IRF2BP2 transcriptional repressor restrains naive CD4 T cell activation and clonal expansion induced by TCR triggering

Cristiane Sécca,* Douglas V. Faget,* Steffi C. Hanschke,* Mayra S. Carneiro,†
Martin H. Bonamino,‡,§ Patricia S. de-Araújo-Souza,*,‡ and João P. B. Viola*,†

Programs of *Cellular Biology and †Molecular Carcinogenesis, Brazilian National Cancer Institute, Rio de Janeiro, RJ, Brazil; ‡Reference Laboratories, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; and §Department of Immunobiology, Fluminense Federal University, Rio de Janeiro, RJ, Brazil

ABSTRACT

CD4 T cell activation and differentiation mechanisms constitute a complex and intricate signaling network involving several regulatory proteins. IRF2BP2 is a transcriptional repressor that is involved in gene-expression regulation in very diverse biologic contexts. Information regarding the IRF2BP2 regulatory function in CD4 T lymphocytes is very limited and suggests a role for this protein in repressing the expression of different cytokine genes. Here, we showed that Irf2bp2 gene expression was decreased in CD4 T cells upon activation. To investigate the possible regulatory roles for IRF2BP2 in CD4 T cell functions, this protein was ectopically expressed in murine primary-activated CD4 T lymphocytes through retroviral transduction. Interestingly, ectopic expression of IRF2BP2 led to a reduction in CD25 expression and STAT5 phosphorylation, along with an impaired proliferative capacity. The CD69 expression was also diminished in IRF2BP2-overexpressing cells, whereas CD44 and CD62L levels were not altered. In vivo, transferred, IRF2BP2-overexpressing, transduced cells displayed an impaired expansion capacity compared with controls. Furthermore, overexpression of IRF2BP2 in differentiated Th cells resulted in slightly reduced IL-4 and pro-TGF-β production in Th2 and iTregs but had no effect on IFN-γ or IL-17 expression in Th1 and Th17 cells, respectively. Taken together, our data suggest a role for IRF2BP2 in regulating CD4 T cell activation by repressing proliferation and the expression of CD25 and CD69 induced by TCR stimuli. J. Leukoc. Biol. 100: 000–000; 2016.

Introduction

CD4 T cells are key players in the adaptive immune response. Upon TCR activation, CD4 T lymphocytes undergo clonal expansion through intense proliferation supported by the IL-2/IL-2R signaling axis and differentiate into diverse effector or regulatory profiles that modulate the immune response. The Th1 profile promotes an effective response against intracellular pathogens and tumors, whereas Th2 is involved in immunity against helminths and allergic responses [1, 2]. The Th17 profile differs from the others discussed herein, in that it does not benefit from IL-2 signaling for expansion. This profile promotes a protective response against extracellular bacterial infections and is an important mediator of inflammatory and autoimmune responses [1, 2]. In addition to the effector profiles, CD4 T cells can differentiate toward a regulatory profile, known as iTregs, that act to counter the response of effector cells [3]. The molecular mechanisms involved in CD4 T cell activation, expansion, and differentiation are crucial for its effector functions and have been studied extensively. However, the entire scenario concerning these mechanisms is not yet fully understood.

The protein IRF2BP2 is a member of the IRF2BP family that includes the IRF2BP1 and IRF2BP2 members, the latter of which is comprised of 2 alternative splicing isoforms in humans (hIRF2BP2-A and hIRF2BP2-B). Originally, IRF2BP proteins were identified as nuclear transcriptional corepressors that were dependent on IRF2; binding to IRF2 results in their recruitment to the DNA, where they can mediate their repressor function [4]. More recently, IRF2BP2 was identified as a transcriptional repressor in several other biologic contexts that did not necessarily require IRF2 participation [5–9].

Although the majority of works available in the literature proposes a role for IRF2BP2 as a repressor in the regulation of diverse genes [4–9], there is some evidence that IRF2BP2 may also act as a positive regulator of gene expression [10, 11]. The mechanisms by which IRF2BP2 mediates its repression or...
induction in the course of gene-expression regulation have not been established and may involve interaction with the DNA and/or with different proteins that vary according to the context. The IRF2BP2 protein displays 2 conserved regions: a zinc finger domain and a RING domain [4]. Both domains have been described as being important for their ability to mediate interactions between different proteins [12, 13] and may contribute to IRF2BP2 transcriptional regulation, possibly through its participation in regulatory complexes. Indeed, this protein has been described as a participant in at least 2 distinct transcriptional repressor complexes, in which it is dependent on either its zinc finger or RING domains [8, 9]. As IRF2BP2 is ubiquitously expressed and has diverse roles described in several biologic contexts [4–11], more studies are necessary to investigate its possible roles in different cell types.

