 Alexa532 (eBioscience, USA), CD16 eFluor450, TIGIT PerCP-eF710 (ThermoFisher, USA), CD38 ECD (Beckman Coulter, USA), CD14 BV711, CD19 BV570, PD-1 BV421, HLA-DR APC/Fire 750 (BioLegend, USA), along with the Live/Dead Fixable Near IR fluorescent dye (ThermoFisher, USA). After this the cells were fixed and permeabilised (eBioscience Fixation and Permeabilization Kit) and stained with the intracellular markers anti- CD4 BV510, CD154 PE/Cy7, TNFa BV785, GM-CSF PE, IFNg FITC, and IL-2 APC (BioLegend, USA). See Table 1 for the specific antibody clones used. The labelled cells were then measured on an Aurora Spectral Analysis Flow Cytometer (Cytek Biosciences, USA).

2.3. Flow cytometry data analysis

The flow cytometric data was analysed using FlowJo software (v10.6.1, Treestar, USA). The gating strategy used to identify the antigen reactive cells is outlined in Fig. 1. Spectral unmixing was calculated using single-stained anti-mouse Ig beads (eBioscience, USA). The negative cutoffs for each individual cytokine/CD154 staining was determined by comparing PPD induced cytokine staining to the background no antigen control, and then applied to the antigen stimulated samples.

The total antigen responsive cells were defined as being CD4+CD154+TNFa+. Cell populations that were negative for this combination but positive for the other cytokines in the antigen stimulated samples were occasionally seen, but gating on them invariably revealed it to be either non-specific staining and/or overlap of non-T cell populations into the CD4 gate. The combinations of the positive staining of the other cytokines and markers on this population was then determined.

The frequency of expression of the different markers/cytokines on the antigen specific CD4 T cells was expressed in two ways. One as a proportion of total CD4 T cells, where the percentage of CD4 T cells CD154+TNFa+ and positive for the other markers was calculated. The other as a proportion of the total antigen specific CD4 T cells.

2.4. Statistical analysis

The two tailed Mann-Whitney *U* test was used to determine differences in subset distributions of the two groups (Graph Pad Software V5.01). The level of statistical significance was set at below 0.05.

3. Results

3.1. Patient demographics, clinical status, treatment

A total of 36 subjects was tested. 18 subjects had active TB and had their blood taken before anti-MTB treatment, while another 18 subjects whose Ziehl-Neelsen sputum staining had already converted negative after 8 weeks of anti TB medications also had their blood taken. Table 2 details their demographic and clinical information. There were no differences in sex, age, or body mass index between the two groups. Blood glucose levels were significantly higher in the treatment group compared to the no treatment group ($p = 0.0085$), but all individual results were within the normal range.

Table 1
Antibody clones used for Immunofluorescent Labelling.

Marker	Fluorochrome	Vendor	Clone
CD14	BV711	Biolegend	63D3
CD19	BV570	Biolegend	HIB19
CD16	eFluor 450	Thermofisher	eBioCB16 (CB16)
CD3	AF532	eBioscience	UCHT1
CD4	BV510	Biolegend	OKT4
CD154	PE-Cy7	Biolegend	24–31
TNFa	BV785	Biolegend	MAb11
IFNg	FITC	Biolegend	4S.B3
IL-2	APC	Biolegend	MQ1-17H12
GM-CSF	PE	Biolegend	BVD2-21C11
CD38	Pe-TexasRed	Beckman	LS198-4-3
HLA-DR	APC/Fire 750	Biolegend	LN3
TIGIT	PerCP-Ef710	Thermofisher	MBSA43
PD-1	BV421	Biolegend	NAT105

