RESEARCH ARTICLE

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The Interplay of *PD-L1*, Kynurenine Pathway, and Vitamin D Shapes an Immunosuppressive Microenvironment in Acute Myeloid Leukemia

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Abstract

Background: Acute myeloid leukemia (AML) is a genetically heterogeneous malignancy. However, the immune microenvironment-including immune checkpoint pathways and metabolic regulators also plays a pivotal role in disease progression. **Aims:** To investigate the immune-metabolic landscape of newly diagnosed AML patients, focusing on the *PDL1*-PD1 and IDO-Kynurenine-AhR pathways, and their association with vitamin D levels. **Setting:** A prospective, observational study conducted at a tertiary care academic hospital. **Materials:** A total of 127 newly diagnosed AML patients were enrolled. Flow cytometry was used to assess PD-L1 expression on blasts and immune T cell subsets. Serum levels of tryptophan, kynurenine, and vitamin D were measured using enzyme-linked Immunosorbent Assay (ELISA) and Chemiluminescent Immunoassay (CLIA). **Statistics:** Correlation analysis and chi-square test/Mann-Whitney U test were applied. A p-value <0.05 was considered significant. **Results:** *PDL1* expression on blasts inversely correlated with CD8+ T cells (p=0.044), indicating immune evasion. CD3+ positively correlated with CD8+ T cells (p=0.007), while CD4+ negatively correlated with CD8+ T cells (p<0.001), suggesting divergent immune roles. Elevated tryptophan/kynurenine correlated with increased PD1+CD4+ T cells (p=0.039), which in turn were associated with higher Treg frequencies (p=0.001). Low vitamin D levels were associated with higher odds of aTregs (OR 2.7; 95% CI 1–7). **Conclusions:** An immunosuppressive microenvironment in AML is driven by PD-L1 expression, kynurenine pathway activation, and low vitamin D levels. These findings suggest potential immunotherapeutic targets and highlight vitamin D's immunomodulatory role.

Keywords: Acute myeloid leukemia- Immuno-metabolic markers- Immune regulation- T cell subsets- Vitamin D

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Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Etiopathogenesis of AML is primarily driven by intrinsic genetic and molecular alterations in myeloid blast cells, the identification of which is helpful in diagnosis, prognosis, treatment, and monitoring of the disease [1]. Additionally, the crosstalk of the myeloid blasts with the bone marrow microenvironment and the various components of immune regulation generates a network of interactions inhibiting T cell function and providing a permissive environment for tumor progression [2]. However, the local environment created by AML cells to promote their survival and prevent their rejection by the host immune system has been relatively under-explored. Malignancies, including AML, employ several immune evasion mechanisms that inhibit the generation or functional execution of anti-tumor immune responses [3]. Several cellular, cytokines, and metabolic factors control the immunological balance in the tumor microenvironment, of which one emerging key factor is Vitamin D.

Several studies have shown an association between low levels of serum vitamin D and incidence, mortality, and clinical outcomes of several types of solid tumors and hematological malignancies, including AML, [4] although without considerable evaluation of its biological mechanisms. Vitamin D and its metabolites have a complex

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role that mediates many functions, as recognised in vitro and in vivo studies. In addition to the classical endocrine role in calcium and bone homeostasis, vitamin D plays two other significant roles: firstly, as a transcription factor that influences central mechanisms of tumorigenesis: growth, cell differentiation, and apoptosis [5] and secondly, as an immune modulator, it has the capability of acting in an autocrine manner in a local immunologic milieu [6]. As the vitamin D receptor (*VDR*) is expressed in immune cells (B cells, T cells, and antigen-presenting cells), and these immunologic cells are all capable of synthesizing the active vitamin D metabolite, vitamin D can modulate the innate and adaptive immune responses [7].

Reports have suggested that, in cancer, cytotoxic T cells (CTLs) are found to be exhausted or inactivated and lose the capacity to initiate an immune response. One prominent factor for this exhaustion is Programmed death ligand-1-Progrsammed death 1 (*PDL1*-PD1) interaction, which sends the inhibitory signals to CTL. *PDL1* is variably expressed (<1% to 80%) in various solid and hematological malignancies; however, it is less well studied in AML [8]. Another essential pathway supporting the immune evasion is the Indoleamine 2,3-dioxygenase-Kynurenine-Aryl hydrocarbon Receptor (IDO-Kyn-AhR) pathway that increases the immune-suppressive T regulatory cells (Tregs) via kynurenine acting on the AhR receptors [9]. IDO1, expressed in 30-70% of malignancies, is associated with high M2-monocytes [10]. The overall balance of these immune activating and suppressive mechanisms determines the final tumor cell proliferation or death. Several genetic, epigenetic, and metabolic factors have been reported to regulate the expression and activity of these two immune pathways in cancer. Vitamin D has been studied as an immune-modulatory factor, modulating these two critical pathways of *PDL1*-PD1 and IDO-Kyn-AHR in conditions of autoimmunity and inflammation in independent studies [11, 12]; however, studies reporting the effect of vitamin D in modulating immune response via these two pathways in cancer, particularly in AML are very scarce.

In this study, we have explored and described the cellular and metabolic immune components of the *PDL1*-PD1 and IDO-Kyn-AhR pathways and identified their interplay and correlation to Vitamin D in a clinical setting of newly diagnosed AML.

Materials and Methods

Patient Recruitment and Data Collection

All newly diagnosed AML patients undergoing routine diagnostic workups were included by convenience sampling after obtaining approval from the institute ethics committee (JIP/IEC/2019/531) and informed consent from the patients. A total of 127 patients with newly diagnosed AML, from 12 to 65 years of age, were recruited over a study period of two years (from August 2020 to July 2022). Patients with acute promyelocytic leukemia, prior treated for AML, and who did not consent for enrollment in the study were excluded. Baseline patient and disease characteristics were collected from clinical records.

