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Defective Treg generation and increased type 3 immune response in leukocyte adhesion deficiency 1

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ABSTRACT

In 15 Turkish LAD-1 patients and controls, we assessed the impact of pathogenic *ITGB2* mutations on Th17/Treg differentiation and functions, and innate lymphoid cell (ILC) subsets.

The percentage of peripheral blood Treg cells, *in vitro*-generated induced Tregs differentiated from naive $CD4^+$ T cells were decreased despite the elevated absolute counts of $CD4^+$ cells in LAD-1 patients. Serum IL-23 levels were elevated in LAD-1 patients. Post-curdlan stimulation, LAD-1 patient-derived PBMCs produced more IL-17A. Additionally, the percentages of CD18-deficient Th17 cells expanded from total or naïve $CD4^+$ T cells were higher. The blood ILC3 subset was significantly elevated in LAD-1. Finally, LAD-1 PBMCs showed defects in trans-well migration and proliferation and were more resistant to apoptosis.

Defects in *de novo* generation of Tregs from CD18-deficient naïve T cells and elevated Th17s, and ILC3s in LAD-1 patients' peripheral blood suggest a type 3-skewed immunity and may contribute to LAD-1-associated autoimmune symptoms.

1. Introduction

Leukocyte adhesion deficiency (LAD) is a rare autosomal recessive immunodeficiency that is classified into three subtypes [1]. Leukocyte adhesion defect 1 (LAD-1) (OMIM #116920) as the most common subtype is estimated to be 1 in 1,000,000 live births [2] caused by mutations in *ITGB2*, the gene encoding the β subunit (CD18) of the β_2 integrins [3]. The genetic defect in *ITGB2* leads to abnormal expression or activity of β2 integrin in leukocytes. CD18 subunit protein acts as an important adhesion protein, mediating adhesion of leukocytes to other cells and extracellular matrix proteins [1]. This disease leads to severe impairment of leukocyte adhesion to the vascular wall and leukocyte

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migration to the sites of infection and inflammation. Integrins are a heterodimeric family of glycoproteins composed of non-covalently linked α and β chains and are divided into β1, β7, and β2 subgroups according to β chains [1,4,5]. The β2 subgroup includes LFA-1 (αLβ2: CD11a/CD18), Mac-1 (αMβ2: CD11b/CD18), P150.90 (αXβ2: CD11c / CD18) [6]. LFA-1 is expressed by leukocytes including peripheral blood lymphocytes, monocytes, and NK cells [7]. LAD-1, which shows autosomal recessive inheritance, is one of the three types of LADs and is the most common [8–10]. LAD-1 patients are divided into two groups according to their CD18 expressions as severe (<1%) and mild form (between 2 and 30%) [11,12]. Patients with the mild form can survive into adulthood with antibiotic prophylaxis and appropriate treatment of other infections; however, patients with the severe form will die within a year unless they are diagnosed and underwent hematopoietic stem cell transplantation [13,14]. Common clinical characteristics of this defect in leucocyte adhesion are the late separation of the umbilical cord, infections (omphalitis), sepsis, periodontitis, oral aphthous and cervical lymphadenopathy. In addition, recurrent fungal and bacterial infections, necrotic ulcers that do not cause pus, impaired wound healing, and leukocytosis are also observed [15,16].

$CD4^+$T helper (Th) cells are members of the adaptive immune system and play an important role in both maintaining immune homeostasis and the defense of the host against infections. In addition to its role in T lymphocyte adhesion and migration, LFA-1 is known to modulate T cell effector functions by affecting the gene expression program during Th cell differentiation. Therefore, LFA-1 is important in various processes including the control of autoimmunity, alloreactivity, allergy, and infection, as well as T cell activation, differentiation, and effector functions [17,18]. Reduced Treg cell number and suppressive function have been described in both $CD18^{-/-}$ mice, and LFA-1-deficient LAD1 patients [19–21]. However, whether *in vitro* Treg generation or expression of surface markers that are required for suppression are impaired in LAD-1 patients has not yet been explored. Additionally, Th17 cells, a subset of $CD4^+$ T cells, have been implicated in some LAD1 patients' mucosal and inflamed skin [22,23]. It is unclear whether aberrant Th17 response is only seen in the inflamed tissue, or whether CD18-deficient T cells differentiate more robustly into Th17 *in vitro*.

Innate lymphoid cells (ILCs) are grouped into 3 subtypes, ILC1, ILC2, and ILC3 based on their function, transcription factor expression, and cytokine signatures, which precede the responses of adaptive T cells, namely Th1, Th2, and Th17. Although a recent study showed LFA-1 expression by human ILC2s is critical for their trafficking to lungs and perhaps even for their development as shown in $ICAM1^{-/-}$ mice [24,25], the number and phenotype of ILC subsets in LAD-1 patients have not been investigated to this day.

LAD-1 deficiency is a disease diagnosed in the neonatal period or soon after, whereby the dysfunction of the neutrophils, innate immunity, is more prominent. Lesions become necrotic because of impaired neutrophil adhesion to the endothelium and impaired chemotaxis. Although in this period, adaptive immunity seems to lag behind, LAD-1 immunological phenotype evolves into a more severe immunodeficiency in patients with delayed bone marrow transplantation compared to the first few months, with contributions from dysregulated adaptive immunity [26]. Thus, in the current study, we investigated the impact of CD18 deficiency on regulatory T (Treg) cells, Th17 cells, and related cytokines, surface suppression markers, and functions. Additionally, we aimed to characterize the ILCs in the peripheral blood of 15 Turkish LAD-1 patients.

2. Materials and methods

2.1. Ethics approval

This study has been approved by the local institutional review board of Erciyes University (Approval number: 2021/17). Informed consent was obtained from all patients/parents involved in the study. All the experiments have been performed according to regulations and guidelines outlined by the Declaration of Helsinki.

2.2. Cell isolation and FACS staining

Peripheral blood (10–15 cc) was taken from patients with LAD-1 and healthy donors after informed consent was obtained. Then, peripheral blood mononuclear cells (PBMCs) were isolated *via* Ficoll-Paque (density 1.077 g / ml) following the manufacturer's protocol (GE Healthcare #GE17–1440-02). The leukocytes at the interface were collected. Naive $CD4^+$ T cells from PBMCs were purified by naïve $CD4^+$ T cell isolation MACS kits (Miltenyi Biotec#130–094-131). Memory $CD4^+$ T cells were isolated using MojoSort Human $CD4^+$ Memory T Cell Isolation Kit (BioLegend#480064). For ILC staining, PBMCs were washed twice and resuspended in staining buffer. ILCs were stained with monoclonal antibodies (mAbs) for 30 min on ice, following 5 min Fc-Block. PBMCs were washed twice and resuspended in staining buffer (2% FBS in Phosphate Buffered Saline (PBS)) and run on FACSAria III. The following mAbs were used: FITC anti-human TCRγδ (clone: B1), FITC anti-human CD94 (clone: DX22), FITC anti-human CD11c (clone: 3.9), FITC anti-human TCRαβ (clone: IP26), FITC anti-human CD1a (clone: HI149), anti-human CD123 (clone: 6H6), FITC anti-human CD303 (clone:201A), FITC anti-human CD14 (clone:63D3), FITC anti-human FcεRIα (clone: NP4D6), FITC anti-human CD34 (clone: 561), Alexa Fluor® 488 anti-human CD19 (clone: HIB19), FITC anti-human CD3 (clone: SK7), PE anti-human CD161 (clone: HP-3G10), APC/Cy7 Anti-Human CD127 (IL—7Ra) (clone: A019D5), Brilliant Violet 421TM anti-human CD117 (c-kit) (clone104D2), PE/Cy7 anti-human CD294 (CRTH2) (clone: BM16), all from Biolegend.

For intracellular cytokine staining, PBMCs were resuspended in RPMI 1640 medium with 10% FBS in each well. Phorbol-Myristate-Acetate (PMA) 50 ng / ml, Ionomycin 1 μg / ml, and Golgi Plug 1 μl / ml were added and incubated at 37° C for 4 h. The cells were first stained with PE / Cyanine7 anti-human CD4 (Biolegend#357410) for the surface marker, and then the following antibodies for intracellular cytokine staining: anti-human interleukin (IL)-22-Percp Cy5.5 (Biolegend#366710), anti-human IL-17A-APC (Biolegend#512334), anti-human Interferon (IFN)-γ-APC-Cy (Biolegend#505849), anti-human Granulocyte-macrophage colony-stimulating factor (GM-CSF)-Pacific Blue (Biolegend#502314), anti-human IL-4 PerCP / Cyanine5.5 (Biolegend#500822) using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Cat#554714, BD).

