Identification of Tribbles-1 as a Novel Binding Partner of Foxp3 in Regulatory T Cells*

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Background: TRIB1, a serine-threonine kinase-like molecule, is a biomarker of chronic antibody-mediated rejection. Results: TRIB1 is highly expressed in Tregs, where its expression correlates with Foxp3, a molecule with which it interacts directly.

Conclusion: TRIB1 is a novel binding partner of Foxp3 in Tregs.

Significance: TRIB1 may play a crucial role in Tregs in cooperation with Foxp3.

In a previous study, we identified TRIB1, a serine-threonine kinase-like molecule, as a biomarker of chronic antibody-mediated rejection of human kidneys when measured in peripheral blood mononuclear cells. Here, we focused our analysis on a specific subset of peripheral blood mononuclear cells that play a dominant role in regulating immune responses in health and disease, so-called CD4+CD25+Foxp3+ regulatory T cells (Tregs). We isolated both human and murine Treg and non-Treg counterparts and analyzed TRIB1 and Foxp3 mRNA expression by quantitative PCR on the freshly isolated cells or following 24 h of activation. Physical interaction between the human TRIB1 and Foxp3 proteins was analyzed in live cell lines by protein complementation assay using both flow cytometry and microscopy and confirmed in primary freshly isolated human CD4+CD25+CD127- Tregs by co-immunoprecipitation. Both TRIB1 and Foxp3 were expressed at significantly higher levels in Tregs than in their CD4+CD25+ counterparts (p < 0.001). Moreover, TRIB1 and Foxp3 mRNA levels correlated tightly in Tregs (Spearman r = 1.0; p < 0.001, n = 7), but not in CD4+CD25- T cells. The protein complementation assay revealed a direct physical interaction between TRIB1 and Foxp3 in live cells. This interaction was impaired upon deletion of the TRIB1 N-terminal but not the C-terminal domain, suggesting an interaction in the nucleus. This direct interaction within the nucleus was confirmed in primary human Tregs by co-immunoprecipitation. These data show a direct relationship between TRIB1 and Foxp3 in terms of their expression and physical interaction and highlight Tribbles-1 as a novel binding partner of Foxp3 in Tregs.

Biomarkers, as defined by the Biomarkers Definitions Working Group, are characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention (1). Biomarkers have been the subject of intense interest over the past decade, with the hope of moving personalized medicine strategies into clinical practice. Our team previously identified Tribbles-1 (for Tribbles homolog 1, also called TRIB1) as a peripheral blood biomarker of chronic antibody-mediated rejection of human kidneys (2).

TRIB1 is one of three Tribbles homologs first identified in the Drosophila (3). In the latter species, Tribbles act as a mitotic inhibitor by blocking the G2 phase of mitosis and facilitating degradation by the proteasome of the String phosphatase, thus impacting the ventral furrow formation (4–6). Tribbles also play an important role in cell cycle progression during morphogenesis (4–6). In mammals, TRIB1 is a serine-threonine kinase-like molecule (7). However, contrary to most kinase proteins, this highly conserved and regulated protein appears to lack catalytic activity but acts rather like a scaffold protein by interacting with other molecules (8). For example, TRIB1 interacts with MEK1, and this interaction leads to the phosphorylation of ERK, resulting in cell survival or proliferation (7). In addition to its potential as a biomarker in transplantation, TRIB1 has also been implicated in several diseases, primarily in cancer (9) and myocardial infarction (10–12).

To further explore the role of TRIB1 in the immune system, we analyzed its expression in human peripheral blood and found it to be expressed by lymphocytes and more particularly in resting B cells and activated monocytes and dendritic cells (2). Here, we set out to explore the potential of TRIB1 role in the
Tribbles-1 Interacts with Foxp3 in Regulatory T Cells