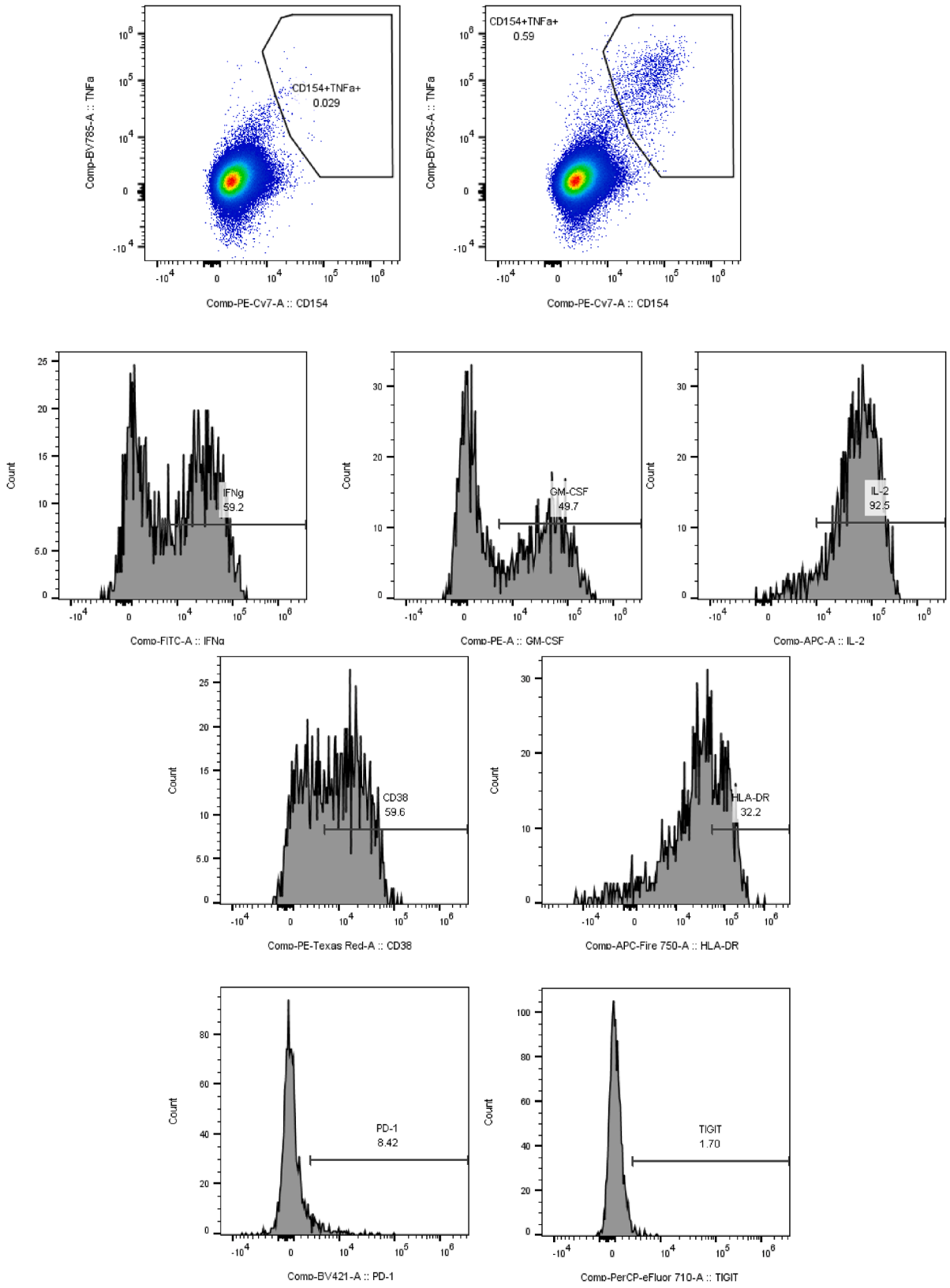


Fig. 1. Flow cytometric gating strategy. Representative flow cytometric plots for an individual subject are shown to detail the gating strategy used. The top row details the CD154 + TNFa+ region for cells gated positive for CD3 and CD4 and negative for the dump markers (Live/Dead fixable dye positive, CD14, CD16, and CD19), stimulated with PPD (right) or with no stimulus (left). The bottom rows show the staining and positive cutoffs of the other markers measured for the CD154 + TNFa+ cells. The second top row details IFNg, GM-CSF, and IL-2 staining. The second bottom row details CD38 and HLA-DR, and the bottom row PD-1 and TIGIT staining. All shown are from the PPD stimulated sample.

Table 2
Subject demographic characteristics.

	Before Treatment (n = 18)	8 Weeks Treatment (n = 18)
Sex, n (%)		
Male	11 (61%)	13 (72%)
Female	7 (39%)	5 (28%)
Age (year, mean)	38	39
BMI (mean)	21.2	21.0
Blood glucose (mean)	104	113

3.2. Frequency of PPD specific CD4 T-cells before and during treatment

The frequency and phenotype of the PPD responding CD4 T cells (defined as CD154+TNFa+) measured as a proportion of total CD4 T cells was determined for each of the subjects. It was found that the average proportion of the PPD specific CD4 T cells was higher in the before treatment group compared to the treatment group, but it did not reach statistical significance (see Table 3). There was no significant difference in the proportion of the individual cytokine positive PPD specific CD4 T cells between the two groups, or of their different combinations. Nor was there any change in the proportion of the immune checkpoint markers PD-1 and TIGIT positive after the 8 weeks of treatment.