For the study of baseline immune markers, multicolor flow cytometry was used to characterize peripheral blood (PB) T cell subsets and *PDL1* on the myeloid blasts. Vitamin D levels were measured by CLIA, and kynurenine and tryptophan levels were measured by ELISA. Expression levels of Vitamin D receptor (*VDR*) and interferon-gamma receptor 1 (*IFNGR*) were studied by Real time polymerase chain reaction (RT-PCR) for a subset of 36 patients with more than 50% circulating blasts in PB and 36 age/gender-matched healthy controls. Sample collection for the study is described in Electronic Supplementary Material 1(ESM 1).

Cell Preparation for Flow Cytometry

The cell suspension was prepared by bulk erythrocyte lysing with ammonium chloride-based lysing reagent (0.15 M NH4cl (8.29 g NH4Cl), 1.0g KHCO3, 37 mg EDTA, and 1L distilled water). 2 ml of PB sample was added to 7-8 ml of RBC lysing reagent in a 15ml falcon tube. Incubation was done for 10 minutes at room temperature, and cells were washed with phosphate-buffered saline (PBS) by centrifugation for 5 minutes at 2500 rpm. The supernatant was discarded, and cells were washed twice with PBS. After the final wash, the cells were re-suspended with PBS of 0.5ml.

Staining and fixation

Before starting, all the buffers were taken at room temperature. 100 µL of the blood sample was added to each of the following tubes to analyze the T cell subsets and characterize *PDL1* on myeloid blasts. The antibody panel for each tube is outlined below. The detailed staining and fixation protocol for all the cell surface and intracellular markers is described in Electronic Supplementary Material 1(ESM 1).

For T regulatory cells: For identification and quantification of T regulatory cells, a panel of CD45-KO, CD3-PB, CD4-FITC, CD25-PC5.5, FOXP3-PE was used. Surface staining antibodies KO-conjugated anti-human CD45 (Beckman Coulter), PB-conjugated anti-human CD3 antibody (Beckman Coulter), FITC-conjugated anti-human CD4 antibody (Beckman Coulter), PC5.5-conjugated anti-human CD25 antibody (eBioscience), APC750-conjugated anti-human PD1 antibody (eBioscience) were added into the tube as per manufacturer's instructions. PE-conjugated anti-human FOXP3 antibody (BD Biosciences) was added at the recommended concentrations after permeabilization.

For Cytotoxic T cells and *PDL1* on myeloid blasts: surface staining antibodies KO-conjugated anti-human CD45 (Beckman Coulter), PB-conjugated anti-human CD3 antibody (Beckman Coulter), FITC-conjugated anti-human CD8 antibody (Beckman Coulter), APC-750-conjugated anti-human PD1 antibody (eBioscience), APC700-conjugated anti-human CD34 antibody (Beckman Coulter), PC7-conjugated anti-human *PDL1* antibody (eBioscience) were added into the tube as per manufacturer's instructions. APC-conjugated anti-human PLCG1 antibody (BD Biosciences) was added to the cell suspension after permeabilization.

Acquisition & Analysis

The sample acquisition was done on a flow cytometer (Navios-AY43297, Beckman Coulter, USA) equipped with a 3-laser (10-color). A minimum of 1,00,000 events were acquired for analysis. The data was analysed using Kaluza version 2.1 software (Beckman Coulter USA). Gating strategy and quantification are described in Electronic Supplementary Material 1(ESM 1) Representative plots for gating and analysis strategy are given in supplementary Figure 1.

In brief, we employed a CD45/ side scatter [SSC] plot with sequential gating to define the T-cell subsets. For one of the representative key populations of aTregs, we initiated our gating strategy by specifying the lymphocyte population by CD45/SSC (Supp. Figure 1A) and isolating CD3 T cells from the lymphocyte population (Supp. Figure 1B). We then sequentially gated CD4+ T cells (from CD3+ T cells, Supp. Figure 1C), CD4+25+ T cells (from CD4+ T cells, Supp. Figure 1D), and finally CD4+25+FOXP3+ T cells (from CD4+25+ T cells, Supp. Figure 1E) to isolate the aTregs population. Detailed gating strategy for all other T cell subsets is described in ESM1.

CLIA (Chemiluminescent Immunoassay)

We utilized CLIA to quantify vitamin D levels by measurement of 25(OH)D metabolite in baseline serum samples of patients with AML [Vitamin D estimation kit (Beckman Coulter, 96 tests)]. Level of Vitamin D was expressed as ng/ml, and a cut-off of < 20ng/ml was used to define low Vitamin D level or Vitamin D deficiency [13].

ELISA (Enzyme-Linked Immunosorbent Assay)

For the estimation of kynurenine and tryptophan levels in serum samples, we employed ELISA method with kynurenine ELISA kit (Abbkine Inc.) and Tryptophan ELISA kit (ELK biotechnology) respectively. Levels were expressed as µg/ml for both kynurenine and tryptophan. High and low levels were defined by the median values observed in our study cohort.

Sample preparation, quality control, and polymerase chain reaction (PCR)

In our study, we aimed to assess the expression of the Vitamin D receptor (*VDR*) and Interferon-gamma receptor (*IFNGR*) gene in peripheral blood mononuclear cell (PBMC) samples for AML cases and age & gender-matched healthy controls. For this, PB samples were collected from patients with > 50% circulating PB blasts, and then a Ficoll-based separation method to isolate the PBMCs was used. Subsequently, we performed RNA extraction (Qiagen) from these PBMCs, ensuring RNA quality by confirming a 260/280 ratio exceeding 1.8 and verifying RNA integrity via formaldehyde gel electrophoresis (Thermo Fisher). Once we had high-quality RNA samples, we proceeded with cDNA synthesis (Takara bio), using 300ng of RNA for each cDNA synthesis reaction. The synthesized cDNA was then stored at -80°C for future use. Finally, we conducted a reverse transcription-polymerase chain reaction (RT-PCR) to measure the expression levels of the *VDR* and *IFNGR* gene using the stored cDNA samples.