A subset of peripheral blood lymphocytes that play a key role in immune regulation, CD4\(^+\)CD25\(^+\)CD127\(^-\) Foxp3\(^+\) regulatory T cells (Tregs). These cells are primordial for maintaining self-tolerance by preventing autoimmunity and also contribute to transplant tolerance as well as to the control of cancerous cells (15, 16). Tregs are characterized by their expression of Foxp3 (Forkhead box P3), an X-linked transcription factor specifically and largely overexpressed in this cell type (17). In mice, Foxp3 is the most reliable marker for Tregs, more so than other well known Treg markers such as CD25 (α-chain of the IL-2), GITR (glucocorticoid-induced TNFR-related protein), and CTLA4 (cytotoxic T lymphocyte antigen 4), which can all be additionally expressed in other types of T cells (17–19). Foxp3 is known to be responsible for the suppressor function of Tregs (20); the retroviral transduction of CD4\(^+\)CD25\(^-\) T cells with a vector containing the Foxp3 gene confers these cells with suppressive functions and a Treg phenotype (20–22). In this work, we explore TRIB1 expression in Tregs and subsequently demonstrate a physical link between TRIB1 and the key Treg marker Foxp3.

**EXPERIMENTAL PROCEDURES**

**Human T Cell Isolation**—Peripheral blood mononuclear cells (PBMC) were prepared by lymphosep-lymphozyte separation media (BioWest, Nuaille, France) gradient centrifugation. CD25\(^{hi}\) cells were isolated using a CD25 MicoBeads II kit for humans (Miltenyi Biotec, Bergisch Gladbach, Germany) and an autoMACS\(^+\) separator. Half of the recommended beads were used to select only the CD25\(^{hi}\) cell population. The CD25\(^{hi}\) cells were then labeled with anti-CD4-PerCP-Cy5.5 (BD Pharmingen, Mountain View, CA), anti-CD127-PE (BD Pharmingen), and anti-CD25-Alexa Fluor 647 (anti-CD25 from Immunotech, Marseille, France) coupled to the fluorochrome using a molecular probe kit from Invitrogen) and sorted using a BD FACSAria\(^+\) Flow Cytometer cell sorter and with FACSDiva\(^+\) software. The negative fraction containing the CD25\(^{lo}\) cells was then labeled with anti-CD4-PerCP-Cy5.5 (BD Pharmingen) and anti-CD25-Alexa Fluor 647 (anti-CD25 from Immunotech coupled to the fluorochrome using a molecular probe kit from Invitrogen) and sorted using the BD FACSAria\(^+\) Flow Cytometer cell sorter with FACSDiva\(^+\) software. Sort gates for CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\)CD127\(^-\) human T cells are shown in Fig. 1A. The purity was systematically >97% for regulatory CD4\(^+\)CD25\(^+\)CD127\(^-\) T cells and 96% for CD4\(^+\)CD25\(^-\) T cells.

**Mouse CD4\(^+\)CD25\(^{hi}\) and CD4\(^+\)CD25\(^{lo}\) T Cell Isolation**—Splenocytes were prepared by using collagenase D and EDTA on a C57Bl6 mouse spleen. CD4\(^+\) T cells were isolated using the biotinylated mouse CD4 T lymphocyte enrichment mixture (BD Biosciences) according to the manufacturer’s instructions. CD4\(^+\) T cells were then labeled with anti-CD4-FITC and anti-CD25-PE (BD Biosciences) and sorted using the BD FACSAria\(^+\) Flow Cytometer cell sorter with BD FACSDiva\(^+\) software. Sort gates for CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^{hi}\) mouse T cells are shown in Fig. 1B. Purity was systematically >93% for CD4\(^+\)CD25\(^{hi}\) T cells and 98% for CD4\(^+\)CD25\(^-\) T cells.

**Mouse CD4\(^+\) T Cell Isolation**—Splenocytes were prepared from C57Bl6 mice. Untouched CD4\(^+\) T cells were isolated using the CD4\(^+\) T cell isolation kit II, (Miltenyi Biotec, Bergisch Gladbach, Germany) and Automacs pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany).

**Human Treg Activation**—1 × 10\(^5\) Tregs (CD4\(^+\)CD25\(^{hi}\)CD127\(^-\)) were cultured in 100 μl of RPMI 1640 medium supplemented with 10% AB serum, 2 mm l-glutamine, penicillin, and streptomycin in each well of a round-bottomed 96-well tissue culture plate. For activation of CD4\(^+\)CD25\(^{hi}\)CD127\(^-\) Tregs, 500 units/ml of recombinant IL-2 (rIL-2), and 20 μl of beads coated with monoclonal antibodies against the CD3 and CD28 cell surface molecules (Dynabeads Human Treg expander kit; Invitrogen) were added per well. Cells were cultured for 0 to 24 h in a humidified incubator at 5% CO\(_2\), 37 °C.