In lymphocytes, IRF2BP2 has been described as a NFAT1 repressor. In the same study, our group demonstrated that IRF2BP2 was capable of binding directly to this transcriptional factor, thus resulting in the repression of NFAT1-mediated transactivation of cytokine genes, such as IL-4 and IL-2 [5]. Moreover, it has been shown that IRF2BP2 is down-regulated in CD4 and CD8 T cells of multiple sclerosis patients. Interestingly, its levels are restored in these cells in patients in clinical remission, thus suggesting a role for IRF2BP2 in controlling its levels are restored in these cells in patients in clinical remission, thus suggesting a role for IRF2BP2 in controlling T cell-mediated disease inflammation [14]. Recently, a gain-of-function mutation in IRF2BP2 was identified to be causative of a familial form of common variable immunodeficiency disorder, unveiling a role for this protein in regulatory pathways that allow a proper antibody response [15]. In the present work, we show that IRF2BP2 has a role in repressing CD4 T cell proliferation and the expression of CD25 and CD69 induced upon TCR activation. Interestingly, IFNβ2p gene expression was down-modulated upon naive CD4 T cell activation, suggesting a role for this protein in maintaining the naive resting state, possibly by enhancing the threshold of the stimulus required for naive T cell full activation. Furthermore, STAT5 phosphorylation and CD25 levels were reduced by IRF2BP2 overexpression in CD4 T cells, suggesting an impairment of the IL-2 signaling pathway. Additionally, we demonstrate a conserved role across species for IRF2BP2 protein by showing its capacity to down-modulate CD25 expression also in human CD4 T cells.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice were bred at the INCA Animal Facility (Rio de Janeiro, RJ, Brazil). B6Ba animals (which express the Cd90.1 variant protein) were bred at the Nucleus of Laboratory Animals of UFF-Niterói (RJ, Brazil) and kept at INCA’s Animal Facility for experimentation. All animal experiments were performed in accordance with the Brazilian government’s ethical and animal experimental regulations. The experiments were approved and conducted according to the animal welfare guidelines of the Ethics Committee of Animal Experimentation from INCA (CEUA Process No. 004/13). For each experiment, same-sex mice were used at 8–10 wk of age.

For the cell-transfer experiments, recipients were irradiated with a sublethal dose of 5 Gy of X-ray radiation, 24 h before i.p. injection of 5 × 10⁶ cells in 300 μl PBS. For irradiation, animals were anesthetized with prior i.p. administration of ketamine (100 mg/kg) and xylazine (10 mg/kg).

**Cell culture**

Primary murine CD4 T cells were cultured in a humidified environment containing 5% CO₂ at 37°C in DMEM, supplemented with 10% FBS, NaHCO₃ (40 mM), NaH₂PO₄ (1 mM), HEPES (10 mM), 2-ME (55 μM), t-glutamine (2 mM), sodium pyruvate (1 mM), MEM vitamin solution (1X), MEM essential and nonessential amino acids solution (1X), penicillin (100,000 U/l), and streptomycin (10 mg/l; all from Thermo Fisher Scientific, Waltham, MA, USA).

**Plasmids**

The retroviral transduction vectors pRV-IRF2BP2 and MSCV-IRF2BP2 were used for murine IRF2BP2 protein overexpression; these plasmids encode reporter genes for the EGF and the DsRed, respectively. Both vectors have an IRES sequence that allows the expression of IFNβ2p and the reporter gene in a bicistronic mRNA. The vectors pRV-IRF2BP2 and pRV-GFP (control empty vector) were a kind gift from Dr. Anjana Rao from LIAI (San Diego, CA, USA). MSCV-IRES-DsRed [16] was a kind gift from Dr. Kevin Bunting from Case Western Reserve University (Cleveland, OH, USA). MSCV-IRF2BP2 was constructed by subcloning the murine Ifn2p gene from pRV-IRF2BP2 by digestion with the BamHI and Xhol restriction enzymes, followed by T4 ligase binding (all from New England Biolabs, Ipswich, MA, USA). The cloned Ifn2p gene has a C-terminal c-Myc tag in both vectors. The vectors pLIRE5EGFP [17] and pLIRE5-hIRF2BP2-A were used for PBMC transfections. For pLIRE5-hIRF2BP2-A construction, a fragment from hIRF2BP2-A was amplified by PCR using pEF.V5.IRF2BP2-A [4] as template and the following primers: 5’ TGA CTG CAG GCA GGT TGT TGG GTT TCG AGG 3’ and 5’ ACC GCT CGA GTC ACG AGT CTC TCT TTT TCA CTT TCA CA 3’.