Statistical analysis

We assessed data distribution using the Shapiro-Wilk test to determine normality. Since clinical data often deviates from normal distribution, we employed non-parametric tests as our data was non normally distributed. For data description, we used median (interquartile range) for non-normally distributed data, and categorical data was described by frequency and percentage. Continuous data were categorized using median-based cutoffs. As no established clinical cut-offs exist for the studied parameters in AML, median values were used to define high and low groups for association analysis.

To examine the link between T cell subsets and immune-metabolic markers (e.g., vitamin D, kynurenine, tryptophan, Kyn/Trp ratio, *PDL1*, Treg cells, cytotoxic T cells, PD1+CD4+ T cells, and PD1+CD8+ T cells), we employed the spearman's correlation and chi-square test/ Fisher's exact test and Man-Whitney -U test, suited for non-normally distributed data.To assess the associations among these components, p-value <0.05 was considered statistically significant.

Relative expression of *VDR* and *IFNGR* was done with $2^{-(\blacktriangle\blacktriangle Ct)}$ (livak method) and GAPDH was used as a housekeeping gene for normalization. We employed the Mann Whitney U test to compare the $2^{-(\blacktriangle\blacktriangle Ct)}$ values of cases and controls. p-value <0.05 was considered statistically significant. Due to limited sample sizes for certain parameters (aTregs, aCTLs, PD1+CD4+ T cells, PD1+CD8+ T cells), the analyses were conducted based on the availability and quality of the corresponding samples. Statistical analysis was conducted using SPSS (v26), with study graphs generated using SPSS and R programming (v4.3) for visualization. Cut-off values for study parameters are presented in Electronic Supplementary Material 1(ESM 1).

Results

Baseline patient characteristics of newly diagnosed AML

A total of 127 patients of newly diagnosed AML, irrespective of their treatment status, were included in the analysis. The median age was 42 years (range, 14-62 years). Gender was equally distributed between male and female. The median total leucocyte count (TLC) was 23.4 x 109/L (range, 0.4- 341.1 x 109/L) with a median peripheral blood blast count of 67% (range, 2-98%). Table 1 summarizes the baseline characteristics of the entire study cohort.

Profile of Immune parameters in newly diagnosed AML patients at baseline

Table 2 summarizes the immune profiling of patients with newly diagnosed AML at baseline. The median level of Vitamin D was 19 ng/ml (range, 3.7 – 50.9 ng/ml). The median *PDL1* expression on AML blasts was 2.1% (range, 0-85.6%). The median proportions of aTregs and aCTLs in the peripheral blood were 10.2% (range, 0 – 47.1%) and 4.45% (range, 0-33.15%).

Table 1. Baseline Characteristics of the Entire Cohort of Newly Diagnosed AML (n= 127)

Characteristic	n=127
Age (years)	Median 42 (range, 14-62)
Age group (n=127)	
12-18 years	13 (10.2%)
18-40 years	49 (38.6%)
> 40 years	65 (51.2%)
Gender (n=127)	
Male	64 (50.4%)
Female	63 (49.6%)
ECOG Performance status (n=120)	
0-1	61 (50.8%)
2	58 (48.3%)
3	1 (0.8%)
BMI (kg/m^2) (n=111)	
<18	18 (16.2%)
18-25	72 (64.9%)
>26	21 (18.9%)
Extramedullary disease (n=123)	
Yes	31 (25.2%)
No	92 (74.8%)
Risk groups (n=111)	
Good risk	25 (22.5%)
Intermediate risk	68 (61.2%)
High risk	18 (16.2%)
TLC (*10^9/L) (n=127)	Median 23.4 (range, 0.4-341.1)
<30	67 (52.8%)
>30	60 (47.2%)
NLR (Neutrophil / lymphocyte ratio) (n =114)	0.67 (range, 0-15.6)
PB Blasts (%) (n =127)	Median 67% (range, 2-98)
cMPO positive blasts (n=117)	
Yes	107 (91.5%)
No	10 (8.5%)
HLA- DR positive blasts (n=108)	
Yes	91 (84.3%)
No	17 (15.7%)
CD34 positive blasts (n=115)	
Yes	76 (66.1%)
No	39 (33.9%)

ECOG, Eastern Cooperative Oncology Group; BMI, body mass index; TLC, total leucocyte count; PB, peripheral; blood; cMPO, cytoplasmic myeloperoxidase

Table 2. Profile of Immune Parameters in Patients with Newly Diagnosed AML at Baseline