**RNA Extraction and Preparation of cDNA**—Cells were resuspended in TRIzol reagent (Invitrogen) and conserved at −80 °C until use. RNA isolation of T cells was performed using the TRIzol method (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined using a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA was reverse-transcribed into cDNA using an RT-Omniscript kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions.

**Real-time Quantitative PCR**—Real-time quantitative PCR was performed in an Applied Biosystems 7900 sequence detection system (Applied Biosystems, Foster City, CA) using commercially available primer and probe sets for human TRIB1, FOXP3, and HPRT (hypoxanthine-guanine phosphoribosyltransferase) (Applied Biosystems: Hs00179769_m1; Hs00203958_m1, and Hs99999909_m1, respectively) and for mouse TRIB1, Foxp3, and HPRT (Applied Biosystems: Mm00454875_m1, Mm00475156_m1, and Mm01545399_m1, respectively). HPRT was used as the endogenous reference gene to normalize RNA starting quantity. Relative expression between a given sample and a reference sample (fabricated using PBMC from a healthy volunteer or with mouse splenocytes) was calculated according to the 2\(^{-ΔΔCt}\) method after normalization to HPRT, with results expressed in arbitrary units.

**Protein Complementation Assay**—The protein complementation assay (PCA) can be used to determine whether two proteins directly and physically interact with each other (23, 24). PCA has already been used to analyze TRIB1 binding partners (8). The technique consists in transfecting cells with two plasmids. One plasmid contains the gene of the first protein of interest followed by the first part of the green fluorescent protein (GFP) gene. The second plasmid contains the gene of the second protein of interest followed by the complementary part of the GFP gene. The transfected cells express the two proteins associated with the two GFP fragments. In the case of a direct physical interaction between the two proteins, the two complementary fragments of the GFP protein become in close proximity to one another, causing activation of the GFP and emission of fluorescence.
Potential interaction between TRIB1 and Foxp3 was analyzed in HeLa and HEK 293 cultured in DMEM medium with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. The V1 plasmid was a eukaryotic expression vector pcDNA 3.1(+) with a 3′-GFP (1) fragment of the GFP gene (amino acids 1–158), and the V2 plasmid was a similar expression vector with a 5′-GFP (2) fragment of the GFP gene (amino acids 159–239) as described previously (8). These plasmids contain an ampicillin resistance gene. The cDNA for TRIB1 (molecule of interest), TRIB1ΔC (molecule of interest with the deletion of the C terminus to assess the role of this fragment in the interaction), TRIB1ΔN (molecule of interest with the deletion of the N terminus to assess the role of this fragment in the interaction), Foxp3 (molecule of interest), MEK1 (positive control of interaction with TRIB1 (7)), or ZIP (negative control of interaction), Foxp3 (molecule of interest), MEK1 (positive control of interaction with Foxp3 (8)) were inserted into the expression vector by making sure that the orientation and the arrangement of the fusions proteins of interest and GFP fragments were optimal to bring the GFP fragments into close proximity in case of interaction.

On day 0, HEK293 cells were seeded in a 24-well plate at a density of 75,000 cells per well in 500 μl of complete medium. On day 1, cells were transfected with PolyFect (Qiagen, Courtabœuf, France). Each transfection was performed in triplicate. For each well, there were 250 ng of V1 plasmid and 250 ng of V2 plasmid in a final volume of 25 μl of serum-free media. 5 μl of PolyFect were added, and the complexes were incubated at room temperature for 5 min. 25 μl of complete medium were added, and then 55 μl of each complex solution were transferred to relevant wells of the cells and incubated overnight for 24 h at 37 °C, 5% CO2. On day 2, cells were washed with PBS, and GFP expression was analyzed by FACS. Several concentrations of Foxp3V1 or TRIB1V2 plasmids (100 or 400 ng) were tested. For microscopy analysis, the experiment was repeated with HeLa cells at a density of 10,000 cells per well in eight-well culture slides. 25 ng of each plasmid, 5 μl of serum-free media, 5 μl of PolyFect were mixed, and then 7.5 μl of complete medium were added. 18.5 μl of the complex solution were transferred to each well. The transfection was made in duplicate. After 24 h of incubation, cells were observed by fluorescent microscopy.

