Deregulated Aiolos Expression in Common Variable Immunodeficiency

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Abstract: Common variable immunodeficiency (CVID), a frequent primary immunodeficiency disease, is characterized, by defects involving T and B cells. The Aiolos transcription factor plays an important role in the control of B cell differentiation and proliferation and is modulated through alternative splicing. In order to assess the role of Aiolos in this human pathology, we analyzed the Aiolos isoforms distribution and expression. We demonstrated that the major Aiolos isoform expressed in PBMC's of healthy donors and CVID patients is hAio1, whereas the rest of the Aiolos isoforms showed homogenous distribution. In addition, we detected three new Aiolos isoforms in CVID patients that were non-detectable in healthy donors. Furthermore, we quantified Aiolos expression in T and B cells from CVID patients, given that both cell populations are altered in this pathology. Our results show an up-regulation of Aiolos expression in T and B cells from CVID patients compared to healthy donors that was confirmed at the protein level. Finally, we show a different cellular distribution of Aiolos in CVID patients compared to that found in healthy donors.

Keywords: Common variable immunodeficiency, Aiolos, Transcription factor.

INTRODUCTION

Common variable immunodeficiency (CVID), a frequent primary immunodeficiency disease, includes a heterogeneous group of diseases characterized by low concentration of serum-immunoglobulin of all switched immunoglobulin isotypes, defective specific-antibody production and increased susceptibility to infections of the respiratory and gastrointestinal tracts with encapsulated bacteria. Subgroups of patients have increased risks of some of the following conditions including splenomegaly, granulomatous lesions, autoimmune disease and tumoral transformation [1]. The basic molecular immunological defect of this pathology is still unknown and probably results from the heterogeneity of the phenotypic features of the disease.

Since CVID patients often present with hypogammaglobulinemia, presumably due to a default in memory and plasma B cells, phenotypic analysis of their lymphocyte subpopulations has primarily focused on B cells [2-4]. Different classifications have been proposed, among then, the classification in four groups: NoB group, with less that 1% of CD19 positive cells; MB1, with a defect in switched B cell memory; MB0, with a defect in switched and non switched B cell memory and, finally, MB2 group, with a phenotype similar to healthy donors.

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Aiolos is a lymphoid transcription factor that plays an essential role during B cell proliferation and maturation. Within adult hematopoietic progenitors, Aiolos is not expressed in pluripotent stem cells but is detected at low levels in multipotent progenitors. It expression is up-regulated as these progenitors become restricted to T and B cell fates. In addition, development of B cell lymphomas was frequently seen among Aiolos-deficient mice [5, 6].

We show an up-regulation of the Aiolos transcription factor in CVID patients as well as a different subcellular localization compared to healthy donors for the first time. This new information should assist in understanding the pathogenesis of CVID and improving therapeutic strategies.

MATERIALS AND METHODOLOGY

Isolation of T and B Cells from Healthy Donors and CVID Patients

Cryopreserved PBMC samples were obtained after informed consent from CVID patients from different hospitals in France, according to a protocol approved by the institutional ethic committee. The patients were not receiving any immunosuppressive drugs at the time of evaluation but were under immunoglobulin substitution therapy. Fresh blood from healthy donors was collected by the Etablissement Francais du Sang and PBMCs were frozen after density gradient isolation. Total RNA was extracted from PBMCs of CVI D and healthy donors. T cells were isolated using positive selection by anti-CD3 Dynal magnetic beads. B cells

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were isolated using Dynal negative isolation kit (Invitrogen). The purity of the isolated T and B cells reached 98%.

RT-PCR

Total RNA extraction was done using Qiagen RNeasy kit according to manufacturers' instructions. RNA (1 μg) was reverse transcribed and amplified by Titanium one step RT-PCR (Clontech). Aiolos forward, 5'-GGCAGCGACATGGAAG-3', Aiolos reverse 5'-TAGCTGATGGCGTTATTGATGG-3'. We used EF1 as internal control with the following primer sequences. EF1 forward, 5'-AAGAATGTGTCTGTCAAGGATGTTC-3', EF1 reverse 5'-GCCTGGATGGTTCAGGATAA-3'. The RT-PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

RT-(q)PCR Analysis

RNA was reversed transcribed and amplified by superscript VILO cDNA synthesis kit (Invitrogen). Quantitative PCR was carried out on an ABI PRISM 7300 detection system. We used pre-developed TaqMan gene expression assay (Applied Biosystems) for quantitative detection of human Aiolos (Hs00232635_m1) and the absolute qPCR Rox mix (Abgene) according to the manufacturers' instructions. Data from triplicates are expressed as normalized expression by using the $2^{-\Delta\Delta Ct}$ calculation method and the Abelson gene (Hs00245445_m1) as a reference gene.