Parameter	Median (Range)
Immune metabolic markers	
Vitamin D (ng/ml) (n=127)	19 (3.7-50.9)
Kynurenine (µg/ml) (n=127)	3.8 (0.39-14.3)
Tryptophan (µg/ml) (n=127)	70.8 (3-237)
K/T ratio (n=127)	0.056 (0.005-0.97)
Immune markers on PB blasts	
PDL1 (blasts) (%) (n=124)	2.1 (0-85.6)
CD34 (%) (n=124)	18.6 (0-97.5)
T cell subset (in PB)	
CD3+ T cells (%) (n=127)	62.6 (4.3-99)
CD3+4+ T cells (%) (n=126)	48.7(15.3-95.4)
T reg. cells (CD4+25+ T cells; Tregs) (%) (n=126)	15 (0.42-58.4)
Activated T reg. cells (CD4+25+FOXP3+ T cells; aTregs) (%) (N=76)	10.2 (0-47.1)
Cytotoxic T cells (CD3+8+ T cells; CTLs) (%) (n=127)	34.5 (4.5-77.6)
Activated Cytotoxic T cells (CD8+PLCG1+ T cells; aCTLs) (%) (n=65)	4.45 (0-33.1)
PD1+ CD4+ T cells (%) (n=38)	34.7 (1-64)
PD1+ CD8+ T cells (%) (n=49)	39.2 (0-77)
CD4/ CD8 (%) (n=126)	1.4 (0.10-11.1)
Tregs/ CTL (%) (n=126)	0.19 (0.00-3.1)
aTregs/aCTLs (%) (N=46)	1.8 (0.06-76.5)

PB, peripheral blood

Correlation and interplay of Immuno-metabolic markers and T cell subsets in newly diagnosed AML at baseline

The correlation of immune-metabolic markers, *PDL1* on blasts, and the T cell subsets is shown in the correlation matrix in Figure 1 and scatter plots in Figure 2. The scatter plots in Figure 2 illustrate the factors with significant correlation; Kynurenine had a positive correlation with tryptophan (n=127, r=0.263, P=0.003) (Figure 2a); Kynurenine was negatively correlated with PD1+CD8+ T cells (n=49 r= -0.313, P=0.028) (fig.2b)., Kynurenine was negatively correlated with CD4+ T cells (n=126 r= -0.229, P=0.010) (Figure 2c), Tryptophan was negatively correlated with CD4+ T cells (n=126 r= -0.235, P=0.008) (Figure 2d). Tryptophan was positively correlated with PD1+CD4+ T cells (n=38 r= 0.336, P=0.039) (Figure 2e).

Our findings demonstrated a negative correlation between CD3+ T cells and PD1+CD8+ T cells (n=49, r=-0.385, P=0.006) (fig.2f). CD3+ T cells had a positive correlation with CD3+8+ T cells (n=127, r =0.239, p=0.007) (fig.2g). Also, a negative correlation was observed between CD4+ T cells and CD8+ T cells (n=126, r=-0.325, P=0.001) (Figue 2h).

PDL1 expression on blast was inversely correlated with the % of CTL in PB (r = -0.18, P = 0.044) (Figure 2i) though with a weak strength of correlation. Tregs were negatively correlated with cytotoxic T cells (n=126, r= -0.143, P=0.11) (Figure 2j). The percentage of Tregs directly correlated with percentage of PD1+CD4+ T cells (r = 0.522, P = 0.001) Figure 2k) and aCTLs (r = 0.379, P =0.002, Figure 2l).

Vitamin D is inversely associated with activated T regulatory cells (aTregs) in AML

We observed a significant link between low levels of

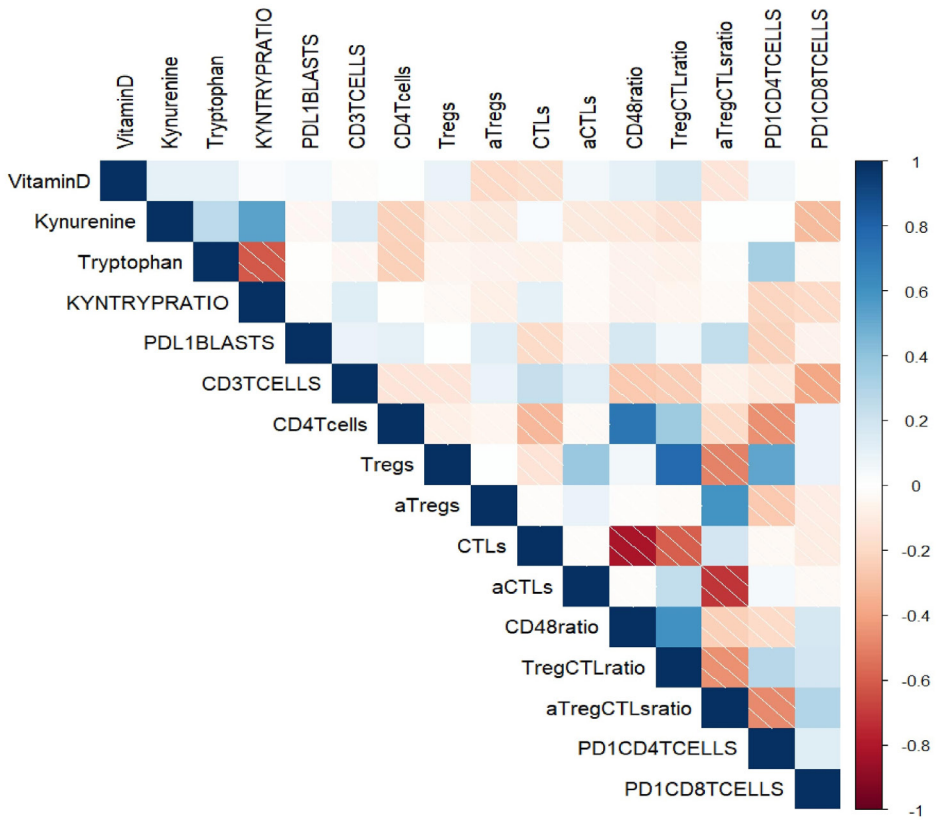

Figure 1. Correlation Matrix Showing Associations among Immune and Metabolic Components in AML. Spearman's test was used. Significant positive and negative correlations are observed between kynurenine, tryptophan, T cell subsets, PD1 expression, Tregs, CTLs, and PDL1+ blasts.

vitamin D and increased aTregs. Specifically, individuals with low vitamin D had a 2.7 times higher likelihood (95% C.I. 1-7) of having elevated aTregs when compared to those with high vitamin D levels (P=0.036) as shown in Table 3 and Figure 3a.