For Treg staining, PBMCs were surface stained with Pe-Cy7 CD4 (Biolegend#357410), then fixed and permeabilized with the True-Nuclear Transcription Factor Staining kit and stained with Alexa Fluor 647 anti-human FOXP3 (Biolegend#320114). The analysis was done by FACSAria III.

2.3. Curdlan stimulation and enzyme-linked immunosorbent assay

Serum samples were collected from the whole blood of both healthy donors and LAD-1 patients. In addition, the PBMCs obtained were counted and equal number of cells were incubated at 37°C for 24 h with 50 μg / ml curdlan. 100 μl of supernatants from wells with and without curdlan were collected and used for ELISA without dilution. Human IL-17A ELISA MAX (Biolegend#433917), Human IL-22 ELISA MAX (Biolegend#434504), Human GM-CSF ELISA MAX (Biolegend#432004), and Human IL-23 ELISA kits (eBioscience#BMS2023–3) were used. Each ELISA experiment was performed in accordance with the manufacturer's instructions using Promega Glomax Multi Detection plate reader.

2.4. Trans-well migration assay

Equal numbers of cells (10^5) resuspended in serum-free RPMI medium were plated in 300 μl/insert. Insert with a pore size of 0.4 μm and

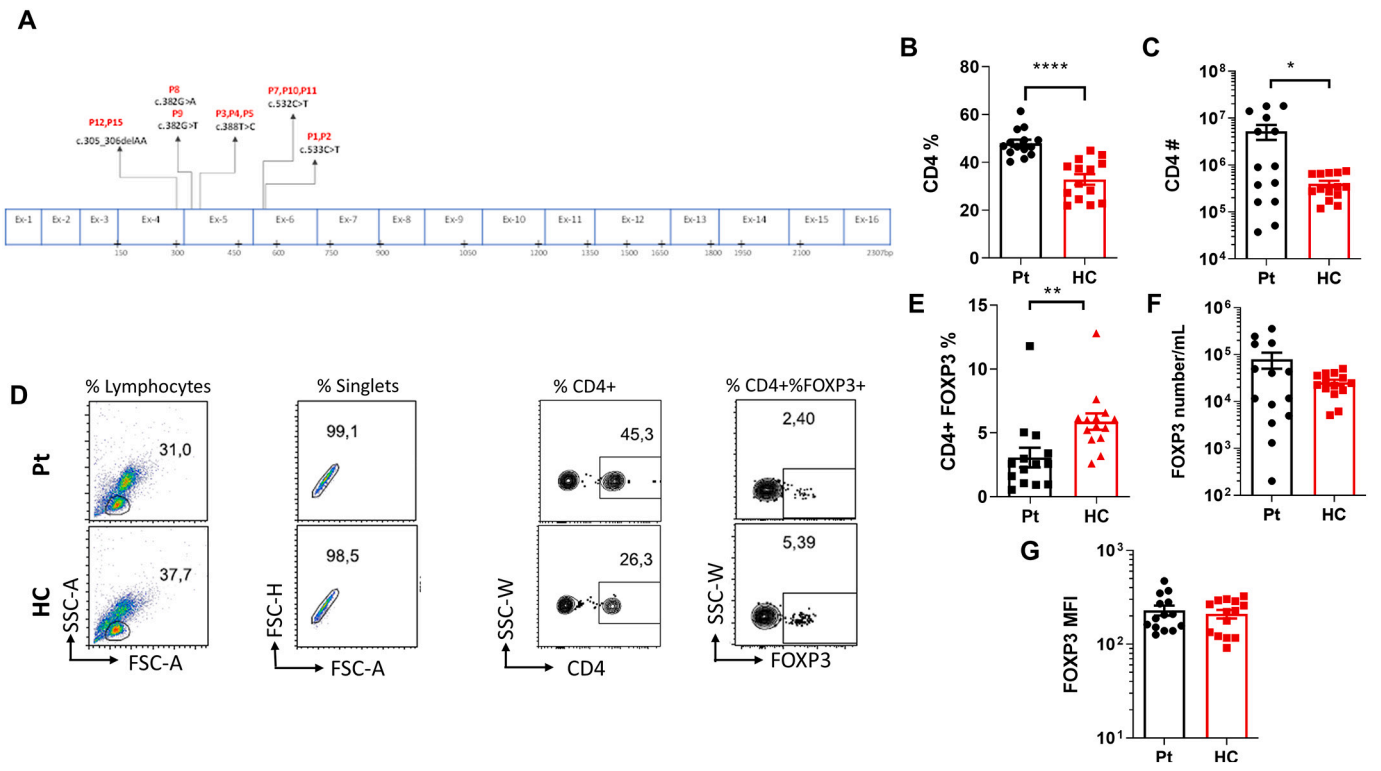


Fig. 1. Elevated number, but reduced frequency (among $CD4^+$) of Treg cells in LAD-1 patient peripheral blood. A) Cartoon of the mutations in *ITGB2* included in this study B) Absolute numbers of $CD4^+$T cells in the peripheral blood of patients with LAD-1 and healthy controls. C) Percentages of $CD4^+$ T cells in the peripheral blood of LAD-1 patients and healthy controls. D) Representative flow charts of $CD4^+FOXP3^+$ cells. (*E*-G) Quantified graphs of $CD4^+FOXP3^+$ cells in the peripheral blood of LAD-1 patients and healthy controls. E) Percentages of $CD4^+FOXP3^+$ F) Absolute number of $CD4^+FOXP3^+$ normalized to ml G) Mean Fluorescent intensity. The samples from fourteen patients and healthy controls were shown. (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM. HC: Healthy Control, Pt: Patient, MFI: Mean fluorescent intensity.

24-well plates were used. 500 ul/well of RPMI medium with 10% FBS was added to the bottom. The cells were incubated at 37°C for 4 h. The remaining cells in the inserts and the ones migrating to the bottom wells were collected into eppendorf tubes and centrifuged at 400 *g* for 5 min, cells were resuspended in PBS with counting beads (Sherotec#ACBP-20-10) and were analyzed on FACSAria III.

2.5. Cell apoptosis and proliferation assay

Equal numbers of PBMCs (10^5) were resuspended in complete RPMI 1640 medium and incubated in 96-well plates, for 24 h with and without anti-CD3 (1 μg/ml) / anti-CD28 (1 μg/ml). Using the FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend# 640922), cells were stained according to the manufacturer's instructions.

For proliferation assays, PBMCs were labeled with Tag it violet dye (Biolegend#425101) according to the manufacturer's instructions and were activated with soluble anti-CD3 (1 μg/ml) and anti-CD3 (1 μg/ml) / anti-CD28(1 μg/ml) for 4 days. The samples were run on FACSAria III for both assays.

2.6. Treg and Th17 differentiation

Differentiation experiments were performed with both naïve (Cat: 130–094-131, Miltenyi Biotec) and memory $CD4^+$ (BioLegend#480064) T cells sorted from peripheral blood of patients and healthy controls. Ninety-six-well plates were coated with anti-human CD3 (1 μg/ml) for 2 h at 37 °C. After 2 washes with PBS, soluble CD28 (1 μg/ml), Tgf-β (5 ng/ml), and IL-2 (50 ng/ml) were added for Treg differentiation and cultured for 3 days. On the 3rd day, the supernatant was taken and replaced with fresh medium with the same cytokines. On day 5, both surface and intracellular cytokine staining was performed.

The following antibodies were used for surface staining and intracellular staining of $FOXP3^+$ cells. Anti-human CD4 FITC / CD25 PE / CD3 PerCP/Cy5.5 (BD#333170), anti-human CD152 (CTLA-4) PE/Cyanine7 (Biolegend#369614), anti-human / mouse / rat CD278 (ICOS) PerCP/Cy5.5 (Biolegend#313517), anti-human KLRG1 (MAFA) APC (Biolegend#368606), anti-human FOXP3 PE (Biolegend#320208), anti-human FOXP3 Alexa Fluor® 647 (Biolegend#320114). Anti-human IFN-γ Pacific Blue (Biolegend#506526), anti-human IL-10 PE/Cyanine7 (Biolegend#501420), anti-human TNF-α PerCP/Cyanine5.5 (Biolegend#502926) anti-human IL-17A-APC (Biolegend#512334), anti-human IL-22-PercpCy5.5 (Biolegend#366710), anti-human GM-CSF-Pacific Blue (Biolegend # 502314) anti-human FOXP3-PE (Biolegend#320208), anti-human FOXP3-Alexa Fluor® 647 (Biolegend#320114).