A large and significant reduction in the total proportion of PPD specific CD4 T cells positive for the activation markers CD38 and HLA-DR was observed in the treatment group. This was due to a highly significant decrease in the PPD specific T cells positive CD38+ HLA-DR+ (Fig. 2), and to some extent, though not statistically significant, a decrease in those CD38-HLA-DR+. The decrease of these subsets as a proportion of CD4 T cells was not specific to any cytokine producing subset, as it was found that those subsets positive for IFNg, IL-2, or GM-CSF had similar significant decreases in the CD38+ HLA-DR+ and reductions in the CD38-HLA-DR+ subsets (Table 3). The proportion of CD4 T cells that were PPD specific and with a phenotype CD38-HLA-DR- was very nearly the same in both the before and during treatment group. This may indicate that at least in the circulation, there is an expansion of PPD specific CD4 T cells with the CD38+ HLA-DR+ and CD38-HLA-DR+ phenotype in active TB infection which is reduced within 8 weeks of commencing successful anti-MTB therapy.

3.3. Phenotype of PPD specific CD4 T-cells before and during treatment

We also compared the frequencies of the different phenotypes of the PPD specific cells as a total population (Table 4). We found no difference in the proportion of PPD specific T cells producing IFNg between the groups, but that there was significantly higher levels of IL-2 and GM-CSF positive in the treatment group compared to the before group. For the different combinations of these three cytokines we found a significant increase after 8 weeks of treatment in PPD specific CD4 T cells GM-CSF+ IFNg-IL-2+. There was also a significant decrease in the percentage of GM-CSF-IFNg+ IL-2-PPD specific CD4 T cells in the treatment group. The immune checkpoint markers PD-1 and TIGIT showed no significant differences between the two groups, either alone or in the combinations of their staining.

For the activation markers CD38 and HLA-DR, we found that after 8 weeks of treatment there was a large and significant decrease in their expression on the PPD specific CD4 T cells compared to the before group (Table 4 and Fig. 3). For the combinations of these markers a striking and significant decrease in the percentage CD38+ HLA-DR+ was seen during treatment, and a corresponding increase in the proportion CD38-HLA-DR-. The particular expression of IFNg, GM-CSF, and IL-2 in the CD38 and HLA-DR subsets of the PPD specific CD4 T cells was determined, and the data is shown in Table 4. Similar to the total CD38 and HLA-DR subsets, all three cytokines had significant differences between the two groups for the CD38+ HLA-DR+ and CD38-HLA-DR- subset.

Table 3
PPD Specific phenotypes as a proportion of total CD4 T cells.