We found a similar link between low vitamin D and elevated cytotoxic T cells (CD8+ T cells; CTLs) with a 2.7 times higher likelihood of low vitamin D levels having elevated CD8+ T cells (P=0.018; 95% CI 1.3 – 5.5) (fig.3b). However, no association was observed between vitamin D and activated CTLs (aCTLs). Table 3 shows the association of vitamin D levels with the key immune components.

Kynurenine is inversely associated with CD3+4+ T cells in AML

Figure 3 shows the significantly associated T cell subsets and immune metabolites in AML. Validating the negative correlation of kynurenine and CD3+4+ T cells (Figure 2c), we found a notable association between low kynurenine levels and a higher proportion of CD3+4+ T cells with an OR of 2.8 (95% C.I. 1.3-5.8), mirroring a similar pattern to their correlation (P =0.005, in Figure 3c). Also, low kynurenine was associated with elevated PD1+CD8+ T cells % (P =0.032) (Figure 3e).

Expression and Association of VDR and IFNGR gene with immune parameters in newly diagnosed AML

Control subjects exhibited approximately 2.4 times higher *IFNGR* expression levels than AML cases, although this difference did not reach statistical significance (P = 0.36). For *VDR*, AML cases had 1.4 times higher expression than the control group. No significant association was observed between levels of expression of *IFNGR* and *VDR* with other immune parameters, as shown in Supplementary Table 1/2.

Proposed Immune-regulation pathway in AML

Figure 4 illustrates the proposed immune regulation pathway in newly diagnosed AML based on the associations and correlations observed in our study between vitamin D, immune-metabolic markers, and the peripheral blood T cell subsets. The interplay of the different immune components determines the final balance of the anti-tumorigenic/pro-tumorigenic immune mechanisms.

Association of immune-metabolic markers with ELN (European Leukemia Net) 2017 risk stratification

In a subset analysis of 71 cases of newly diagnosed AML in which complete risk stratification was available, we studied the association between immune parameters and ELN 2017 risk stratification. [14] Key findings were: poor-risk AML patients showed a significantly higher proportion of aTregs (28.3%) compared to good (8.7%) and intermediate (5.1%) risk groups (p = 0.046). There was a non-significant trend toward higher PD-L1 expression in the poor-risk group (7.1%) compared to the good