**Co-immunoprecipitation of Nuclear Proteins**—The co-IP was performed on freshly isolated human Tregs, with the nuclear complex co-IP kit (Active Motif) according to the manufacturer's instructions. The protocol was run in two parts, with the first part for the isolation of the nuclear proteins and the second part for the co-immunoprecipitation itself. For the isolation of the nuclear proteins, the cells were washed with phosphatase inhibitor and centrifuged, and the pellet was frozen at −80 °C until use, and then the nuclei were isolated, and finally the nuclear fraction was digested and collected following the manufacturer's instructions. The protein concentration was determined by BCA assay (Interchim, Montluçon, France) following the manufacturer’s instructions. For the co-immunoprecipitation, on day 1, 120 μg of protein extract was incubated overnight at 4 °C with either 5 μg of mouse anti-Foxp3 monoclonal antibody (clone ab450, Abcam, Paris, France), or with mouse IgG as a negative control, in low IP incubation buffer (without NaCl or detergent). On day 2, protein G-Sepharose 4 fast flow (GE Healthcare) was added to the protein/antibody mix and incubated for 1 h at 4 °C on a rotator. The proteins were then immunoprecipitated by centrifugation at 4000 rpm for 30 s at 4 °C and washed with low IP wash buffer. The bead pellet was resuspended in 15 μl of 2X reducing loading buffer and boiled at 95–100 °C for 5 min.

**Western Blot**—The co-IP samples were loaded onto a 10% SDS-PAGE gel and run at 200 V. The proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P membrane) (Millipore) at 200 mA for 2 h using a tank transfer. The membrane was blocked by incubation for 30 min at 37 °C with shaking in 2% bovine gelatin (in PBS, 0.1% Tween 20). The membrane was then incubated with the anti-TRIB1 rabbit polyclonal antibody (AP7726b, Abgent, San Diego, CA) overnight at 4 °C with shaking. The next day, the membrane was washed and incubated for 1 h at room temperature with shaking with an HRP-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA). This antibody was used as the secondary antibody in chemiluminescent Western blot assays using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). The revelation was made by using a Luminesse image analyzer (Fujifilm) and the Image Reader LAS-4000 and multigauge software (version 3.0; Fujifilm, Tokyo, Japan).

**Lentivirus Production**—The pUltra lentiviral vector was obtained from Addgene (plasmid 24129). The human Trib1 cDNA was cloned into the XbaI-BamHI sites, downstream of the 2a peptide coding sequence, in frame with the enhanced GFP sequence, allowing for the bicistronic expression of enhanced GFP and TRIB1 from the human ubiquitin C promoter. To obtain virus, 293T cells were cotransfected with the vector plasmid (pUltra or pUltra Trib1), the packaging plasmid psPAX2 and the envelop plasmid pMD2G (addgene plasmids 12259, 12260), as described previously (48). The titers of viral supernatants were determined by infecting 293T cells, and FACs analysis was performed based on detection of GFP expression.

**Lentiviral Infection of Mouse CD4+ T Cells**—Freshly isolated mouse CD4+ T cells were plated in a 24-well plate at 300,000 cells per well in 1 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. 30 units/ml of recombinant IL-2 was added, and Dynabeads® Mouse T-Activator CD3/CD28 (Invitrogen) was also added at a ratio of one bead for one cell. On day 2, the medium was replaced by fresh medium and new Dynabeads® mouse T-activator CD3/CD28 at a 1:1 ratio. CD4+ T cells were infected by spinoculation for 2 h at room temperature at 1000 rpm with various lentiviruses at different multiplicities of infection (MOI).

**Viability Test**—On day 2 after infection, CD4+ T cells were activated using PMA and ionomycin for 5 h, and then the viability of cells was analyzed using BD horizon 450 (BD Biosciences) labeling.

**Proliferation Test**—One day after infection, Dynabeads® mouse T-activator CD3/CD28 were removed, and the medium was replaced by new medium. On day 4 after infection, the medium was replaced by fresh medium with 30 units/ml of IL2.
and Dynabeads® mouse T-activator CD3/CD28 was added at a 1:1 ratio. Cells were then labeled with CellTrace™ violet cell proliferation kit (Invitrogen) in accordance with the manufacturer’s instructions. On day 7, proliferation of cells was analyzed by cytometry using LSR II (BD Biosciences) and FACS DIVA software (BD Biosciences).