For Th17 differentiation, naïve or memory $CD4^+$ T cells were cultured with TGF-β (2.5 ng/ml), IL-23 (20 ng/ml), IL-1β (10 ng/ml), and IL-6 (25 ng/ml) in 96-well plates coated with anti-human CD3 (1 μg/ml) supplemented with soluble anti-human CD28. On the 5th day, the medium was refreshed with a new medium containing IL-2 (1 ng/ml) and IL-23 (20 ng/ml) and cultured for 7 more days. On the 12th day, cells were activated for 4 h with Phorbol-Myristate-Acetate (PMA) 50 ng/ml, Ionomycin 1 μg/ml, Golgi Plug 1 μl/ml, and intracellular staining was performed using anti-human IL-17A-APC (Biolegend#512334).

2.7. Statistics

Data were analyzed with Graph Pad Prism software, version-8. Two-tailed, unpaired Student's *t*-test or Mann-Whitney t-test was used depending on normality analyses for pairwise comparisons. For multiple comparisons, Kruskal–Wallis non-parametric test was used with Dunn's post-test analyses for significance analyses. *P* value <0.05 is accepted as

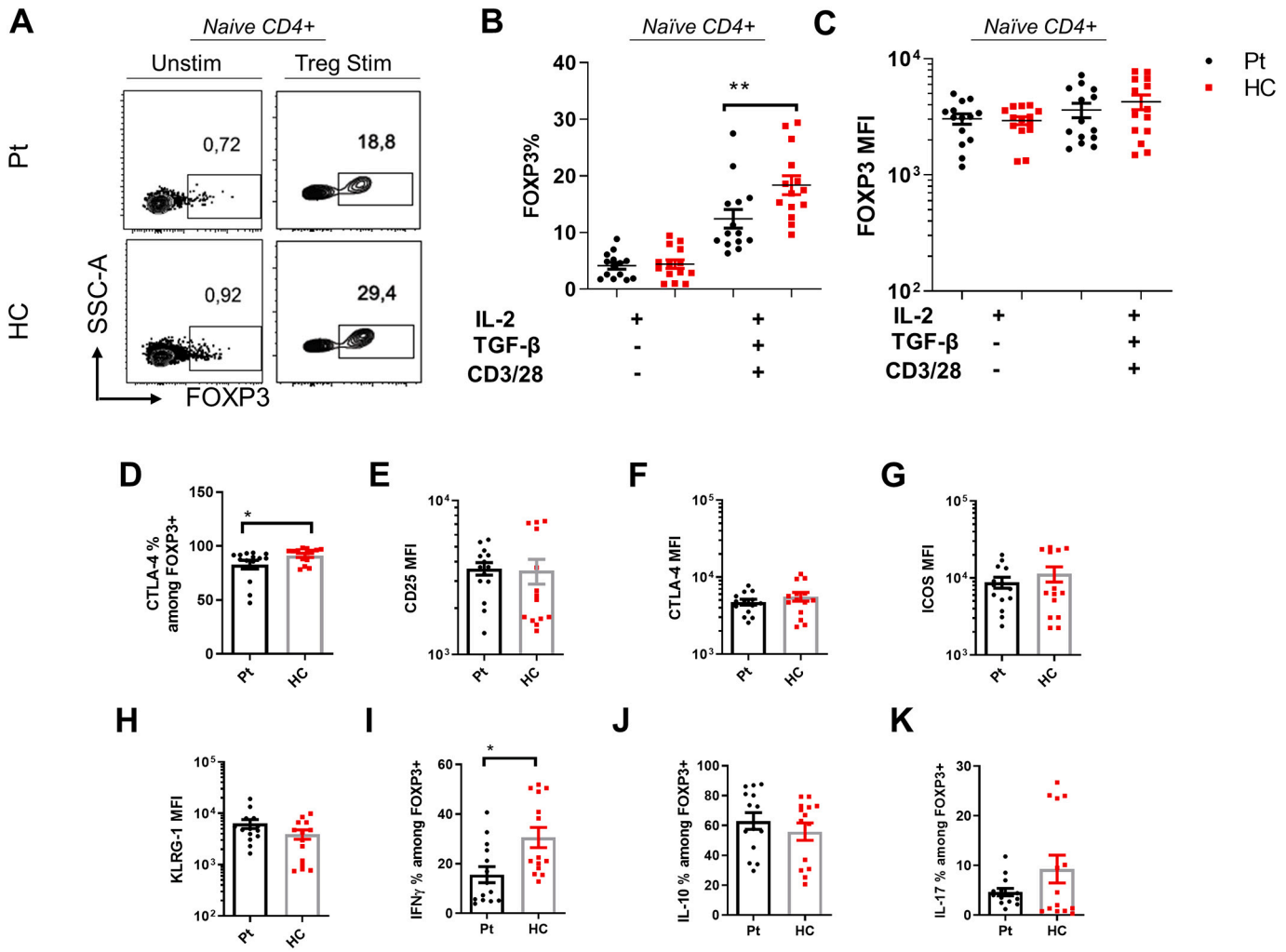


Fig. 2. Impaired *in vitro* Treg differentiation of CD18-deficient naïve CD4$^+$ T cells. Representative flow plots are shown. (A-C) Bead-sorted naïve human CD4$^+$ T cells from patients with LAD-1 and healthy control peripheral blood were harvested and differentiated into Treg cells for 5 days. A) Lymphocytes were gated and charted as SSC-A *versus* FOXP3 plots. Patient (up), Control (down) B) Percentage of FOXP3$^+$ cells in unstimulated (left) and stimulated (Treg polarizing) (right) conditions. C) MFI of FOXP3$^+$ cells in unstimulated (left) and stimulated (right) conditions. (D—K) Surface expressions of CD25, CTLA-4, ICOS, KLRG-1 after iTreg differentiation and IFN-γ, IL-10, IL-17A expressions after PMA/ ionomycin stimulation were performed by intracellular staining and analyzed by flow cytometry. D) Percentages of FOXP3$^+$CTLA4$^+$ cells E) MFI of FOXP3$^+$CD25$^+$ cells F) MFI of FOXP3 + CTLA4+ cells G) MFI of FOXP3$^+$ICOS$^+$ cells H) MFI of FOXP3$^+$KLRG1$^+$ cells I) Percentages of FOXP3$^+$IFNγ$^+$ cells J) Percentages of FOXP3$^+$IL-10$^+$ cells K) Percentages of FOXP3$^+$IL-17$^+$ cells. The samples from seven patients and healthy controls were run in duplicate. (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM. HC: Healthy Control, Pt: Patient, MFI: Mean fluorescent intensity.

statistically significant.

3. Results

3.1. Clinical phenotypes and laboratory findings of patients with LAD-1

A total of 15 Turkish patients (six males and nine females) with LAD-1 and 15 sex- and age-matched healthy controls (HC) were included in this study. LAD-1 defects were confirmed by clinical findings, and flow cytometric staining of LFA-1 (Table 1, Supplemental Tables 1 and 2). The mutations in eleven patients have been previously identified by sequencing and ten of those were point mutations which led to single amino acid change (Table 1, and Fig. 1A). P12 had two nucleotide deletions which led to frameshift and a premature stop codon [14]. Five of the mutations (P1, P2, P7, P10, P11) were in exon 6, five of them (P3, P4, P5, P8, P9) were in exon 5, and two of them (P12, P15) were in exon 4. P8 and P9 with mutations c.382G > A, c.382G > T [1,6,13,27–35], respectively, were originally described by Yaz *et al*, and incorrectly labeled as in exon 4, which are located in exon 5 [14]. Three of the patients (P6, P13, P14) were diagnosed by flow cytometric lack of CD18 expression and clinical presentations. Five of the patients were siblings born in two unrelated families. Two of them were related children, the others were unrelated. The demographic characteristics of the patients and healthy donors were shown in Table 1 and Supplemental Table 1. Omphalitis, delayed umbilical cord separation, pneumonia, sepsis, and abscesses were common clinical symptoms in our patients. Two patients had autoimmune diseases (type I diabetes). The median age of the patients was 5 years. Two of the patients received ustekinumab treatment, nine patients received hematopoietic stem cell transplantation.