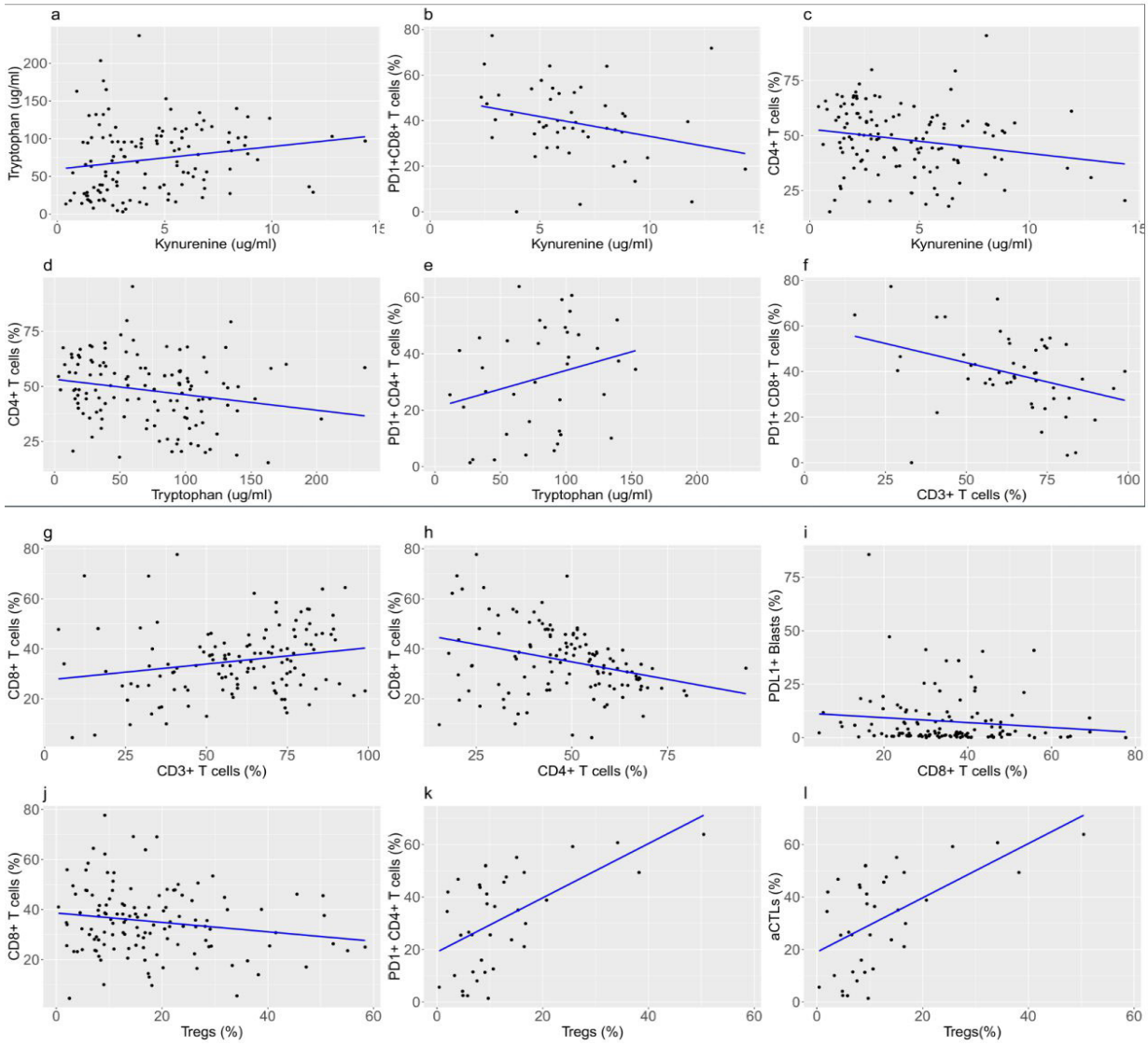

Figure 2. Scatter Plots for Correlation of Immune Components a. Kynurenine and Tryptophan are positively correlated (r=0.263, p=0.003) b. Kynurenine and PD1+CD8+ T cells are negatively correlated (r= -0.313, p=0.028) c. Kynurenine and CD4+ T cells are negatively correlated (r= -0.229, p=0.010) d. Tryptophan and CD4+ T cells are negatively correlated (r= -0.235, p=0.008) e. Tryptophan and PD1+CD4+ T cells are positively correlated (r= 0.336, p=0.039) f. CD3+ T cells and PD1+CD8+ T cells are negatively correlated (r=-0.385, p=0.006) g. CD3+ T cells and CD8+ T cells are positively correlated (r =0.239, p=0.007) h. CD4+ T cells and CD8+ T cells are negatively correlated (r=-0.325, p<0.001) i. CD8+ T cells and PDL1+Blasts are negatively correlated (r = -0.18, p = 0.044) j. Tregs and CTLs are negatively correlated (r= -0.143, p=0.11) k. Tregs and PD1+CD4+ T cells are positively correlated (r = 0.522, p = 0.001) l. Tregs and aCTLs are positively correlated (r = 0.379, p = 0.002). Spearman test was used to analyze the correlation between immune components.

(2%) and intermediate (1.75%) risk groups (p = 0.35). Median vitamin D levels were lower in the poor-risk group (14.4 ng/ml) than in the good (17.1 ng/ml) and intermediate (16.6 ng/ml) groups, though the difference was not significant. Overall, these results suggest a link between adverse genetic risk group and adverse immunosuppressive phenotypes.

Discussion

Acute myeloid leukemia (AML) is a heterogeneous disease traditionally defined by cytogenetic and molecular abnormalities intrinsic to leukemic blasts. However, immune dysregulation within the bone marrow and peripheral blood microenvironment is increasingly recognized as a critical factor influencing disease progression and prognosis [15]. Our study is among the first to comprehensively assess the interplay between blast *PDL1* expression, T cell subset frequencies, and serum levels of immunometabolic regulators kynurenine, tryptophan, and vitamin D in newly diagnosed AML. We observed an inverse correlation between *PDL1* expression on peripheral blood (PB) myeloid blasts and cytotoxic CD8+ T lymphocytes (CTLs), suggesting immune evasion via checkpoint signaling. We found low vitamin D to be associated with higher odds of activated Tregs