% of CD4	Before Treatment (n = 18)	8 Weeks Treatment (n = 18)	p-value
CD154 + TNFa+	0.825 (0.408–1.27)	0.580 (0.385–0.875)	0.4476
CD154 + TNFa + IFNg+	0.326 (0.206–0.886)	0.281 (0.172–0.534)	0.4864
CD154 + TNFa + GM-CSF+	0.378 (0.193–0.684)	0.337 (0.229–0.545)	0.7727
CD154 + TNFa + IL2+	0.512 (0.276–0.927)	0.4265 (0.308–0.784)	0.7193
CD154 + TNFa + CD38	0.219 (0.111–0.305)	0.083 (0.054–0.148)	***0.0272***
CD154 + TNFa + HLA-DR	0.249 (0.117–0.417)	0.067 (0.044–0.1373)	***0.0071***
CD154 + TNFa + PD-1	0.116 (0.062–0.534)	0.060 (0.031–0.120)	0.0987
CD154 + TNFa + TIGIT	0.022 (0.011–0.030)	0.021 (0.014–0.030)	0.9813
CD154 + TNFa + GM-CSF + IFNg + IL-2+	0.230 (0.125–0.519)	0.209 (0.143–0.417)	0.9129
CD154 + TNFa + GM-CSF + IFNg + IL-2-	0.030 (0.013–0.098)	0.009 (0.006–0.035)	0.0746
CD154 + TNFa + GM-CSF + IFNg-IL-2+	0.061 (0.035–0.113)	0.081 (0.050–0.117)	0.3347
CD154 + TNFa + GM-CSF + IFNg-IL-2-	0.015 (0.005–0.037)	0.008 (0.006–0.015)	0.1987
CD154 + TNFa + GM-CSF-IFNg + IL-2+	0.067 (0.032–0.193)	0.037 (0.018–0.100)	0.2482
CD154 + TNFa + GM-CSF-IFNg + IL-2-	0.027 (0.008–0.086)	0.008 (0.006–0.034)	0.0534
CD154 + TNFa + GM-CSF-IFNg-IL-2+	0.092 (0.063–0.131)	0.096 (0.060–0.129)	0.8328
CD154 + TNFa + GM-CSF-IFNg-IL-2-	0.107 (0.030–0.182)	0.057 (0.031–0.088)	0.1124
CD154 + TNFa + CD38 + HLA-DR+	0.097 (0.043–0.199)	0.020 (0.012–0.039)	***0.0029***
CD154 + TNFa + CD38 + HLA-DR-	0.086 (0.044–0.207)	0.049 (0.039–0.115)	0.2894
CD154 + TNFa + CD38-HLA-DR+	0.171 (0.045–0.242)	0.048 (0.030–0.102)	0.0625
CD154 + TNFa + CD38-HLA-DR-	0.334 (0.157–0.645)	0.388 (0.208–0.632)	0.5628
CD154 + TNFa + PD-1 + TIGIT+	0.012 (0.005–0.034)	0.012 (0.006–0.021)	0.6895
CD154 + TNFa + PD-1 + TIGIT-	0.101 (0.052–0.220)	0.042 (0.024–0.111)	0.1089
CD154 + TNFa + PD-1-TIGIT+	0.007 (0.005–0.014)	0.008 (0.005–0.011)	0.8693
CD154 + TNFa + PD-1-TIGIT-	0.516 (0.338–1.104)	0.489 (0.293–0.751)	0.4619
CD154 + TNFa + CD38 + HLA-DR + IFNg+	0.063 (0.022–0.128)	0.012 (0.006–0.028)	***0.0032***
CD154 + TNFa + CD38 + HLA-DR-IFNg+	0.048 (0.018–0.071)	0.023 (0.008–0.076)	0.2479
CD154 + TNFa + CD38-HLA-DR + IFNg+	0.097 (0.021–0.161)	0.024 (0.016–0.067)	0.0832
CD154 + TNFa + CD38-HLA-DR-IFNg+	0.147 (0.077–0.432)	0.179 (0.109–0.434)	0.6221
CD154 + TNFa + CD38 + HLA-DR + GM-CSF+	0.059 (0.028–0.110)	0.012 (0.006–0.031)	***0.0076***
CD154 + TNFa + CD38 + HLA-DR-GM-CSF+	0.039 (0.015–0.075)	0.024 (0.010–0.064)	0.2749
CD154 + TNFa + CD38-HLA-DR + GM-CSF+	0.071 (0.020–0.166)	0.031 (0.018–0.064)	0.0922
CD154 + TNFa + CD38-HLA-DR-GM-CSF+	0.165 (0.079–0.364)	0.228 (0.140–0.424)	0.2232
CD154 + TNFa + CD38 + HLA-DR + IL-2+	0.074 (0.025–0.108)	0.010 (0.007–0.032)	***0.0036***
CD154 + TNFa + CD38 + HLA-DR-IL-2+	0.047 (0.034–0.112)	0.038 (0.028–0.097)	0.7017
CD154 + TNFa + CD38-HLA-DR + IL-2+	0.081 (0.033–0.193)	0.037 (0.021–0.077)	0.0954
CD154 + TNFa + CD38-HLA-DR-IL-2+	0.266 (0.121–0.523)	0.310 (0.162–0.591)	0.3843

Median (25–75%ile) shown. p-Value for Mann-Whitney *U* test comparison between before and during treatment groups.