3.2. Characterization of induced and thymic Treg cells in patients with LAD-1

Treg cells restrain the activity of Th subsets and their cytokine production, as well as eosinophil, basophil, mast cell and inflammatory cell activity/migration into tissues and thus show an anti-inflammatory effect [36,37]. Therefore, we first examined the effects of mutations in the β2 integrin gene on Treg cell number in the patients. The LAD-1 patient

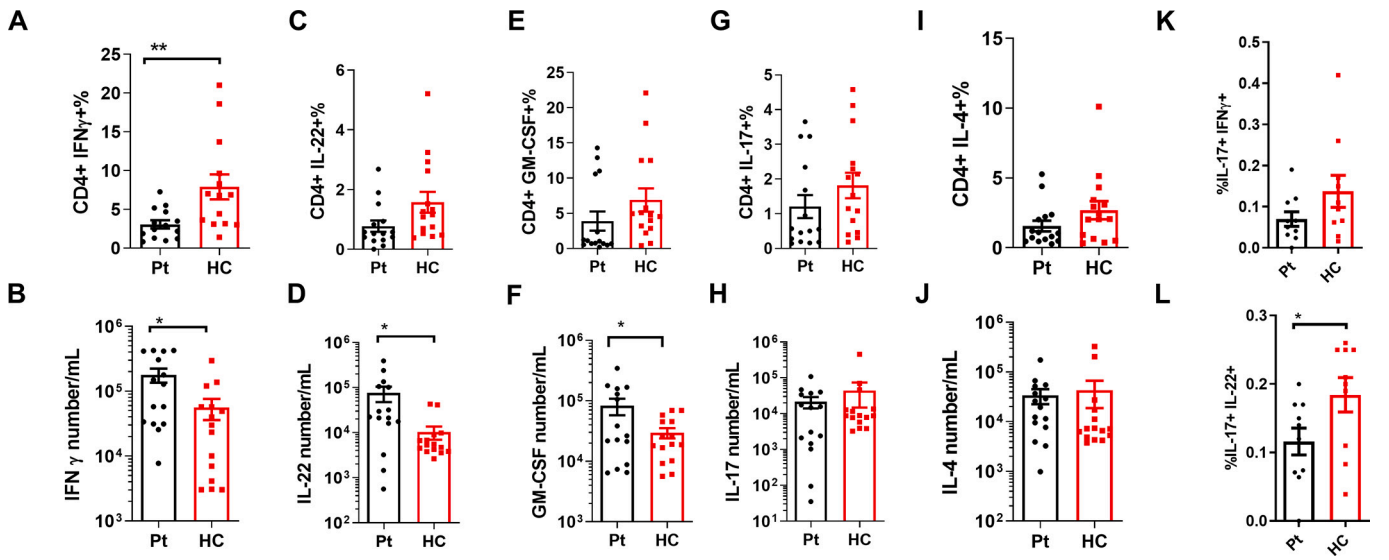


Fig. 3. Cytokine production by LAD-1 Patient derived CD4$^+$ T cells. Cytokine production by CD4$^+$ T cells after stimulation with peripheral PMA ionomycin in patients with LAD-1 and healthy controls. A) Percentages of CD4$^+$IFNγ$^+$ cells B) Absolute number of CD4$^+$IFNγ C) Percentages of CD4$^+$IL-22$^+$ cells D) Absolute number of CD4$^+$IL-22$^+$ cells E) Percentages of CD4$^+$GM-CSF $^+$ cells F) Absolute number of CD4$^+$GM-CSF$^+$cells G) Percentages of CD4$^+$IL-17 cells H) Absolute number of CD4$^+$IL-17$^+$ cells I) Percentages of CD4$^+$IL-4$^+$ cells J) Absolute number of CD4$^+$IL-4$^+$ cells. K) Percentages of CD4$^+$IL-17$^+$ IFNγ$^+$ cells L) Percentages of CD4$^+$IL-17$^+$ IL-22 cells. Absolute numbers were normalized to ml. The samples from five patients and healthy controls were run in duplicate, and five patients were run as single well. (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM. HC: Healthy Control, Pt: Patient.

peripheral Treg cells (and non-Treg T cells) have been confirmed to have reduced CD18 and CD11a expression (Supplemental Fig. 1A-B). We gated first on peripheral CD4$^+$ T cells; there was a significant increase in the percentage (among lymphocytes) and absolute number of CD4$^+$ T cells in LAD-1 patients compared to healthy controls consistent with a defect in exit from blood to tissues (Fig. 1B-C). The percentages of CD4$^+$FOXP3$^+$ Treg cells (among CD4$^+$) in the patients' bloodstream were significantly lower, however; the absolute numbers and but not the mean fluorescent intensity (MFIs) of FOXP3 were slightly (but not significantly) higher, possibly due to impaired extravasation (Fig. 1D-G). To determine whether these changes are due to altered Treg differentiation efficacy or impaired tissue homing, we magnetically selected naive or total CD4$^+$ T cells from PBMCs obtained from patients and HCs. Equal number of total CD4$^+$ T cells were differentiated into Treg for 5 days. FOXP3 intracellular staining was then performed and analyzed. *Ex vivo* differentiated Treg frequency of the patient-derived total CD4$^+$ T cells was found to be significantly higher than that of HCs (Supplemental Fig. 1C-E). We repeated similar differentiation experiments with magnetically selected naive CD4$^+$ T cells to assess whether *de novo* differentiation or Treg expansion is affected. Interestingly, when naïve T cells are used, the patient's iTreg cell frequency was significantly lower in culture compared with HCs, suggesting that *de novo* differentiation rather than expansion of Treg cells is negatively impacted by LFA-1 deficiency (Fig. 2A-C). We also evaluated the surface markers critical for suppressive functions such as CD25, CTLA-4, ICOS, and KLRG-1 after iTreg differentiation. The percentages of CTLA-4 expressed on iTreg were significantly reduced (Fig. 2D), but other surface markers were comparable to HCs (Fig. 2E-H, Supplemental Fig. 2). Finally, we quantified the cytokines produced by iTreg cells *via* intracellular cytokine staining. Those experiments revealed that iTreg cells of the patients produced significantly less IFN-γ but similar levels of TNF-α, IL-10, and IL-17 compared with those of HCs. (Fig. 2I-K, Supplemental Fig. 3A). Interestingly, flow cytometric TBET-staining of patient-derived peripheral Treg cells revealed slightly elevated T-BET expression (Supplemental Fig. 3B). These results collectively suggested that the peripheral blood Treg to CD4 ratio, as well as *de novo* iTreg generation, may be negatively impacted by LFA-1 deficiency.

3.3. Characterization of Th17 cells in patients with LAD-1

LFA-1 deficiency was previously shown to impact Th subsets [38], therefore, in order to compare the distribution of peripheral T cell subsets in patients with LAD-1 and HCs, we examined the cytokine production by CD4$^+$ T cells after stimulation with PMA ionomycin (Fig. 3, Supplemental Fig. 3C). The percentage of IFN-γ produced by CD4$^+$ T cells in patients with LAD-1 significantly decreased, suggesting a Th1 activity defect by LFA-1 deficient T cells (Fig. 3A), however, despite this reduction, the absolute number of IFN-γ $^+$ CD4$^+$ T cells in the peripheral blood was increased consistent with an increase in CD4$^+$ T cells in the blood (Fig. 3B). Moreover, T-BET staining revealed reduced protein expression of TBET by patient derived CD4$^+$ T cells consistent with the reduced IFN-γ production (Supplemental Fig. 3D). In addition, GM-CSF $^+$ CD4$^+$ and IL-22$^+$ CD4 $^+$ T cell absolute numbers (but not percentage) also increased significantly in the peripheral blood of LAD-1 patients (Fig. 3C-F). IL-17$^+$ and IL-4$^+$ CD4$^+$ T cell frequency and absolute numbers were comparable, as shown by similar percentages between LAD-1 patient and HC samples. (Fig. 3G-J). Lastly, frequency of IL-17 $^+$ IFN-γ $^+$ double producer CD4$^+$ T cells were comparable, whereas IL-17 $^+$ IL-22$^+$ double producers were slightly but significantly lower in the LAD-1 patient peripheral blood (Fig. 3K-L).

Because elevated Th17 response was previously shown in the oral mucosa of LAD-1 patients [22], we further tested possible dysregulated Th17 cell response in *ex vivo* cultures. Magnetically selected total CD4$^+$ T cells from LAD-1 patients and healthy controls were polarized into Th17 cells *in vitro*, and IL-17A staining was performed. We observed elevated Th17 cell frequency in such cultures of patient-derived CD4$^+$ T cells (Fig. 4A-B), suggesting that LFA-1 deficiency may lead to augmented Th17 cell generation/or expansion.