Western Blot

Western blot was carried out as previously described [7].

Peptide Synthesis and Antibody Production

A specific peptide of the Aiolos amino acid sequence 368-384 was synthesized on an automated multiple peptide synthesizer (AMS422, Abimed, Langefeld, Germany) using the solid phase procedure and standard Fmoc chemistry. Purity and peptide composition were confirmed in reverse-phase HPLC and by amino acid analysis in a Beckman 6300 amino acid analyser. The peptide, glutaraldehyde-coupled to KLH *via* N-terminal lysine, was injected into outbreed New Zeeland rabbits. Serum was collected 7-10 days after the last injection.

Immunofluorescence and Confocal Analysis

Immunofluorescence and confocal analysis was carried out as previously described [8]. Briefly, PBMC from CVID patients or healthy donors were fixed with 1% paraformaldehyde for 5 min, permeabilized and then incubated with polyclonal anti-Aiolos antibody for 2h in PBS/BSA at room temperature. FITC-secondary antibody was added and incubated for 1h at room temperature. After several washing steps, samples were mounted and analyzed. In each experiment, we analyzed six CVID patients and seven healthy donors. The anti-Aiolos antibody has been previously described [9]. The anti-Bad antibody was from Abcam (Cambridge, UK).

RESULTS AND DISCUSSION

Characterization of Aiolos Isoforms in CVID

Given that the Aiolos transcription factor is involved in B cell maturation and that B cells are affected in CVID, we decided to quantitatively analyze Aiolos expression. We analyzed the expression pattern of Aiolos by PCR in PBMCs of individuals diagnosed with CVID (4 MB0, 7 MB1, 4 MB2 and 4 NoB). Table **1** summarizes the phenotypic analysis of B and T cells from 19 CVID patients, as well as healthy donors. Table **2** shows the clinical data of CVID patients and healthy donors. PCR analysis of healthy donors reveals the presence of 6 Aiolos isoforms (Fig. **1A**): hAio1, hAio2, hAio 3/4, hAio5 and hAio-del (4, 5,6) [10-12]. Long isoforms, with at least three zinc fingers, can bind efficiently to DNA, while shorter versions behave as dominant negative isoforms upon heterodimerization. These short isoforms are normally expressed at low levels, compatible with their potential regulatory role on the activity of the predominant isoform. No major differences were observed in the distribution of isoforms between healthy donors and CVID samples. Interestingly, some Aiolos isoforms were differentially expressed in CVID patients and not detected in healthy donors (Fig. **1A**). The isoforms differentially expressed in CVID patients might correspond to the isoforms described by Caballero *et al*. [13] in cell lines derived from B and T lymphoid malignancies. The new isoform expression pattern described by Caballero *et al.* [13] was not associated with particular cell or sample types. The new isoforms were also observed in B cells from individuals diagnosed with systemic lupus erythematosus and rheumatoid arthritis, two common autoimmune diseases. Aio1 was the predominant isoform detected in this work [13], similarly to our results.

In our CVID samples, the expression of the isoforms not detected in healthy donors is not associated with any particular group of patients. On the other hand, hAio1 was the isoform with the most variability among patient groups (Fig. **1A**). A similar up-regulation of hAio1 was observed in cells from chronic lymphocytic leukaemia patients [11] as well as in the cases described by Caballero *et al*. [13].

Our results did not reveal any significant over-expression of Aiolos dominant negative isoforms in CVID patients compared to healthy donors. The imbalance observed between dominant negative and wild type isoforms might be involved in the pathogenesis of CVID by disturbing Aiolos subcellular localization and its association with HDAC-containing complexes [13].