**Statistics**—The nonparametric Mann-Whitney test was used for comparison between two groups, and the nonparametric Kruskal-Wallis test was performed for comparison of more than two groups. The nonparametric Spearman test was used for correlation analysis of <30 samples, and the parametric Pearson test was used for correlation analysis of >30 samples. Values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (****) were considered significant.

**RESULTS**

**Increased Expression of Tribbles-1 in Human and Mouse CD4+CD25+CD127− Tregs Compared with Their CD4+CD25− Non-Treg Counterparts**—We isolated CD4+CD25+CD127− Tregs and CD4+CD25− non-Treg counterparts from

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**FIGURE 1.**

A, sort parameters for isolation of the human CD4+CD25− (up) and CD4+CD25−CD127− (down) T cells also called regulatory T cells. The *left panels* show the gate eliminating the CD4+ T cells and selected specifically the CD4+ T cells (FSC mean Forward Scatter). The *right top panel* shows the gate for selecting the CD4+CD25− T cells from the CD4+ fraction previously selected. The *right bottom panel* shows the gate for selecting the CD4+CD25−CD127− T cells from the CD4+ fraction previously selected. B, sort parameters for isolation of the mouse CD4+CD25− and CD4+CD25high T cells (int mean intermediate). The different populations were isolated after a CD4+ T cell enrichment of mouse splenocytes. The panel displays only CD4+ T cells. CD4+ T cells were eliminated from this population by the enrichment procedure and then the population was gated for CD4+ T cells. The *left gate* shows the sorting of the CD4+CD25− T cells. The *right gate* shows the sorting of the CD4+CD25high T cells.
human PBMC using flow cytometry and a cell sorter (Fig. 1A) and measured the level of TRIB1 by quantitative RT-PCR. The expression of the well known Treg marker, Foxp3, was also analyzed in parallel. TRIB1 mRNA, similar to Foxp3, was expressed at significantly higher levels in human CD4+CD25highCD127− T cells compared with their CD4+CD25+CD127− counterparts (Fig. 2A) (p < 0.001 for TRIB1 and Foxp3). Moreover, a significant correlation was observed between TRIB1 and Foxp3 mRNA expression in human regulatory T cells (r = 1.0; p = 0.0004; Fig. 2A) but not in CD4+CD25− T cells, suggesting a strong link between the regulation of TRIB1 and Foxp3 expression in the Tregs. These results were confirmed in C57Bl6 mice where, again, an increase in TRIB1 expression after 1 to 2 h of activation, and then the level of TRIB1 returned definitively to a basal level. However, the expression of Foxp3 increased after two hours, then decreased and again increased after 16 h of activation (Fig. 3) (p = NS).

**TRIB1 Interacts with FOXP3 in Human Adherent Cell Lines**—We next analyzed the potential direct physical interaction between the TRIB1 and Foxp3 molecules. Adherent cells lines (HEK293 cells or HeLa cells) were co-transfected with plasmids containing the two genes of interest (TRIB1 and Foxp3) or other genes used as controls (see “Experimental Procedures”). Direct physical interaction between the two plasmid-encoded proteins led to the emission of GFP fluorescence, which

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**TRIB1 Is Transiently Regulated upon Treg Activation**—Given the correlation between TRIB1 and Foxp3 mRNA in Tregs, we wanted to know whether these two molecules are regulated in the same way during Treg activation. This was not the case as human Tregs activated with IL-2 and anti-CD3/anti-CD28 beads displayed only a transient increase in TRIB1 expression after 1 to 2 h of activation, and then the level of TRIB1 returned definitively to a basal level. However, the expression of Foxp3 increased after two hours, then decreased and again increased after 16 h of activation (Fig. 3) (p = NS).