We also measured the levels of IL-23, GM-CSF, IL-17, and IL-22 cytokines in the sera of HC and LAD-1 patients by ELISA. The levels of IL-23 in the serum of the patients were found to be increased significantly. The levels of other cytokines (GM-CSF, IL-17A, IL-22), however, were comparable to that of HCs (Fig. 4C-F).

Lastly, we compared the levels of secreted cytokines IL-23, GM-CSF, IL-17A, and IL-22 by PBMCs in the supernatants collected after stimulation with 50 μg/ml curdlan overnight (Fig. 4G-J). IL-17A production

Table 1
Clinical and molecular features of patients with leukocyte adhesion deficiency type 1.

Patient Ids	Age/sex	Nucleotide change	Amino acid change	Zygosity	Exon	CD18 (%)	Main clinical presentation	Novelty (Reference)
P1	5 years/F	c.533C > T	p.Pro178Leu	Homozygous	6	0%	Omphalitis, delayed cord separation, recurrent skin and lung infections	[1,6,44–46]
P2	5 years/F	c.533C > T	p.Pro178Leu	Homozygous	6	10%	Omphalitis, delayed cord separation,recurrent lung infections	[1,6,44–46]
P3	3 years/M	c.388 T > C	p.Tyr130His	Homozygous	5	19%	Omphalitis, delayed cord separation, diaper dermatitis, ulcerated wound, atopic dermatitis, typhilitis	[13]
P4	5 years/F	c.388 T > C	p.Tyr130His	Homozygous	5	0.6%	Delayed cord separation, Rec. RTI, pneumonia (Parainfluenza type 3, Inf.A) Recurrent Bacteremia, Gingivitis, perianal ulcers Rec. Typhlitis, Tip I DM	[13]
P5	7 years/F	c.388 T > C	p.Tyr130His	Homozygous	5	0.4%	Moniliasis, Rec. RTI, Recurrent urinary infection, Parainfluenza type 3, Influenza A) Rec. bacteremia, gingivitis, perianal ulcers Tip I DM, meningomyelocele, developmental dysplasia of the hip	[13]
P6	22 month/M	Not tested	x	x	x	0%	Recurrent infections. Requiring hospitalization and leukocytosis	x
P7	5 years/F	c.532C>T	p.Pro178Ser	Homozygous	6	5%<	Delayed cord separation, perianal abscess, poor wound healing, necrotizing skin infection	[1,47]
P8	21 years/M	c.382G > A	p.Asp128Asn	Homozygous	5	3%	Rec. RTI, pneumonias, gingivitis, recurrent skin infections	[1,14]
P9	5 years/M	c.382G > T	p.Asp128Tyr	Homozygous	5	2%	Recurrent abscess, delayed umbilical detachment	[1,6,13,27–35]
P10	17 years/F	c.532C > T	p.Pro178Ser	Homozygous	6	2–30%	Recurrent skin abscess, Rec. pneumonia, Tooth deformity due to recurrent periodontitis, Chronic anemia	[1,47]
P11	15 years/M	c.532C > T	p.Pro178Ser	Homozygous	6	2–30%	Recurrent skin abscess, Recurrent pneumonia, tooth deformity due to recurrent periodontitis, Pyoderma gangrenosum, Chronic warts on the hands	[1,47]
P12	3 years/F	c.305_306delAA	Lys102Serfs*39	Homozygous	4	1%	Omphalitis, delayed cord separation, recurrent skin infections	[1,14]
P13	3 years/M	Not tested	x	x	x	0.9%	Omphalitis, delayed cord separation, severe otitis media, mastoiditis disseminating to the petrous bone, temporal bone destruction perianal, perineal necrotic lesions, typhlitis	x
P14	5 years/F	Not tested	x	x	x	5%<	Recurrent Pneumonia, inflammatory bowel disease, typhlitis	x
P15	4 months/F	c.305_306delAA	Lys102Serfs*39	Homozygous	4	4.4%	Omphalitis, delayed cord separation,ulcerated diaper dermatitis	x

by LAD-1 patient PBMCs was significantly increased after curdlan stimulation. Simultaneously, curdlan stimulation was applied to PBMCs from our P3 and P4 patients before and after treatment with ustekinumab, and the above cytokine levels were measured in the supernatants, but no difference was observed between before and after treatment (not shown). Collectively, these results suggest that LAD-1 patient serum and immune profile may be skewed to a heightened type 3 immune response and that tissue antigen presenting cells and phagocytes may contribute to this response.

3.4. Characterization of ILCs in patients with LAD-1

ILCs undertake similar functions as Th cells until the adaptive immune system is activated and continue to help effector cells even after the activation of adaptive immunity. In previous studies, the expression of β1 and β2 integrin receptors on ILCs have been shown [25]. Therefore, we explored whether LFA-1 deficiency would impact human ILC subsets differentially. ILC gating strategies were adopted from our previous publications (Fig. 5A) [39–41]. Similar to Treg cells, reduced LFA-1 expression has been confirmed in the total and subsets of patients' ILCs by CD18 and CD11a staining (Supplemental Fig. 4A-B). The percentage of ILC3s among total ILCs, and the absolute number of the ILC3 significantly increased in the peripheral blood of LAD-1 patients compared with HCs (Fig. 5B-I). Frequencies of ILC1 and ILC2 in LAD-1 patient peripheral blood were comparable to those of HCs. Additionally, we FACS-sorted total ILCs (Lineage-CD161 $^{+}$ CD127^{+}) from the peripheral blood of four patients and healthy controls. From this material, real-time qPCR was performed in technical replicates (Fig. 5J). We observed elevated *IL23R* and comparable levels of *IL22*, *CCR6*, *IL17A*, *TBX21,* and *GATA3* in patient-derived ILCs compared with healthy donor-derived ILCs. Collectively, these data suggest an elevated type 3 immune response in LAD-1 patients.

3.5. Migration, proliferation and apoptosis defect in the blood of patients with LAD-1

The most prominent pathophysiological features of patients with LAD-1 are abnormal adhesion, migration, and chemotaxis disorders in leukocytes. [42] Therefore, we conducted a trans-well migration experiment to see whether lymphocytes with increased frequencies in the peripheral blood had migration defects. We added an equal number of patient and healthy control lymphocytes to inserts in media without FBS and then placed them in 24 well plates containing complete media with FBS medium. After 4–5 h, we analyzed the lower wells and upper insert. We showed that the number of cells migrating across the trans-well was significantly decreased in patients with LAD-1 (Fig. 6A).

We next compared the *ex vivo* proliferative capacity of PBMCs isolated from patients with LAD-1 and control blood. After labeling lymphocytes with Tag-it-violet, we cultured them for 4 days and stimulated them with anti-CD3 or anti-CD3 / CD28. We observed a significant decrease in the proliferation of CD3/CD28-activated LAD-1 patients' lymphocytes (Fig. 6B-C).