We further quantify Aiolos expression in T and B cells of CVID, given that both populations seem to be altered in this human pathology. Aiolos expression was analyzed by a-PCR in T and B cells of CVID patients and healthy donors and Aiolos expression levels are compared among the four phenotypic groups (Fig. **1B**). T cells from MB1 and MB2 patient groups show strong up-regulation of Aiolos expression compared to healthy donors. T cells from patients within the MB0 group show a moderate up-regulation of Aiolos expression and finally, the NoB group shows a slightly higher Aiolos expression than healthy donors (Fig. **1B**). Regarding the expression of Aiolos in B cells from CVID patients, we also observed a strong up-regulation of Aiolos, except in patients of the NoB group that have non-significant levels of B cells (Fig. **1B**). It is interesting to notice that Aiolos expression is upregulated in both T and B cells of CVID patients (Fig. **1C**), suggesting an alteration in both cell populations. Taken together, these results show that T and B cells express higher total levels of Aiolos in CVID patients compared to healthy donors. Our results show a quantitative and qualitative

Table 1. Characteristics of the Four Groups of Patients and Healthy Donors Used in this Study

CVID Patients	Sex	Age	Group	Years Since Onset	Years Since Diagnosis
P1	F	75	MB0	64	17
P2	F	21	MB0	21	21
P3	M	36	MB0	31	10
P4	M	28	MB0	17	1
P5	F	25	MB1	7	5
P6	F	50	MB1	4	4
P7	F	49	MB1	19	19
P8	F	36	MB1	35	0
P9	F	55	MB1	7	2
P10	F	67	MB1	16	3
P11	F	54	MB1	24	24
P12	M	56	MB2	4	0
P13	M	28	MB2	11	0
P14	F	33	MB2	6	1
P15	F	19	MB2	1	0
P16	M	41	NoB	41	38
P17	F	33	NoB	3	0
P18	F	62	NoB	58	26
P19	F	16	NoB	16	5

HD	Sex	Age
HD1	F	50
HD2	F	48
HD3	M	53
HD4	F	24
HD5	F	50
HD6	M	19
HD7	F	38
HD8	F	23
HD9	M	40

anomaly concerning Aiolos expression, in contrast to chronic lymphocytic leukaemia (CLL), which shows only a quantitative anomaly in Aiolos expression.

Aiolos is Overexpressed in CVID

The increase in Aiolos mRNA expression in CVID patients was also confirmed at the protein level, as shown in Fig. (**2A**). All patients show an up-regulation of Aiolos expression at the protein level, compared to healthy donors. Fig. (**2B**) shows the densitometric analysis of the Aiolos expression shown in Fig (**2A**). Similarly, Aiolos up-regulation has been observed in patients from CLL [11], but the consequences of this up-regulation are not yet known. Aiolos has been implicated in the control of Bcl-2 expression in T cells [9] but its implication in B cells has not been demonstrated [14]. In our patients, Bcl-2 expression seems independent of Aiolos levels (data not shown). The susceptibility of T cells to undergo apoptosis is controlled by the Bcl-2 family proteins [15]. Over-expression of Bcl-2 and Bcl-x_L enhances survival of T cells that are induced to undergo apoptosis. However, Di Renzo and co-workers [16] did not find any difference in Bcl-2 and Bcl-x_L expression in CD4 or CD8 T cells between CVID patients and normal donors, in support of our observation in the present study.

Aiolos also appears to inhibit the threshold of BCR activation by modulating tyrosine kinase phosphorylation and calcium release to the cytoplasm [5, 14]. A slight imbalance between dominant negative and anti-oncogenic Aiolos isoforms might be involved in the pathogenesis of CVID by

Table 2. Phenotype of the Four Groups of Patients and Healthy Donors Used in this Study