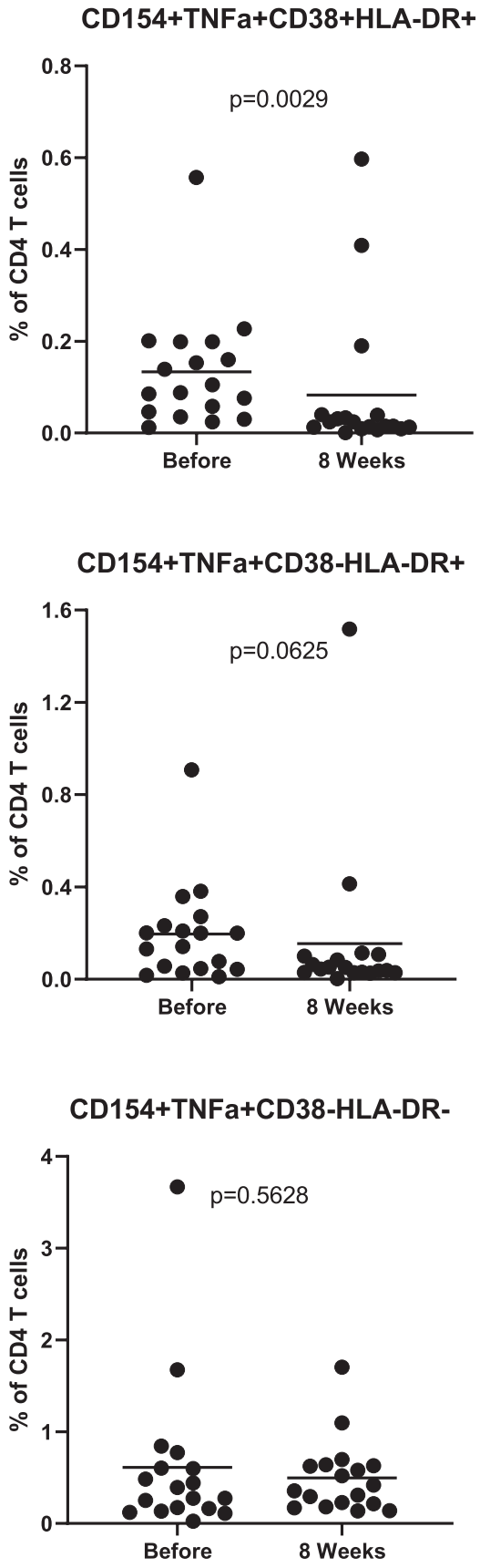


Fig. 2. Proportion of total CD4 T cells PPD specific and CD38 + HLA-DR+, CD38 + HLA-DR−, or CD38-HLA-DR-. Individual subject data shown for before and 8 Weeks treatment groups. p-values for Mann-Whitney *U* test comparisons between the before and after treatment groups are displayed.

Table 4
Phenotype frequency of PPD Specific CD4 T cells.

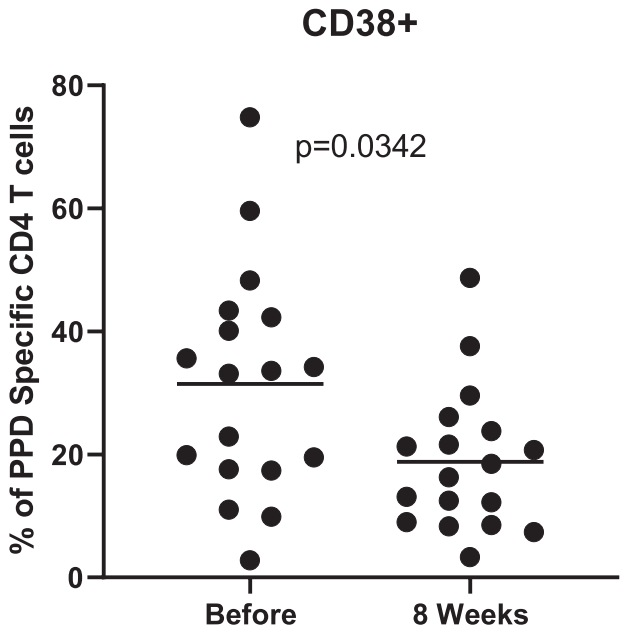
% of CD4 + TNFa + CD154+	Before Treatment (n = 18)	8 Weeks Treatment (n = 18)	p-value
IFNg+	59.5 (47.3–73.7)	55.6 (47.3–66.5)	0.4198
GM-CSF+	52.5 (47.2–61.0)	61.3 (55.7–66.1)	***0.0224***
IL2+	72.9 (53.5–83.8)	84.1 (71.6–89.3)	***0.0402***
CD38	33.4 (17.6–42.6)	17.4 (8.9–24.4)	***0.0342***
HLA-DR	32.6 (24.1–46.3)	14.5 (8.0–29.8)	***0.0064***
PD-1	13.4 (9.2–23.1)	11.2 (6.8–14.4)	0.2481
TIGIT	2.1 (1.4–5.0)	3.2 (2.0–5.4)	0.4018
GM-CSF + IFNg + IL-2+	34.9 (23.4–43.8)	41 (37.0–47.3)	0.0907
GM-CSF + IFNg + IL-2-	4.4 (2.6–7.9)	2.5 (1.2–4.7)	0.0891
GM-CSF + IFNg-IL-2+	9.3 (5.1–16.4)	13.1 (10.2–18.0)	***0.0379***
GM-CSF + IFNg-IL-2-	1.8 (0.9–4.3)	1.6 (1.1–2.6)	0.6900
GM-CSF-IFNg + IL-2+	9.1 (6.1–17.4)	8.1 (4.8–9.9)	0.2055
GM-CSF-IFNg + IL-2-	4.9 (2.1–8.2)	1.6 (0.8–6.2)	***0.0321***
GM-CSF-IFNg-IL-2+	12.3 (9.7–16.9)	16.5 (10.6–24.5)	0.2112
GM-CSF-IFNg-IL-2-	15.6 (7.9–18.2)	9.4 (7.6–13.2)	0.1537
CD38 + HLA-DR+	14.0 (7.4–22.8)	3.7 (1.6–12.3)	***0.0046***
CD38 + HLA-DR-	14.1 (5.8–18.7)	11.0 (6.9–14.8)	0.4197
CD38-HLA-DR+	17.2 (10.6–24.9)	10.1 (6.3–15.4)	***0.0308***
CD38-HLA-DR-	50.5 (33.0–63.1)	72.3 (55.6–84.5)	***0.0038***
PD-1 + TIGIT+	1.3 (0.4–2.8)	1.9 (1.0–4.0)	0.2342
PD-1 + TIGIT-	11.4 (7.9–19.8)	8.2 (4.6–12.7)	0.0861
PD-1-TIGIT+	1.1 (0.5–1.7)	1.2 (0.8–1.7)	0.4008
PD-1-TIGIT-	85.7 (75.6–90.2)	87.7 (82.5–91.8)	0.2967
CD38 + HLA-DR + IFNg+	8.9 (4.7–15.5)	2.4 (1.0–7.8)	***0.0040***
CD38 + HLA-DR- IFNg+	5.8 (3.8–8.7)	3.5 (2.7–6.0)	0.1360
CD38-HLA-DR + IFNg+	9.7 (5.6–15.3)	4.8 (3.4–9.2)	***0.0444***
CD38-HLA-DR-IFNg+	27.7 (16.7–37.7)	37.7 (26.5–52.5)	***0.0379***
CD38 + HLA-DR + GM-CSF+	9.1 (3.8–13.0)	2.3 (0.8–8.4)	***0.0048***
CD38 + HLA-DR-GM-CSF+	7.1 (2.6–8.2)	3.6 (3.0–7.4)	0.3115
CD38-HLA-DR + GM-CSF+	10.0 (5.4–13.6)	6.2 (3.1–9.0)	0.1054
CD38-HLA-DR-GM-CSF+	24.5 (15.1–35.4)	46.3 (27.4–52.6)	***0.0002***
CD38 + HLA-DR + IL-2+	10.2 (5.1–12.3)	2.7 (1.1–8)	***0.0032***
CD38 + HLA-DR-IL-2+	9.4 (4.5–12.8)	10.2 (5.1–12.3)	0.8820
CD38-HLA-DR + IL-2+	12.2 (8.4–16.3)	7.8 (4.3–10.8)	0.0722
CD38-HLA-DR-IL-2+	38.4 (23.9–44.0)	61.1 (42.0–73.5)	***0.0008***