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was analyzed by flow cytometry and microscopy. For the FACS analysis, the assay was performed on HEK 293 cells as these cells produce a large amount of proteins. For microscopy analysis, the assay was performed on HeLa as these cells spread out on the culture plate, thus facilitating observation of intracellular fluorescence localization. By flow cytometry analysis, we showed that TRIB1 and Foxp3 interacted together (Fig. 4A). Indeed, the number of fluorescent cells increased by 27% compared with the untransfected cells ($p < 0.01$). The strength of the interaction was similar to the positive control, which is the previously reported strong interaction between TRIB1 and MEK1 (7). Moreover, the negative control gave results similar to those of untransfected cells. When we tested other concentrations of plasmids, we showed that the interaction between TRIB1 and Foxp3 decreased with lower quantities of Foxp3V1 or TRIB1V2 plasmids. Furthermore, an increase in Foxp3V1 or TRIB1V2 plasmids did not increase the level of fluorescent cells, probably due to a lack of the interacting partner (Fig. 4C). Indeed, a large increase in one of the two proteins would generate a lack of the other protein, and therefore, no increase in interaction would be possible. The absence of an increased in the level of fluorescent cells could also be due to saturation.

The analysis by microscopy on HeLa cells showed that the interaction took place in the nucleus (Fig. 4B). This nuclear interaction could explain why the increase in fluorescence was no longer significant upon deletion of the N terminus fragment of TRIB1, which was reported to be necessary for the nuclear import of the molecule (25). However, the deletion of the C terminus of TRIB1 does not impact the interaction between TRIB1 and Foxp3, which is significantly increased compared with the untransfected cells ($p < 0.05$) and similar to the interaction between the entire TRIB1 and Foxp3.

**TRIB1 Interacts with FOXP3 in Freshly Isolated Human Regulatory T Cells**

Given that TRIB1 and FOXP3 were found to directly interact in a human cell line, we wished to confirm such an interaction in primary human Tregs. For this purpose, we performed co-immunoprecipitation experiments on nuclear extracts of freshly isolated human Tregs with an anti-Foxp3 antibody. As shown in the Fig. 5A, blotting of TRIB1 on the nuclear extract precipitated with anti-Foxp3 identified a clear band representing the TRIB1 protein. This band was strongly reduced in the negative control (co-IP performed using mouse IgG, Fig. 5A), confirming a direct interaction between the TRIB1 and Foxp3 proteins in freshly isolated human CD4$^+$CD25$^+$CD127$^-$ Tregs. The effi-
The efficiency of the anti-TRIB1 antibody was also tested in a Western blot using extract from transfected cells with a plasmid containing the TRIB1 gene followed by the GFP gene or with a plasmid containing the GFP gene alone (Fig. 5B).

TRIB1 Blocks CD4⁺ T Cell Proliferation—After infection of mouse CD4⁺ T cells, analysis of viability showed no difference between the cells infected with the control lentivirus and with the TRIB1 lentivirus (data not shown). The percentage of infected cells determined by FACS analysis of GFP fluorescence was similar between the negative control lentivirus pULTRA and the TRIB1 lentivirus. Two different MOIs were tested, and there were no significant differences between an MOI of 10 or 50. At an MOI of 10, 63% of cells were GFP⁺ with the negative control and 9.5% with the TRIB1 lentivirus. At an MOI of 50, 64% of cells were GFP⁺ with the negative control and 10.2% with the TRIB1 lentivirus.

When we analyzed the proliferation of the GFP⁺ cells, we observed that the cells infected by TRIB1 lentivirus proliferated...
much less than those infected by the negative control lentivirus. Indeed, we found that there is a greater proportion of TRIB1-infected cells that divided once compared with the negative control-infected cells. After this first cell division, the TRIB1-infected cells no longer divided, unlike the negative control-infected cells that continued to divide (Fig. 6). We also observed a difference between the morphology of the TRIB1-infected cells and that of the pULTRA-infected cells. Indeed, with the pTRIB1 lentivirus, the population with high FSC (Forward Scatter) and low SSC (Side Scatter) was not observed, suggesting that the cells were blocked in different phases of the cell cycle (Fig. 7).

**DISCUSSION**

Here, we report on TRIB1, a molecule implicated in various forms of cancer (26–29) plasma lipid homeostasis, myocardial infarction (10, 30, 31), and inflammation states (8, 32, 33). We previously demonstrated this molecule as a PBMC and intra-graft biomarker of transplant rejection in an animal model as well as in humans. These data were confirmed in PBMC by Alvarez et al. (34). These results led us to further explore TRIB1 expression and function in a key subtype of PBMC with a transversal role in regulating immune responses, so-called Tregs. Given that Tregs are characterized by their expression of Foxp3, we searched for a potential relationship between Foxp3 and TRIB1.