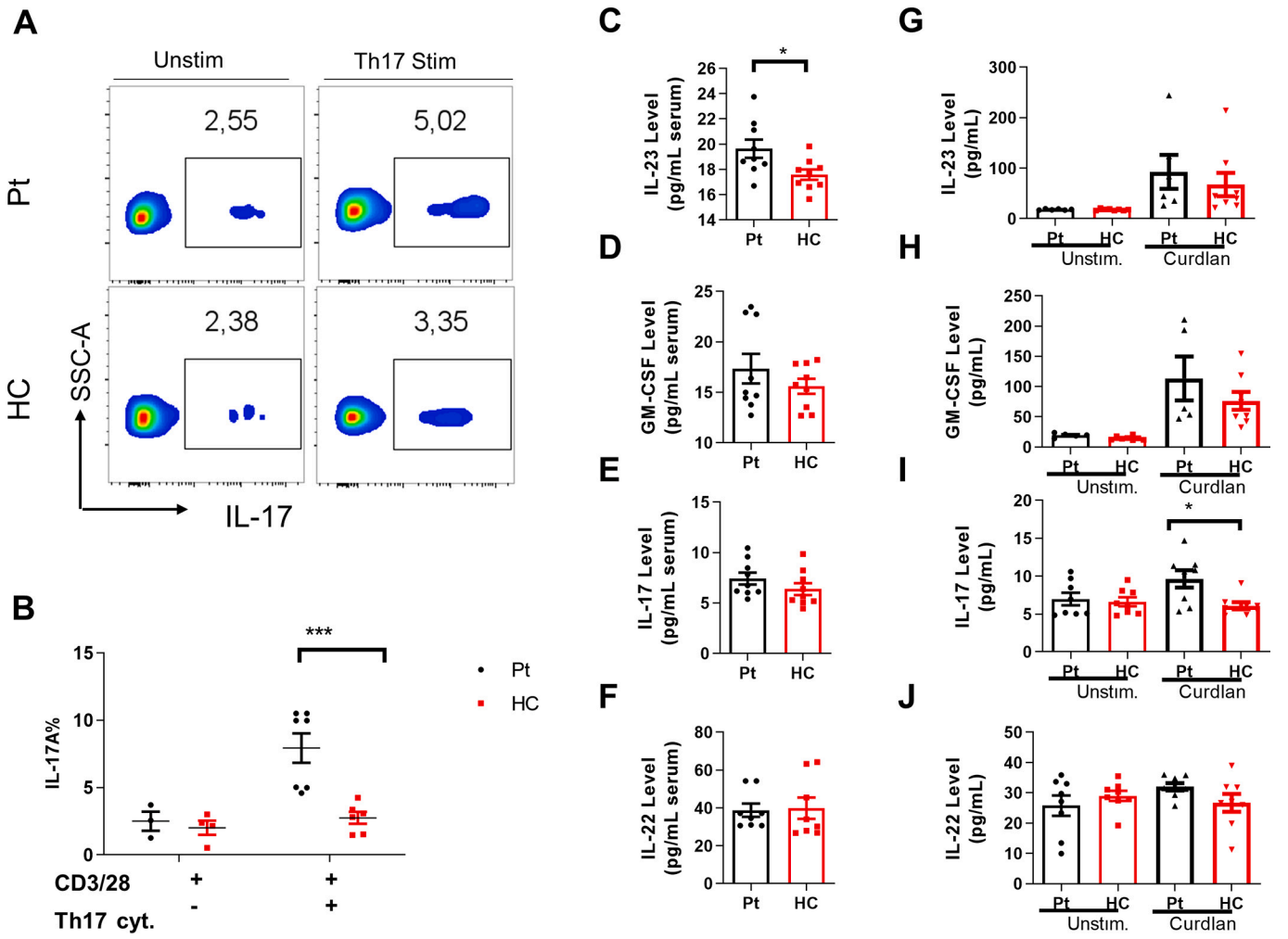


Fig. 4. Increased *ex vivo* Th17 differentiation/expansion of LAD-1 patients derived $CD4^+$ T cells and elevated IL-23, IL-17A in the patient serum and culture, respectively. (A-B) Magnetically selected total $CD4^+$ T cells from LAD-1 patients and healthy controls were polarized into Th17 cells *in vitro*, and IL-17A staining was performed. A) Lymphocytes were gated and charted as SSC-A *versus* IL17-A plots. Patient (up) Control (down) B) Percentage of IL-17A+ cells in unstimulated (left) and stimulated (right) conditions. The samples from three patients and healthy controls were run in duplicates for most conditions, and individually in Th0 condition. (C-J) We measured the levels of C) IL-23 D) GM-CSF E) IL-17 and F) IL-22 cytokines in the sera of HC and LAD-1 patients by ELISA, $n = 7–8$ for patients and healthy controls. (G-*J*) The levels of cytokines, $n = 5–8$ for patients and healthy controls G) IL-23 H) GM-CSF I) IL-17A J) IL-22 secreted by PBMCs were compared in the supernatants collected after stimulation with 50 μg/ml curdlan overnight. (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM. HC: Healthy Control, Pt: Patient.

Additionally, PBMCs isolated from LAD-1 patients were more resistant to activation-induced apoptosis after overnight stimulation with anti-CD3 / CD28 compared with those of healthy controls (Fig. 6D-E, Supplemental Fig. 5). Collectively, these data show that LFA-1 deficiency results in impaired migratory capacity of PBMCs, reduced proliferation capacity, and resistance to activation-induced cell death possibly due to impaired TCR signaling.

4. Discussion

In this study, we tested for the first time whether CD18 deficiency in humans impacted *in vitro* Treg and Th17 differentiation, and *in vivo* peripheral blood ILC populations. We also characterized Treg, Th17, ILC cell numbers, cytokine profiles, migration, and proliferation properties of PBMCs in 15 Turkish LAD-1 patients and age- and sex-matched HCs.

The genetic variants of *ITGB2* in 11 LAD-1 patients were identified by whole-exome sequencing. The patients were diagnosed based on absent/reduced CD18 protein levels, genetic analyses and clinical /and laboratory data. The CD18 subunit of β2-integrin LFA-1 is linked to the cytoskeleton and contains 8 extracellular domains. Four epidermal growth factors like domain (EI-4), hybrid domain (H), plexin-semaphorin-integrin (PSI), β tail, and finally the BI domain [4]. Although many single nucleotide changes in the *ITGB2* gene cause a disruption in its function, more mutations were detected in the highly conserved VWFA domain. This domain is encoded by exons 5–9 and has enzymatic activity [43]. Ten of the mutations described in this study lie between exons 5–9.

The role of LFA-1 on murine Treg cell generation and function has been the subject of several animal and *in vitro* studies [17–21,48,49,51]. Treg cells have been shown to express LFA-1 (CD11α/CD18) [19,20]. An *in vitro* study showed that the addition of soluble anti-LFA-1 antibodies augmented induced Treg generation from Tg4 T conv cells *ex vivo* [18]. $CD18^{-/-}$ mice were shown to bear a reduced number of thymic and peripheral Treg cells and have reduced *in vitro* and *in vivo* suppressive capacity.

The impact of LFA-1 on human Treg cell function has previously been shown by Tran *et al.* [21]. Similar to the reduced suppressive capacity of $CD18^{-/-}$ murine Treg cells, human Treg cells from LAD1 patients, as well as human Tregs treated with anti-human CD11α or CD18 monoclonal antibodies had impaired suppressive ability *in vitro* [21]. The