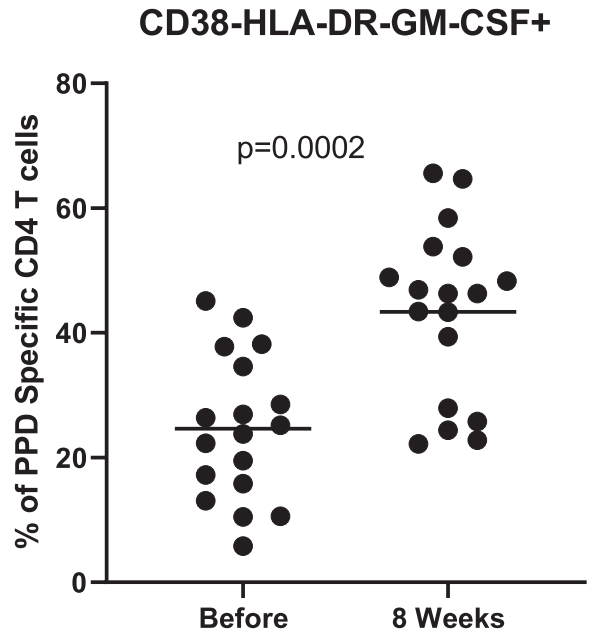
Median (25–75%ile) shown. p-Value for Mann-Whitney *U* test comparison between before and during treatment groups.

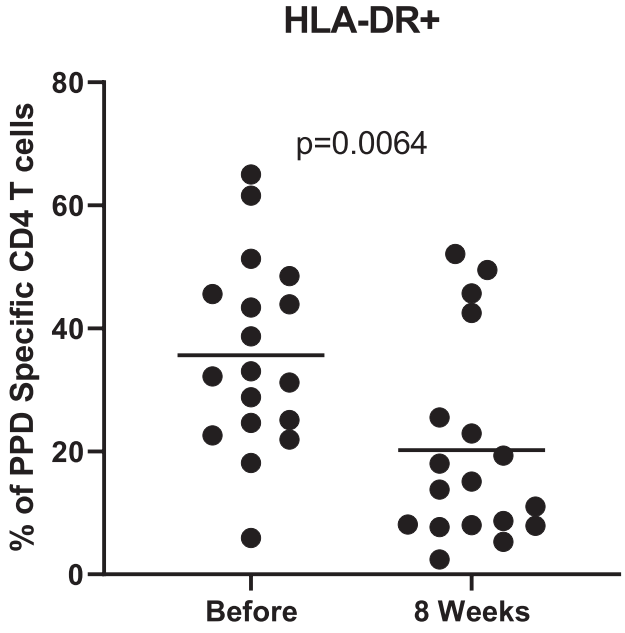
There was also a significant decrease in the percentage of CD38-HLA-DR+ IFNg+ PPD specific CD4 T cells in the treatment group. The largest changes following treatment of the PPD specific CD 4T cells was for the CD38-HLA-DR-GM-CSF+ and CD38-HLA-DR-IL-2+ subsets (Fig. 4). In the case of the CD38-HLA-DR-GM-CSF+ cells the proportion increased from 25% in the before treatment group, to half of all the PPD specific CD4 T cells after 8 weeks of treatment. The frequency of the CD38-HLA-DR-IL-2+ cells also had a significant increase in the treatment group.