CVID Patients	Group	B Cells						T Cells			
		$CD19^+$		$IgD^+/CD27^-$	$IgD^+/CD27^+$	$IgD^-/CD27^+$	$CD21^-/CD38^+$	$CD3^+$		$CD4^+$	$CD8^+$
		%	/mm^3	%	%	%	%	%	/mm^3	%	%
P1	MB0	19,6	384	94	6	1	7	56	1098	23	31
P2	MB0	14,3	201	97	1	1	1	72	1014	33	32
P3	MB0	12	227	98	1	0	6	75	1421	27	46
P4	MB0	13,8	103	95	2	1	3	75	560	37	25
P5	MB1	16,9	253	81	18	0	38	70	1048	60	7
P6	MB1	17	279	63	30	5	9	77	1264	38	39
P7	MB1	16,8	164	52	46	2	48	61	597	41	17
P8	MB1	18,3	299	65	32	2	5	68	1111	29	36
P9	MB1	14,6	183	69	28	1	15	68	851	48	16
P10	MB1	14,1	204	84	13	2	17	71	1026	53	16
P11	MB1	17,5	277	81	17	1	2	67	1061	46	17
P12	MB2	7,2	120	65	13	8	3	81	1354	44	21
P13	MB2	5,8	129	60	13	22	2	81	1805	43	33
P14	MB2	9,7	126	63	22	11	3	77	999	52	24
P15	MB2	10,6	146	61	26	9	5	77	1060	41	21
P16	NoB	0						90	1522	61	27
P17	NoB	0,8						88	1740	33	45
P18	NoB	0,5						85	1097	42	22
P19	NoB	0						78	2058	39	29
HD (n=50)	median	11	202	67	14,5	14,5	2	74	1418	45	25
	min	57	85	34	6	4	0,4	61	542	34	15
	max	20,8	623	87	49	40	7	85	2598	63	39

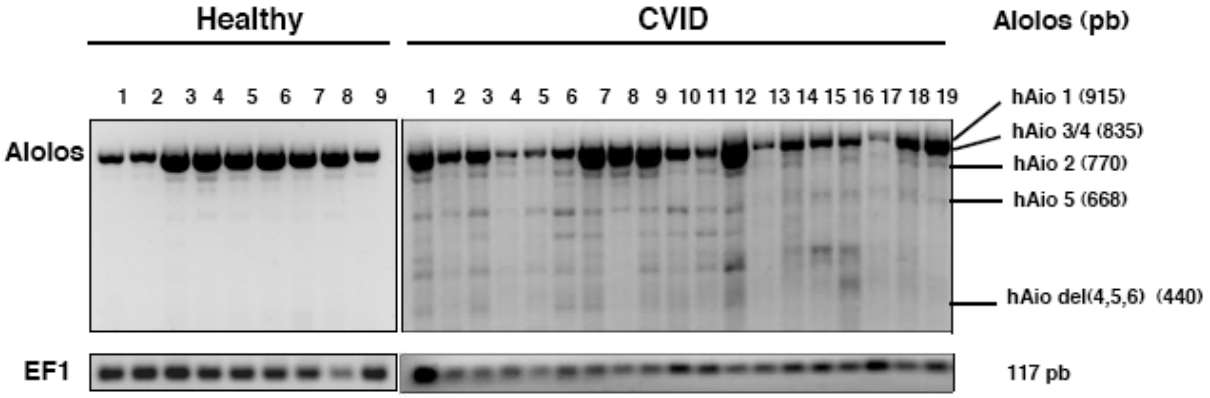
disturbing Aiolos sub-cellular localization and its association with HDAC-containing complexes [17].

Based on our results, we can't conclude whether Aiolos over-expression is the cause or the consequence of the CVID pathology. We do not exclude the possibility that the mechanisms involved in Aiolos over-expression might involve mRNA half-life stabilization or even histone modifications, since the parameters implicated in the regulation of Aiolos are largely unknown. We have shown that DNA methylation of Aiolos promoter directs Aiolos silencing and chromatin status in tumour cell lines while expression of Aiolos in primary cells is mainly regulated by histone modifications [7, 11]. The up-regulation of Aiolos in CLL seems independent of euchromatin markers modification throughout the Aiolos promoter [11]. In addition, it has also been shown that Ikaros and NF-κB transcription factors are critical for Aiolos promoter activity *in vitro* and *in vivo* [7].