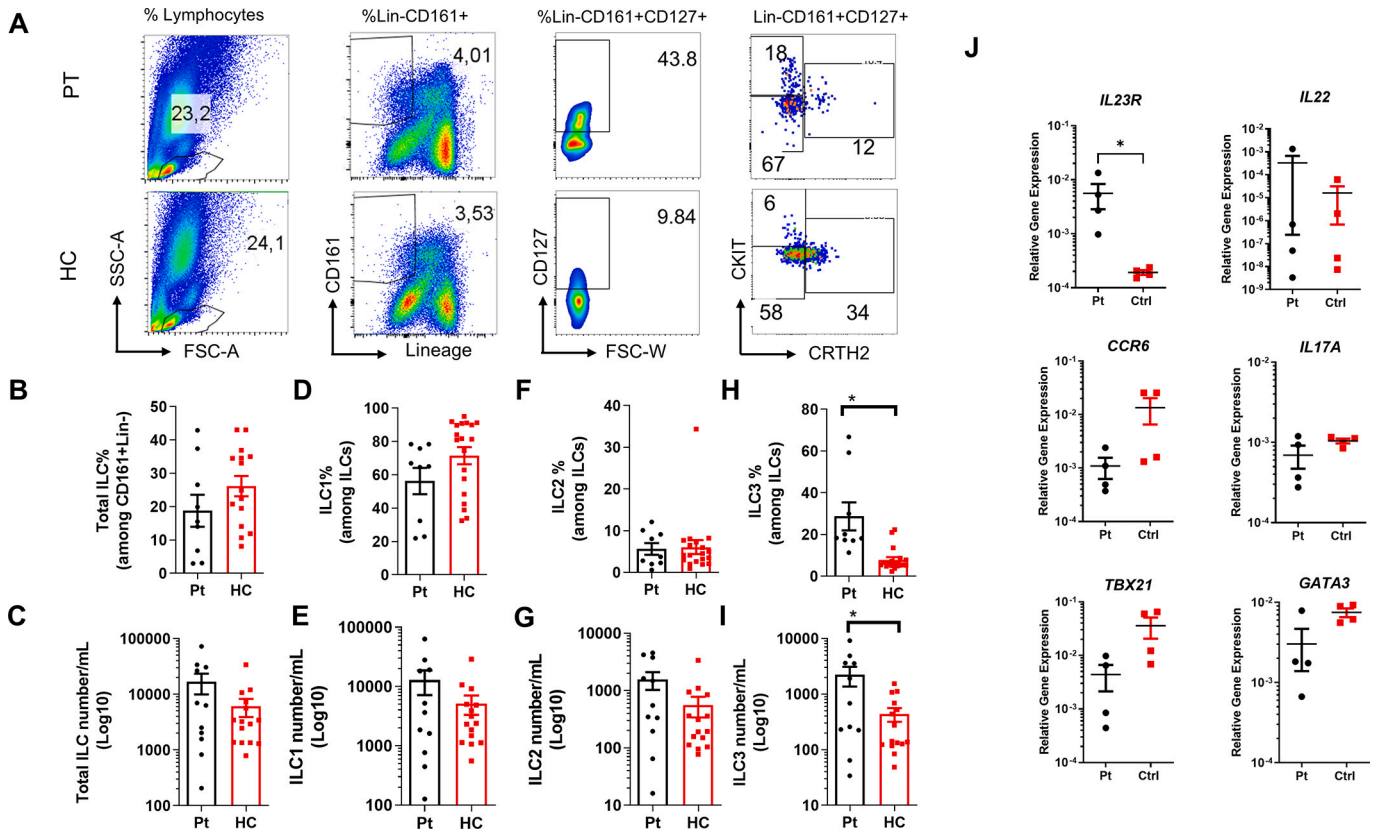


Fig. 5. Elevated number of ILC3s in the peripheral blood of LAD-1 Patients. A) Gating strategy for human ILCs, representative plots for control and patient blood. Total ILCs were gated as CD127$^+$CD161$^+$CD3$^-$Lin$^-$ cells, ILC3s as cKit$^+$CRTH2$^-$CD127$^+$CD161$^+$CD3$^-$Lin$^-$, ILC2s as CRTH2$^+$CD127$^+$CD161$^+$CD3$^-$Lin$^-$, and ILC1s as cKit$^-$CRTH2$^-$CD127$^+$CD161$^+$ CD3$^-$ Lin$^-$. (B, D, F, H) Percentage of total or subsets of ILCs in the peripheral blood of patients with LAD1 or healthy controls per ml of peripheral blood. The samples from nine patients and fifteen healthy controls were shown. (C, E, G, I) Absolute number of total or subsets of ILCs in the peripheral blood of patients with LAD-1 or healthy controls per ml of peripheral blood. J) Peripheral blood samples from 4 patients with LAD-1 and healthy controls were stained and gated for sorting of ILCs. Lin-CD161 $^+$ CD127$^+$ cells were taken as total ILC. Total ILCs (Lin-CD161 $^+$ CD127$^+$) from patients with LAD-1 and control donors were used to evaluate the expression of various genes associated with ILCs. Results are expressed as fold change over the mean of respective mRNA levels in controls. RNA expression normalized to 18S RNA. Pt; patients with LAD-1 ($n = 4$); HC; healthy controls ($n = 4$). (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM.

same study also revealed that LFA-1-dependent suppression of T cells by Tregs was mediated through ICAM1 on DCs. In Tran *et al.*'s work, the impact of LFA-1 deficiency on *in vitro* Treg generation was not studied. Our data revealed that in LFA-1-deficient LAD-1 patients' Treg cell frequency in the peripheral blood is reduced although their numbers appear to be elevated, consistent with their inability to extravasate into tissues. However, a recent study by Klaus *et al.*, using Treg specific CD18 deletion revealed that in mice, LFA-1 may be dispensable for extravasation of Treg cells [50]. Thus, future studies investigating Treg numbers in LAD-1 patient tissues or those in humanized mice (with respect to LFA-1 and ICAM) will provide more definitive answers. Importantly, our data on Treg differentiation from naive CD4$^+$ T cells showed reduced *de novo* Treg generation *in vitro*, also supporting the observed reduced frequency of Treg cells (among all CD4$^+$ T cells) in LAD-1 patients. This suggests a Treg cell-intrinsic defect due to CD18 deficiency in differentiation pathway, and perhaps may be better observed in a differentiation setting that would involve antigen-presenting cells.

Additionally, our data revealed that CD18-deficient and sufficient human Treg cells show comparable CTLA-4, ICOS, CD25, and KLRG1 expression on a per-cell basis as shown by MFI values, suggesting that earlier reports of the impaired suppressive capacity of CD18-deficient Tregs could perhaps be due to problems in other suppression mechanisms, or impaired migration into tissues. A recent study by Klaus *et al.* in CD18^{flox}Foxp3^{Cre} mice revealed normal tissue homing of Treg cells to non-lymphoid tissues but their shorter interactions with DCs which may contribute to the impaired suppressive activity of CD18$^{-/-}$ Treg cells [50]. Thus, impaired Treg tissue homing, and impaired interactions, as well as reduced differentiation may contribute to the immune dysregulation in LAD-1 patients.

LFA-1's involvement in *in vivo* murine Th17 cell differentiation has been assessed in CD18$^{-/-}$ mice in the context of the EAE model [51]. This study revealed that upon immunization with MOG_{35-55}, the draining lymph nodes contained numerically fewer Th17 cells (both polyclonal, and antigen-specific), although the frequency of Th17 was not statistically different [51], suggesting that polarization can still be intact. Antigen-presenting cell (APC)-derived Th17-polarizing cytokines have also been assessed in β2-integrin deficient mice. CD11b$^{-/-}$ APCs produced significantly higher IL-6, which in turn resulted in augmented Th17 cells in CD11b-deficient mice [52]. Similar to CD11b$^{-/-}$ (Mac-1 deficient) APCs, CD18$^{-/-}$ (LFA-1-deficient) APCs also had elevated secretion of IL-6, suggesting that both β1 and β2 integrins are regulating IL-6 production (along with other cytokines). However, despite this dysregulation in IL-6 production, CD11c^{Cre}CD18^{flox} mice were partially protected from EAE [53], and Th17 cell frequency in the CNS of such mice was comparable to the control mice. The potential impact of CD18 on Th17 cell differentiation could also be seen in a study by Sing K *et al.* who employed a murine model of LAD1 carrying a hypomorphic variant of the CD18 gene, CD18hypo PL/J. Treg cells from CD18hypo PL/J mice, compared with that of WT, converted to Th17 more rapidly *in vitro* and *in vivo*, and this could also be mimicked *in vitro* by the addition of anti-CD18 into Treg -DC cultures. Like complete CD18KO mice, and CD11b-deficient mice, IL-6 and IL-23 levels in the peripheral lymphoid organs of