4. Discussion

The frequency and phenotype of PPD specific CD4 T cells was measured and compared between groups of TB patients before and after 8 weeks of anti-MTB treatment. We found that when considered as a proportion of total CD4 T cells, there was a significant decrease in the CD38+ HLA-DR+ PPD specific population during treatment, while there was little or no change in the CD38-HLA-DR- population. This decreasing population did not have a specific IL-2, IFNg, or GM-CSF







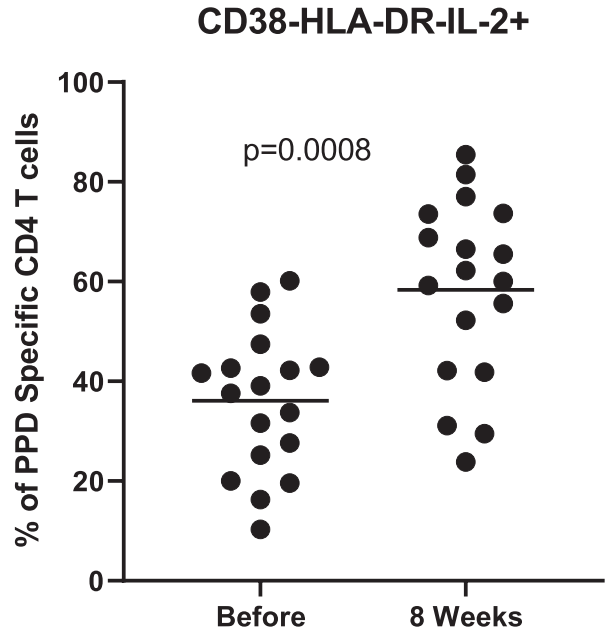


Fig. 3. Proportion of total PPD specific CD4 T cells positive for CD38 and HLA-DR. Individual subject data shown for before and 8 Weeks treatment groups. p-values for Mann-Whitney *U* test comparisons between the before and after treatment groups are displayed.

Fig. 4. Proportion of total PPD specific CD4 T cells CD38-HLA-DR-GM-CSF+ or CD38-HLA-DR-IL-2+. Individual subject data shown for before and 8 Weeks treatment groups. p-values for Mann-Whitney *U* test comparisons between the before and after treatment groups are displayed.

producing phenotype. We also found no difference in PD-1 and TIGIT immune checkpoint expression. When looked at as a proportion of the total PPD specific population we found that there was a significant decrease in the proportion of cells expressing CD38 and HLA-DR and a significant increase in these cells expressing IL-2 and GM-CSF after 8 weeks treatment. The majority of the increase in these cytokine expressing cells was in the CD38-HLA-DR- population. No differences were measured in the expression of two immune checkpoint markers PD-1 and TIGIT as a proportion of total PPD specific CD4 T cells.

Previously, flow cytometry and ICS has been used to determine if there are differences in the phenotype of MTB specific CD4 T cells between latent and actively infected subjects [5–8]. There have also been some studies looking at the effects of anti-MTB treatment on MTB specific CD4 T cell phenotype in patients. Kim et al measured the expression of the cytokines IFNg and TNFa in peripheral blood CD4 T cells stimulated with peptide mixes of MTB specific proteins ESAT-6, CFP-10, and TB7.7 in patients before and 6 months after the start of treatment [10]. Like our results at 8 weeks treatment, they found no difference between the before and treatment groups in the proportion of CD4 T cells that made IFNg following MTB peptide stimulation, but they did find a significant decrease in those that made TNFa. We also found on average a decrease in the TNFa producing PPD specific CD4 T cells, but in our study it was not statistically significant. A longitudinal study of cryopreserved peripheral blood mononuclear cells was performed by

Ahmed et al, and it followed the treatment of individuals from before they started till 6 months later [11]. At 9 weeks, which corresponds closely to the time of treatment we studied, they found like us a significant decrease in CD38 and HLA-DR expression as measured as the proportion of total MTB specific CD4 T cells. Interestingly, they also found that the bigger the decrease in CD38 and HLA-DR expression between prior treatment and Week 9 of treatment, the quicker an individual would reach sputum culture negativity. Again, similar results to ours were also found by Adekmabi et al. [8], where decreases in CD38 and HLA-DR expression on MTB specific CD4 T cells corresponded with successful anti-MTB therapy.