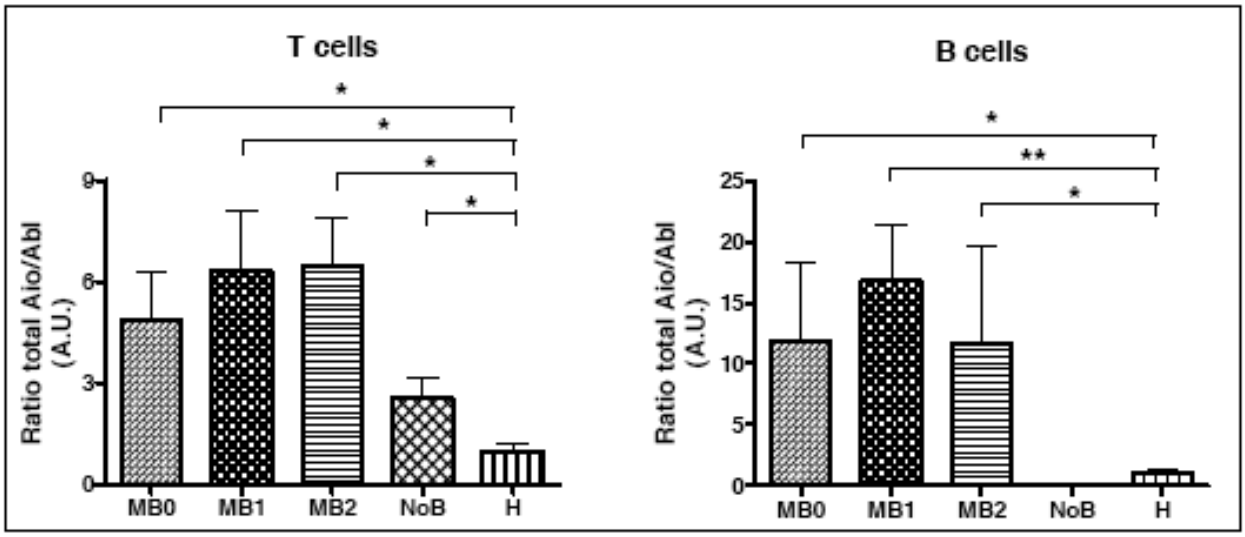
It is well known that the regulation of protein localization can be used to control cell signalling pathways, therefore, we further analyzed the subcellular localization of Aiolos in PBMC by confocal microscopy (Fig. **2C**). We observed that cells from healthy donors show lower Aiolos labelling compared to cells from CVID patients. Interestingly, there is a clear difference in Aiolos localization between normal and disease cells. While healthy cells display condensed nuclear staining, CVID patients' cells display Aiolos staining in the nucleus and throughout the cytoplasm. Staining with an anti-Bad antibody display similar distribution in controls and CVID patients. To date, Pax5, SLP-65 and Ikaros [7, 18, 19] are the only transcription factors described that control Aiolos expression either directly or indirectly. Therefore, these proteins are likely candidates to observe for modifications of expression, localization and association with HDAC complexes in CVID patients [17].

For the first time, we demonstrate that misregulation of Aiolos protein localization is associated with an immunological pathology such as CVID, with corresponding increases in Aiolos message and protein levels. The exact causes and consequences of Aiolos up-regulation in CVID patients are yet to be determined. We furthermore suggest that Aiolos could be included in the molecular diagnosis of CVID together with btk, CD40L and SH2D1A [20].

(**A**)



(**B**)



(**C**)