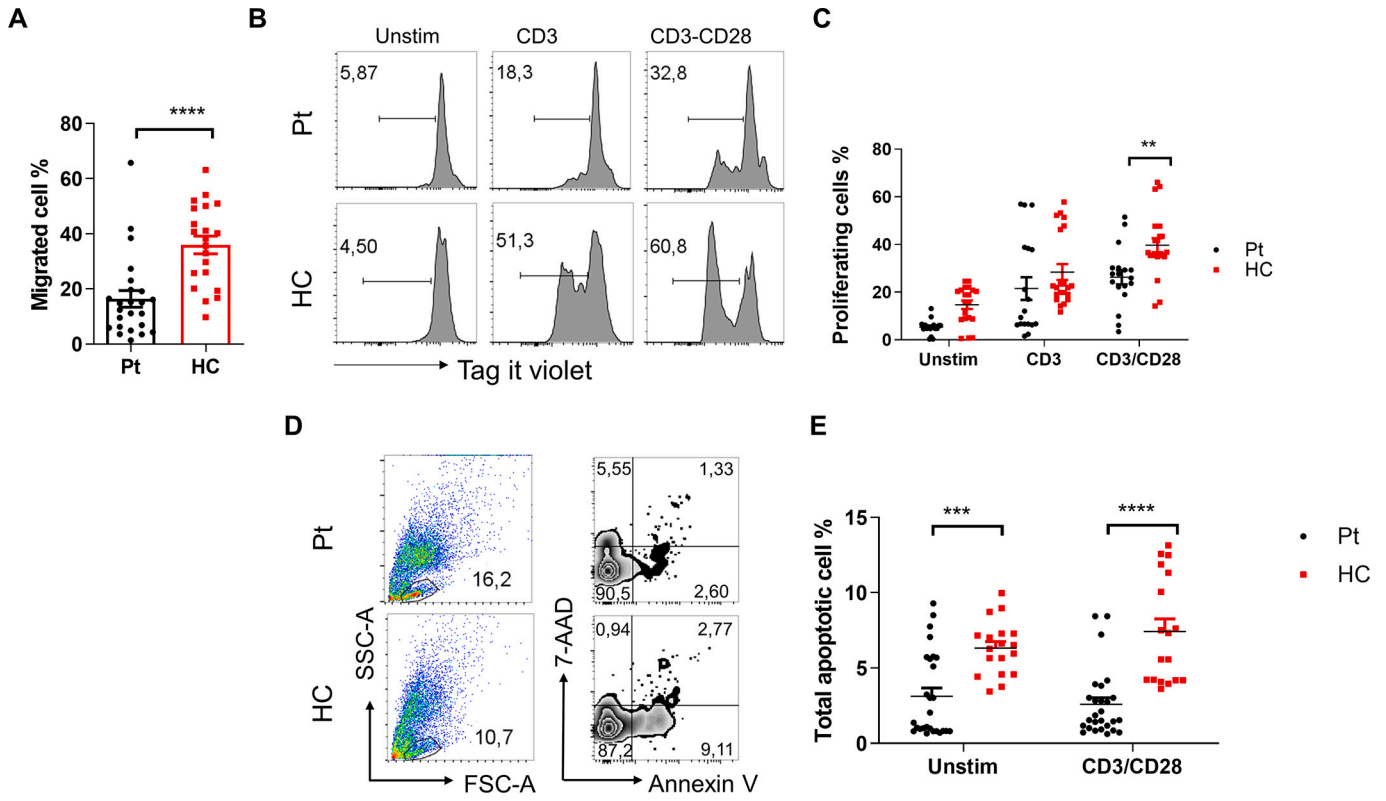


Fig. 6. Reduced impaired transwell migration of CD18-deficient PBMCs, and impaired proliferation of CD18-deficient T cells and their resistance to anti-CD3/CD28 activation induced cell death. A) Migration of equal numbers of patients with LAD-1 and healthy control lymphocytes into the medium with FBS was tested after 4–5 h. The cell migrating to the bottom of the transwell was compared to the total cell. The samples from eight patients and seven healthy controls were run in triplicates. B) Lymphocytes of patients and controls were labeled with Tag-it-violet and activated with CD3 or CD3/CD28 for 4 days in complete medium, cell proliferation was measured by flow cytometry. Percentages of proliferating cells were shown as flow plots. The samples from six patients and seven healthy controls were run in triplicates. C) and quantified bar graphs D-E) PBMCs from the LAD1 patients and controls were stimulated in triplicate overnight with or without CD3/CD28 (1 μg/ml) and cells were stained with ANNEXINV and 7AAD. D) A representative flow chart is shown and combined patients' data are shown in E) as bar chart. The samples from nine patients and six healthy controls were run in triplicates. (*) indicates $P < 0.05$, (**) $P < 0.01$ (***) $P < 0.001$, (****) $P < 0.0001$. The error bars show +/− SEM. HC: Healthy Control, Pt: Patient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CD18hypo compared with CD18wt PL/J mice were augmented. This anti-inflammatory potential of LFA-1 in in myeloid cell types including monocytes, macrophages and dendritic cells have been shown in the context of signaling pathways including TLR9 [54,55].

The abovementioned data regarding a more Th17-favoring environment could also be observed in LAD-1 patients' mucosal and skin tissues if not in blood, perhaps generally in other neutrophil disorders where their numbers are reduced in tissues [23]. Based on such observations, ustekinumab, targeting p40, was used in some LAD-1 patients presenting with features of IBD. Our own data with the LAD-1 cohort provide evidence that Th17 cells are elevated in LAD-1 patient peripheral blood-derived *ex vivo* Th17 differentiation cultures. Additionally, our results from peripheral blood serum, or supernatants from curdlan-activated PBMCs, show elevated IL-23, and IL-17 production by patient's cells, respectively. It is noteworthy that serum IL-23 levels are significantly elevated, whereas PBMCs when stimulated showed comparable levels of IL-23, suggesting that the sources of serum IL-23 might be tissue resident but not circulating macrophages/and antigen presenting cells. The data presented herein also supports the use of ustekinumab, perhaps other more selective IL-23 blockers currently in the trials (such as guselkumab) in the treatment of mucosal ulcers and LAD-1-asociated IBD.

Association of a milieu favoring type-3 immunity (especially elevated IL-23) in LFA-1 deficiency is further supported by our finding that peripheral blood ILC3s and IL-23R expression are also elevated in LAD-1 patients. Our results did not reveal a change in the number of ILC2s. An earlier report in mice demonstrated that β2 integrins are important in ILC2 recruitment to the lungs during *Alternaria* infections and that this migration is LFA-1 dependent, which requires further studies as to whether this is the case for human ILC2s or other ILC subsets' tissue trafficking. Additionally, a study by Lei et al. has recently shown that ILC2 development and function rely on intrinsic ICAM-dependent signals as ICAM1$^{-/-}$ mice have reduced ILC2 numbers and cytokine production [24]. Our data on the comparable number of ILC2s in LAD-1 patients and controls suggest that perhaps in humans the dependence of ILC2s on ICAM-1 may not be absolute or mediated by the interaction of ICAM-1 with other ligands. High and moderate cell surface expression of β1 and β2 integrins, respectively, by human ILC2s have recently been shown [25]. Studies with CD18$^{-/-}$ mice revealed that β2-integrin expression on macrophages leads to inflammasome activation, and subsequent IL-1β production, which indirectly leads to ILC3-mediated IL-22 production and resistance to *C. rodentium*-induced lethal colitis [56]. It would be invaluable to analyze ILC numbers and phenotype in the intestinal and mucosal tissues of LAD-1 patients. Since levels of ILC3s (or ILC precursors) appear to be elevated in the peripheral blood, their access to tissues may be restricted. Given the importance of ILC3s in barrier immunity, antimicrobial defense, and gut specific tolerance, as well as microbiota specific RORγt^{+} Treg induction, reduction of ILC3s, as well as Treg cells in tissues may contribute to barrier breach and chronic inflammation in LAD-1 patients [57,58].

Our results also revealed a reduction in Th1-cell derived cytokine IFN-γ (percent) by both conventional CD4^{+} T cells, and Treg cells. Reduced IFN-γ production by CD18$^{-/-}$ T cells were previously reported by others in mice in the context of colitis *in vivo* model, as well as in cell

culture [59,60].

Our data also showed that CD18$^{-/-}$ PBMCs showed significantly reduced trans-well migration from a serum-free media to FBS. This migration is independent of ICAM-1, and possibly against endogenous ligands such as sphingosine-1 or other chemokines present in the serum. Nevertheless, a reduced migration of LFA-1-deficient PBMCs was observed. Additionally, our data also revealed reduced T-cell proliferation, when PBMCs were stimulated with anti-CD3 and anti-CD28. Such proliferation defect has been reported by Varga et al. by CD18$^{-/-}$ T cells especially in cultures where dendritic cells are present [59] and others using anti-CD11a or anti-CD18 antibodies [61]. Consistent with reduced TCR-signaling due to impaired synapse formation, LAD-1 patient-derived PBMCs stimulated with anti-CD3/CD28 also showed a resistance to activation-induced cell death. Overall, such abnormal TCR-mediated signaling due to LFA1-deficiency may contribute to the autoimmunity features documented in LAD-1 patients.

This study also has some limitations. Although 15 patients were included in total, due to different time windows for sample collection, and transplantation of some patients, some assays were performed with a fraction of patients. Secondly due to low number of peripheral ILCs, cytokine levels could not be measured. Additionally, ICAM-1 mediated migration of PBMCs could not be tested. Lastly, FOXP3 staining in blood did not include CD127. Nevertheless, this report has also some strengths. Our study provides a detailed analysis of Treg and Th17 cells in 15 LAD-1 patients, and compares *ex vivo* differentiation into these lineages, and provides the first detailed investigation of ILC subsets in LAD-1 patients along with detailed cytokine profile, proliferation capacity of CD18$^{-/-}$ T cells and migration of PBMCs. The data presented herein may help explain the complex immunological symptoms of LAD-1 patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2023.109691.

Author contributions

AE, SE, and EU conceptualized the entire study. SE, YH, SH, HA, and AE performed the experiments. SE and AE wrote the manuscript. SH, EA, OK, SBE, EY, HC, HA, EY, AO, EU, MK, MHC, SSK, AD, FG, NG, MYK, AOO, SB, AM, SNG, IR, SK, EFD, KAI cared for the patients, provided the samples, contributed to interpretation of the data, critical reading and revisions of the manuscript. AE provided funding. All of the authors read the manuscript, contributed intellectually.

Declaration of Competing Interest

The authors declare no competing interest.

Data availability

Data used to support the findings of the current study are available from the corresponding author upon request.

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