The regulatory mechanisms of Tregs are principally due to Foxp3. Moreover, Foxp3 expression is regulated by complex mechanisms but also via its interaction with other molecules, acting as a transcription factor. The expression of Foxp3 (Fork-head box P3) is highly regulated by many transcription factors that bind one of the four regulating regions of the Foxp3 gene (35–37). In addition to regulating its own expression, Foxp3 also regulates the transcription of other molecules by interacting with various proteins such as NFAT (nuclear factor of activated T cells (38, 39)), NF-κB (nuclear factor-κB (39)) and Runx1/AML1 (40). These interactions lead to regulation of cytokine production, including IL-2, IL-4, and IFN-γ, as well as the regulation of Treg marker expression, including CD25 and CTLA-4 (41, 42), thereby influencing their suppressive activity. Foxp3 can thus be considered as a master regulator of Tregs.
Here, we show that TRIB1 is expressed at significantly higher levels in freshly isolated human Tregs compared with their non-Treg counterparts. These results were confirmed in mice. Moreover, we also found the expression of TRIB1 and Foxp3 to be strongly correlated in freshly isolated human Tregs but not after polyclonal stimulation. This could be due to an interaction of TRIB1 with Foxp3 only at the resting state of Tregs. This suggested that TRIB1 and Foxp3 may be involved in regulating common pathways and could even physically interact in resting conditions. Interestingly, TRIB1 mRNA was only transiently up-regulated by polyclonal stimulation, unlike Foxp3 mRNA, which had a cyclic up-regulation. Given the correlation between the two molecules in freshly isolated Tregs, we thus asked whether these two molecules may interact directly. For this, we used the protein complementation assay. Using this technology, we showed that TRIB1 and Foxp3 interact together and that this interaction is as strong as that reported for TRIB1-MEK1 (7). This interaction was no longer significant when TRIB1 was deleted of its N terminus domain. Such a nuclear interaction fits well with the fact that the N terminus domain of TRIB1 is particularly important for transporting TRIB1 to the nucleus (3, 43) and the fact that Foxp3 is principally expressed in the nucleus (17). This interaction was confirmed by fluorescent microscopy subsequent to the PCA, which again clearly showed the interaction between TRIB1 and Foxp3 in the nucleus. By contrast, when TRIB1 was deleted of its C terminus domain, no difference was observed as compared with the full TRIB1. These data indicate that the C terminus domain is not involved in the interaction of TRIB1 with Foxp3.

As the PCA experiment consists in transfecting cell lines with plasmid, we wanted to confirm the TRIB1 and Foxp3 interaction in more physiological conditions. To this end, we performed co-IP starting from the nuclear extract of freshly isolated human CD4+CD25+CD127− Tregs. This analysis served to confirm the PCA results, demonstrating that TRIB1 and Foxp3 interact together in the nucleus.

The interaction between these two molecules has not been reported previously and may have an impact on Treg function. TRIB1 is known to act on various pathways by binding with diverse proteins (26, 44) and is reported to be implicated in cell proliferation (7, 27, 45, 46) and to have anti-proliferative effects (47) that could influence Treg biology or function. The analysis of mouse CD4+ splenocyte proliferation using lentiviral infection led to the same conclusion. Indeed, we showed that overexpression of TRIB1 in CD4+−infected T cells results in a defect in proliferation. Moreover, we also observed, by morphological analysis, that the TRIB1-infected cells seem to be blocked in different phases of the cell cycle. These results are consistent with those observed in Drosophila where it was shown that Tribbles slows G2 progression in a specific cell type at a precise time point and also that the overexpression of Tribbles did not impact the first division but rather the following divisions (4, 5). Moreover, we noted that the TRIB1-infected CD4+ T cells also had an impact on noninfected cells by impairing the proliferation of the CD4+ T cells. Tregs have a detrimental impact on cancer development but, on the contrary, they are key to preventing autoimmune diseases, and we could hypothesize that TRIB1 plays a role in the anergic state of Tregs.

Thus, the impact of the interaction between TRIB1 and Foxp3 on the regulatory T cell proliferation may either be detrimental or beneficial, depending on the pathway involved. The impact of TRIB1 on Treg function and the consequences of such require further exploration.

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