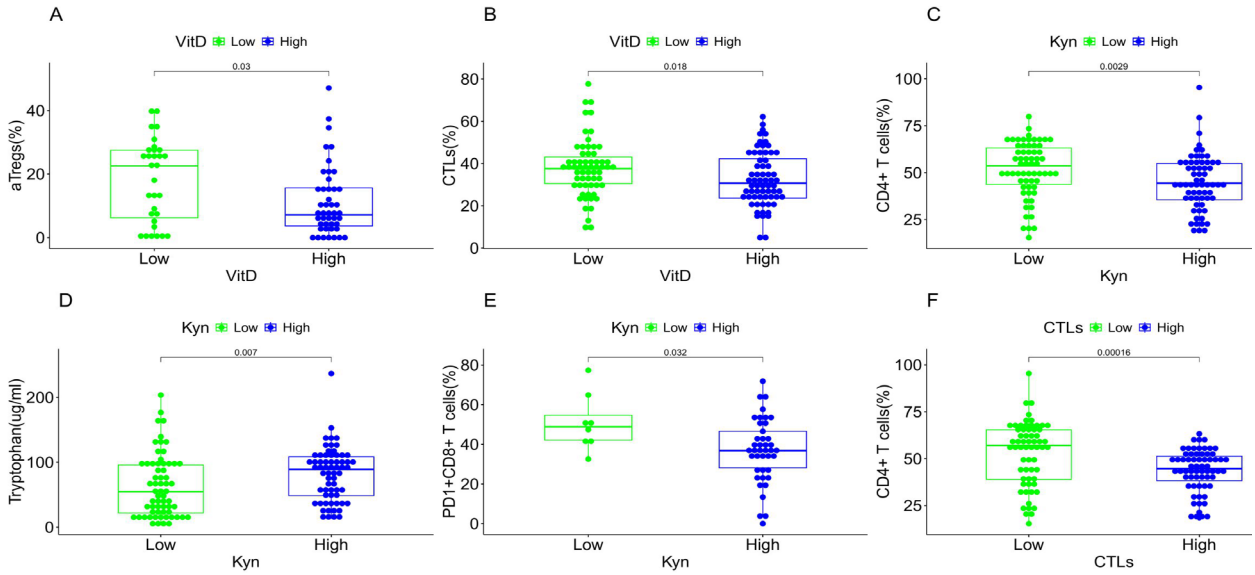

Figure 3. Association of T Cells and Immune Metabolites in AML: A. Association of Vitamin D and aTregs (CD4+25+FOXP3+) showing low vitamin D group having higher aTregs % [22.5% (range, 0-39.9)] compared to high vitamin D [7.2% (range, 0-47.1)] (p=0.03) B. Association of Vitamin D and CTLs (CD8+ T cells) showing low Vitamin D group having increased CTLs % [37.6% (range, 9.6-77.6)] compared to high vitamin D [30.7% (range, 4.5-62.1)] (p=0.018) C. Association of Kynurenine and CD 4+ T cells showing low Kynurenine group having increased CD 4+ T cells % [53.6 % (range, 15.3-79.9)] compared to high kynurenine group [44.3 % (range, 17.8-95.4)] (p=0.0029) D. Association of Kynurenine and Tryptophan showing high Kynurenine group having increased Tryptophan level [89 µg/ml (range, 13-237)] compared to low kynurenine group [54.6 µg/ml (range, 3-204)] (p=0.007) E. Association of Kynurenine and PD1+CD8+ T cells showing low Kynurenine group having increased PD1+CD8+ T cells % [48.8% (range, 33-77) compared to high kynurenine group [36.7% (range, 0-72)] (p=0.032) F. Association of CTLs and CD 4+ T cells showing low CTLs group having increased CD 4+ T cells % [57% (range, 15.3-95.4)] compared to high CTLs group [44.6% (range, 17.8-63.2)] (p=0.00016).

(aTregs) and CTLs in PB, potentially contributing to the immunosuppressive environment in AML. Additionally, an inverse relationship between PB CD4+ and CD8+ T cell proportions indicates opposing regulatory dynamics.

Our cohort had a median age of 42 years (range 14–62), similar to other Indian AML studies, but younger

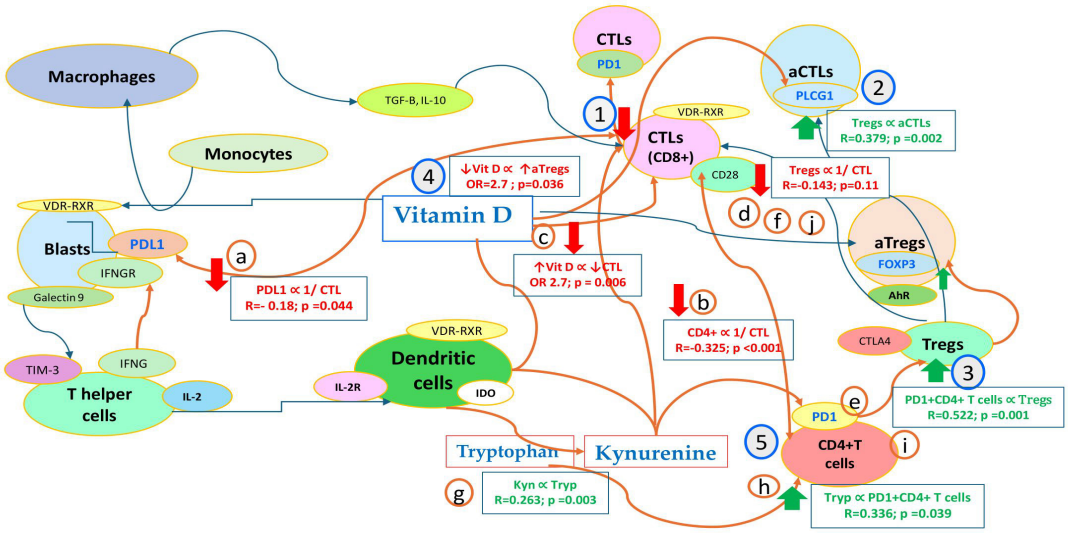

Figure 4. The Proposed Immune Regulation Pathway in Newly Diagnosed AML Showing the Interplay between Vitamin D, Immune-Metabolic Markers, and T Cell Subsets. (1) Factors causing a decrease in PB cytotoxic T cells (CTLs) are (a) an increase in the expression of PDL1 on blast cells, (b) an increase in CD4+ T cells, (c) higher serum levels of Vitamin D, (d) and an increase in Tregs. (2) however, activated cytotoxic T cells (aCTLs) directly correlate with Tregs, with higher Tregs leading to increased aCTLs. (3) Factors causing an increase in PB Tregs are (e) higher PD1+CD4+ T cells and (f) low CD8+ CTLs, while (4) aTregs are stimulated by low Vitamin D. (5) Additionally, (g) increased kynurenine is related to increased tryptophan, (h) which in turn correlates with elevated PD1+CD4+ T cells, (i) and these elevated PD1+CD4+ T cells increase the Tregs, (j) and eventually the Tregs levels lead to a decrease in CTLs. Thus, the interplay of the different immune components determines the final balance of the anti-tumorigenic immune mechanisms.

Table 3. Association of Vitamin D with Key Immune Components

Parameters		PDL1 (2.19%) (n=124)		P	K/T ratio (0.056) (n=127)		P	aTregs (10.2%) (n=76)		P	Odds ratio (OR) with 95% CI	CTLs (34.5%) (n=127)		p	Odds ration (OR) with 95% CI	aCTLs (4.5%) (n=65)		P
		Low (n=59)	High (n=65)		Low (n=66)	High (n=6 1)		Low (n=38)	High (n=38)			Low (n=63)	High (n=64)			Low (n=32)	High (n=33)	
Vitamin D (20 ng/ml) (n=124)	Low (n=61)	27 (45.8%)	34 (52.3%)	0.46	33 (50%)	29 (47.6 %)	0.7 8	11 (29%)	20 (52.7%)	0.036	2.7 (1- 7)	23 (36.5%)	39 (61%)	0006	2.7 (1.3- 5.5)	15 (46.9%)	14 (42.4%)	0.71
	High (n=63)	32 (54.2%)	31 (47.7%)		33 (50%)	32 (52.4 %)		27 (71%)	18 (47.3%)			40 (63.5%)	25 (39%)			17 (53.1%)	19 (57.6%)	

than Western cohorts. Cytogenetic risk distribution was consistent with prior literature, with most patients falling into the intermediate-risk category [16-18].