We have shown previously that GM-CSF is measurable in MTB specific CD4 T cells, and that there are differences in its expression in active and latent TB infection [7]. In this study we found that there was a significant increase in the proportion of PPD specific CD4 T cells making GM-CSF after 8 weeks treatment, mainly in the CD38-HLA-DR- population. Recent studies have led to an understanding of the important role of GM-CSF in TB. Gonzalez-Juarrero et al have shown that in a mouse model deletion of the gene for GM-CSF mice results in uncontrolled MTB growth and increased mortality after pulmonary infection [12]. In another study using TNFa knockout immunocompromised mice, it was found that blocking GM-CSF with antibodies during isoniazid/rifampicin chemotherapy comprised bacterial control and lead to more inflammation and increased numbers of intracellular *M. tuberculosis* bacilli [13]. They also found *in vitro* blocking of GM-CSF promoted an anti-inflammatory M2 macrophage phenotype along with increased MTB infection of these cells. Finally, Robinson et al found in that in another mouse model of TB, CD4 T cells are the main source of GM-CSF within the lung as the infection progresses, and that transfer of GM-CSF producing effector CD4 T cells was protective against disease, while transferring CD4 T cells from GM-CSF knockout mice exacerbated disease [14]. These studies give support to the idea that the higher proportion of GM-CSF producing PPD specific CD4 T cells we found after 8 weeks treatment indicate an important role for this cytokine in the clearing response against MTB infection.

Our study is the first to investigate the expression of the immune checkpoint molecule TIGIT on PPD specific T cells in active and treated TB subjects. Overall, we found little expression of this immune checkpoint marker on the PPD specific CD4 T cells, with no significant difference between the before and 8 week treatment groups. We also investigated the expression of another important immune checkpoint marker PD-1 on the MTB specific CD4 T cells. Previously, it has been shown that individuals who have latent TB infection have higher PD-1 expression on these cells compared to BCG immunised but not MTB exposed individuals [15], but that there is no difference in PD-1 expression on PPD specific CD4 T cells between active and latently infected subjects [16]. It was also found that there was no change in PPD specific CD4 T cell PD-1 expression after completing treatment [17]. These results do fit with our data that found no change in PD-1 expression on the PPD specific CD4 T cells after 8 weeks treatment. PD-1 expression is thought to be an indicator of T cell exhaustion in viral diseases [18] and also in MTB [19]. This lack of change in PD-1 expression may be due to the treatment removing the antigen load driving the immune response, which would be expected to have little effect on the already exhausted PD-1 positive T cells.

In our study we found that there was a significant and particular decrease in the CD38 and HLA-DR positive PPD specific CD4 T cells after 8 weeks of treatment, while the PPD specific CD38-HLA-DR- proportion of total CD4 T cells remained the same. It has been recently shown that there is a high expression of CD38 and HLA-DR on tissue resident memory CD4 T cells at the site of active TB infection [20]. This does correlate with the high level of the expression of the same markers we found on the circulating PPD specific CD4 T cells in circulation in the untreated subjects. The loss of these cells with treatment could reflect the decrease in bacterial burden driving their activation. It also may be that the remaining CD38-HLA-DR- CD4 T cells indicate a protective phenotype against MTB. Unfortunately, our study lacks the data to clearly answer these questions, but we believe it is something worthy of follow up investigation. Altogether, our data does add to the growing evidence that measuring CD38 and HLA-DR on PPD specific CD4 T cells may be a good early indicator of treatment efficacy.

There are some limitations of our study. Due to funding and logistics, it was only possible to do a cross sectional study of the before and 8 weeks of treatment groups, rather than a longitudinal follow up. Subject numbers were small, and we were only able to look at one time point of treatment. Future studies will hopefully provide more information by following the course of treatment on individual patients in more detail and correlating them with tests of bacterial burden and treatment efficacy.

In conclusion, we found clear and striking changes in the quantity and phenotype of CD4 T cells specific for MTB in TB patients during the early stages of anti-MTB therapy. Measurement of these changes may provide a new clinical correlate of the success or not of anti-MTB treatment in TB patients.

Ethics Statement

Subjects were recruited from Dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia. This took place from 01 November to 30 November 2019 under Ethics Approval Number 113/EA/FK-RSUDZA/2019, Fakultas Kedokteran Universitas Syiah Kuala Rumah Sakit Umum Daerah Dr Zainoel Abdin. All subjects were adults and gave written informed consent for study participation.

CRediT authorship contribution statement

Herry Priyanto: Conceptualization, Investigation, Writing - review & editing. **Edmond Chua:** Investigation, Writing - review & editing. **Paul Hutchinson:** Conceptualization, Investigation, Writing - original draft. **Jusak Nugraha:** Conceptualization, Writing - review & editing. **Muhammad Amin:** Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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