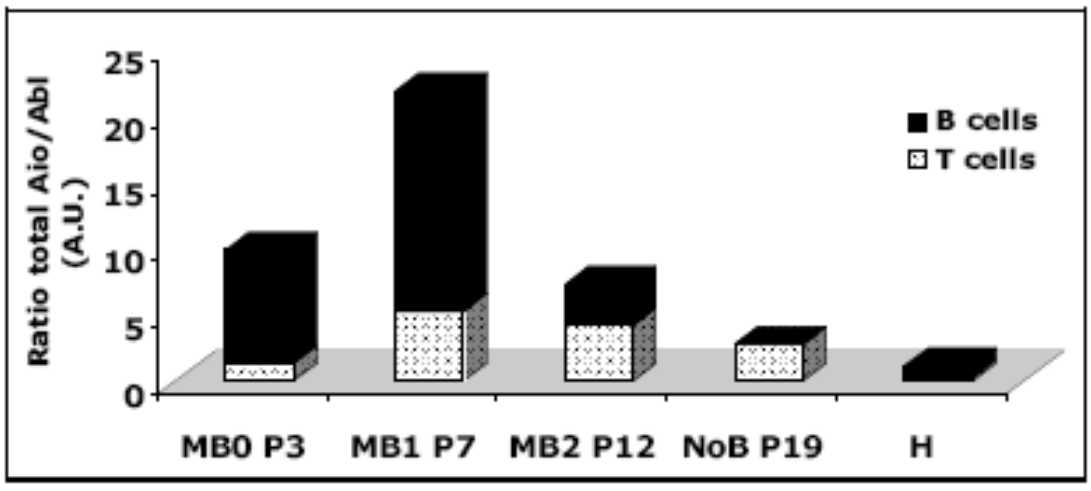
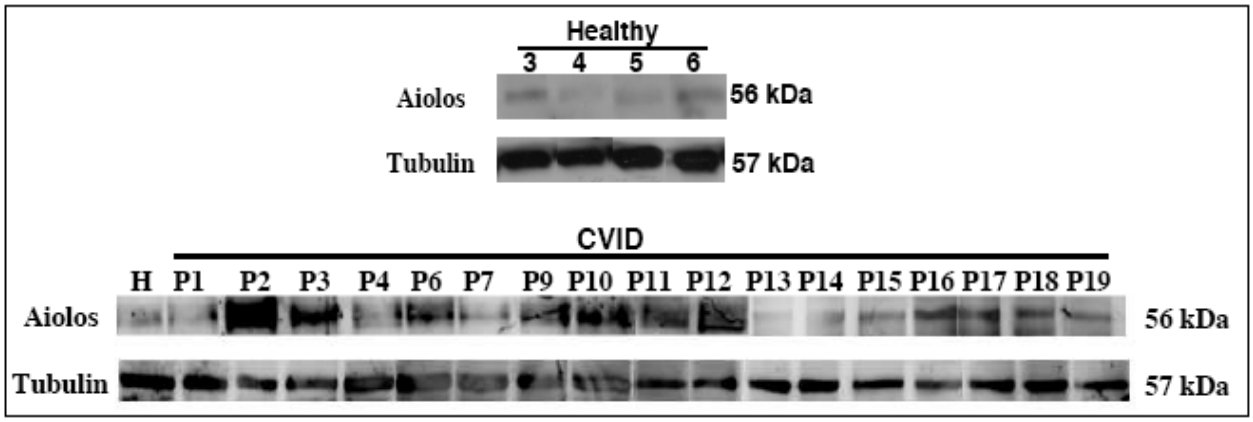
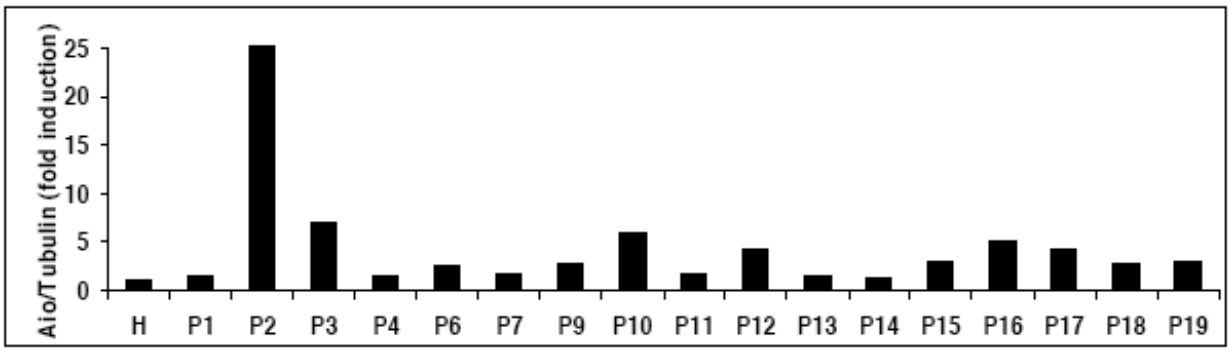


Fig. (1). Aiolos is up regulated in CVI patients at the mRNA level. (**A**) Representative results of Aiolos isoforms expression in healthy donor (H) and CVID patients after RT-PCR amplification of PBMC RNA. Elongation factor 1 (EF1) was used as internal control. The new Aiolos isoforms are marked by arrows and boxed. (**B**) Ratio of total Aiolos versus Abl expression in T and B cells after RT-q-PCR of the four groups of CVID patients. We analyzed four patients of each group and five healthy donors. (**C**) Ratio of total Aiolos versus Abl expression in T and B cells of patients 3, 6, 15 and 19. We used Prism4.0c (GraphPad Software, San Diego) for statistical analysis. Non parametric test with Mann-Whitney test was performed and statistical significances was set at $p \leq 0.05$ with the following degrees: *$0.01 < p \leq 0.05$, **0.001. Abl, kinase Abl used as internal control; A.U, arbitrary units.