PDL1 has been extensively studied in solid tumors and lymphomas, but its role in AML is less well defined. In our study, the median *PDL1* expression on PB blasts was 2.19% (range 0–85.6%). While prior studies report variable cutoffs and correlations between mRNA and protein expression [19, 20]; our findings align with its role in impairing CD8+ T cell activity. Unlike previous reports linking *PDL1* with regulatory T cells (Tregs) [21], we did not observe associations between blast *PDL1* expression and Tregs or serum vitamin D, kynurenine, or tryptophan levels [22, 23].

We found that kynurenine levels were directly proportional to tryptophan, consistent with their origin via IDO1-mediated tryptophan metabolism. Kynurenine promotes Treg differentiation through the aryl hydrocarbon receptor (AhR)-FOXP3 axis. In our data, higher kynurenine and tryptophan levels were inversely associated with CD4+ T cell proportions in PB. Notably, low kynurenine levels were linked to 2.8-fold higher odds of increased CD4+ T cells. Although we found no direct associations with Tregs or activated Tregs (aTregs), likely due to smaller sample size, kynurenine correlated positively with PD1+CD4+ T cells, which in turn were associated with Tregs. This aligns with studies demonstrating PD1-mediated expansion of Tregs and immune tolerance [10, 24].

Conversely, and unexpectedly, kynurenine levels inversely correlated with PD1+CD8+ T cells. This contrasts with its known role in promoting CD8+ T cell exhaustion, possibly reflecting the complex, context-dependent nature of the kynurenine pathway and T cell differentiation stages [25, 26].This contrasting finding is not directly explicable but may be related to an extended web of effects of the kynurenine pathway through interaction and modification of activity in many other transduction systems. Moreover, PD1 expression may vary during the naive-to-effector CD8 T cell transition and at different stages of T cell differentiation [27].

Vitamin D, a key immunomodulator, was deficient in most of our cohort (median: 19 ng/ml). We found low vitamin D levels were significantly associated with increased frequencies of aTregs (OR = 2.7) and CTLs (OR = 2.7). Although activated CTLs were lower in vitamin D-deficient patients, the association was not significant, suggesting that vitamin D deficiency may lead to an accumulation of naive or incompletely activated CD8+ T cells. These findings reflect the dualistic nature of vitamin D in immune modulation—supporting immune tolerance via Tregs, while also potentially promoting CD8+ T cell activation in some contexts [28-30]. Interestingly, although vitamin D levels did not correlate with *PDL1*, kynurenine, or tryptophan, their immune impact appears mediated through *VDR* (vitamin D receptor) signaling [31]. While AML patients showed 1.4-fold higher *VDR* expression compared to controls, and controls had 2.4-fold higher *IFNGR* expression, neither difference reached statistical significance. *IFNGR*, known to regulate *PDL1* via IFNG signaling, was not associated with immune

parameters in our cohort, suggesting a disconnect between receptor expression and functional outcomes in AML.

Our data support the established immunosuppressive role of Tregs and highlight the low frequencies and possible dysfunction of CTLs in AML [32, 33]. The median CD4/CD8 ratio of 1.4 in PB was comparable to other AML studies. These findings align with previous reports showing elevated Tregs and impaired CTL responses in AML, which contribute to immune escape and poor prognosis [34].

Some limitations of our study were the lack of data on the immune profile from corresponding marrow samples, the small number of cases for some individual immune parameters, and the absence of profiling of other components of the wider immune network, such as macrophages, natural killer cells, B lymphocytes, and other additional immune cytokines, such as IFNγ. Also, the clinical outcome data for the treated cases is being collected and updated and, after a sufficient follow-up, will be included in a subsequent report.

In conclusion, our study reveals novel insights into the immunometabolic landscape of AML, particularly the interplay between checkpoint ligand expression, T cell subset dynamics, and serum levels of vitamin D and kynurenine pathway metabolites. The observed associations between low vitamin D, increased Tregs, and altered CD8+ T cell profiles suggest that vitamin D supplementation may have potential as an adjunctive immunomodulatory therapy in AML. Further investigations with larger cohorts and mechanistic studies are warranted to validate these findings and explore therapeutic interventions targeting immune dysregulation in AML.

Author Contribution Statement

Study conceptualization & methodology: Amit Choudhary, Ramya Ramesh, Smita Kayal. Clinical Data collection: Amit Choudhary, Ramya Ramesh, Smita Kayal, Prasanth Ganesan, Biswajit Dubashi. Lab work and analysis: Amit Choudhary, Ramya Ramesh, Rakhee Kar, Rajesh NG, Prakash Babu Narasimha. Manuscript writing: Amit Choudhary, Ramya Ramesh, Smita Kayal, Prakash Narasimha. Review and editing: Smita Kayal, Prakash Babu Narasimha, Rakhee Kar, Prasanth Ganesan. Final approval of manuscript: by all authors.

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Ethics approval

Institute Ethics approval was taken before commencement of the study (JIP/IEC/2019/531)

Availability of data & material

Will be made available on request

Study registration in any registration dataset

This study was not registered in any clinical trial or study registration database.

Consent to participate

Informed consents were taken from the patients before participation and taking the samples and clinical data for the study.

Conflict of interest

All authors declare no conflict of interest.

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