(**A**)



(**B**)



(**C**)

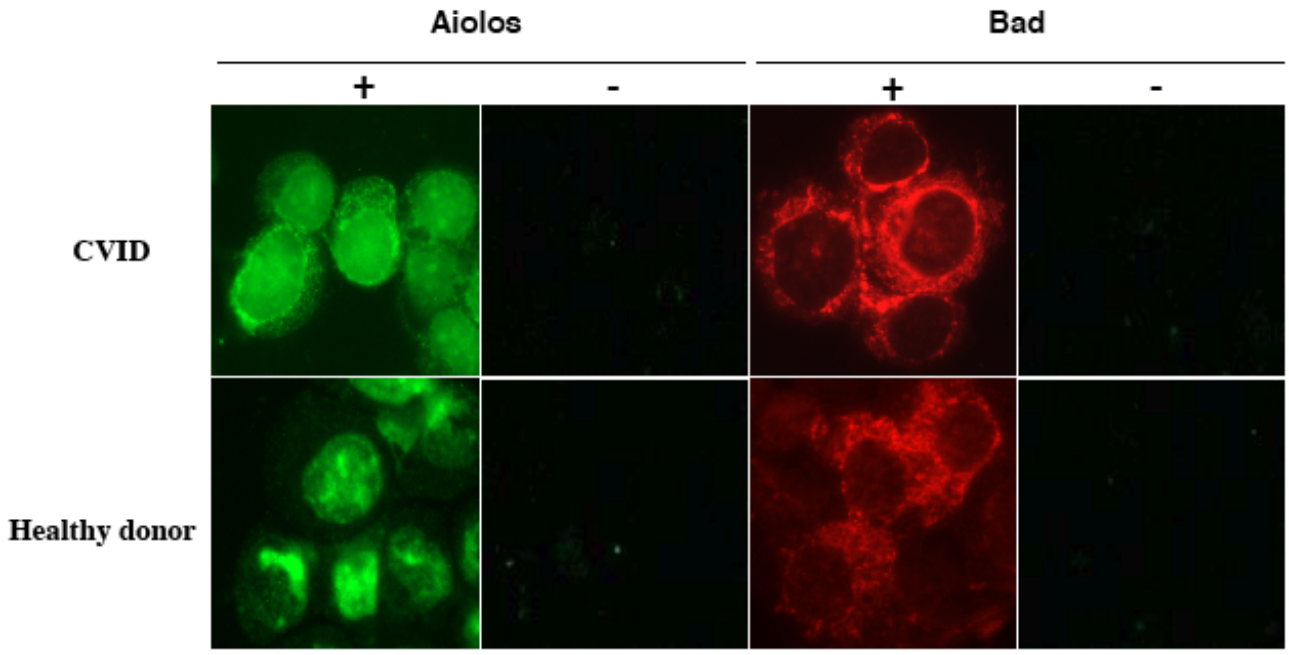


Fig. (2). Aiolos is up regulated in CVID patients at the protein level in PBMC cells. (**A**) Western blot analysis of Aiolos expression in healthy donors and CVID patients. Tubulin expression was used as internal control. Molecular weight of the proteins is shown. (**B**) Densitometric analysis of results on Fig. (**2A**). Similar results were obtained in three independent experiments. (**C**) Localization of Aiolos in healthy donors and CVID patients. Samples were stained with anti-Aiolos (rabbit polyclonal Ab), anti-Bad antibody (purified polyclonal Ab) or a pre-immune rabbit serum followed by a fluorescence secondary antibody (anti-rabbit FITC or Cy3) and analyzed by confocal microscopy. Similar results were obtained in three independent experiments. In each experiment, we analyzed six CVID patients randomly selected (one from MB0 group, two from MB1 group, two from MB2 group and one from NoB group) and seven healthy donors.

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