Next, this fragment was cleaved by PchI and Xhol and cloned into pcDNA-hIRF2BP2-B [5] between the same restriction sites, generating pcDNA-hIRF2BP2-A (5’ restriction sites). Finally, hIRF2BP2-A cDNA was extracted from pcDNA-hIRF2BP2-A by the cleavage of BamHI and Xhol and cloned into pLIRE5-EGFP between flgRl and Sol restriction sites.

**CD4 T cell isolation and differentiation**

Primary CD4 T cells were isolated by negative selection (Dynal beads system; Thermo Fisher Scientific) from peripheral lymph node macerates (inguinal, axillary, brachial, and cervical). CD4+ cell purity was verified by PCR using pEF.V5.IRF2BP2-A [4] as template and the following primers: 5’ Bgl II and Sal I restriction sites. The cloned Irf2bp2 gene was AMF biocistronic mRNA. The vectors pRV-IRF2BP2 and pRV-GFP (control empty vector) were a kind gift from Dr. Anjana Rao from LIAI (San Diego, CA, USA). MSCV-IRES-DsRed [16] was a kind gift from Dr. Kevin Bunting from Case Western Reserve University (Cleveland, OH, USA). MSCV-IRF2BP2 was constructed by subcloning the murine Ifn2p gene from pRV-IRF2BP2 by digestion with the BamHI and Xhol restriction enzymes, followed by T4 ligase binding (all from New England Biolabs, Ipswich, MA, USA). The cloned Ifn2p gene has a C-terminal c-Myc tag in both vectors. The vectors pLIRE5EGFP [17] and pLIRE5-hIRF2BP2-A were used for PBMC transfections. For pLIRE5-hIRF2BP2-A construction, a fragment from hIRF2BP2-A was amplified by PCR using pEF.V5.IRF2BP2-A [4] as template and the following primers: 5’ TGA CTG CAG GCA GGT TGT TGG GTT TCG AGG 3’ and 5’ ACC GCT CGA GTC ACG AGT CTC TCT TTT TCA CTT TCA CA 3’.

Next, this fragment was cleaved by PchI and Xhol and cloned into pcDNA-hIRF2BP2-B [5] between the same restriction sites, generating pcDNA-hIRF2BP2-A (5’ restriction sites). Finally, hIRF2BP2-A cDNA was extracted from pcDNA-hIRF2BP2-A by the cleavage of BamHI and Xhol and cloned into pLIRE5-EGFP between flgRl and Sol restriction sites.
and 100 μg/ml anti-IFN-γ (XMG 1.2) antibodies. After activation, the cells were expanded on a daily basis by adding the same volume of fresh media alone (for Th17 cell cultures) or fresh media supplemented with 20 U/ml recombinant IL-2 for the other cultures. All of the recombinant cytokines were from PeproTech (Rocky Hill, NJ, USA).

Recombinant retroviral vector production and primary CD4 T cell transduction

The Phoenix ecotropic packaging cell line (Phoenix-EO) was transiently transfected with the retroviral vector plasmids by calcium phosphate precipitation for 24 h. The virus-containing cell supernatant was collected 48 h after transfection and concentrated by overnight centrifugation at 6000 rcf at 4°C. After concentration, the recombinant retroviruses were resuspended in fresh media, supplemented with 8 μg/ml Polybrene (Fluka Chemie, Buchs, Switzerland), and used immediately for spin infection of activated CD4 T primary lymphocytes at 900 rcf for 1:40 h at room temperature. Before the addition of virus, conditioned lymphocyte media were collected and reserved at 37°C. After spin infection, the conditioned media were returned to the cells and incubated for an additional 24 h before washing for removal of noninfecting viral particles. This time point was considered d 0 of the retroviral transduction protocol. The efficiency of transduction was assessed by GFP analysis by flow cytometry. For the transduction protocol, primary CD4 T cells were obtained and activated as described above. After transduction, the cells were expanded on a daily basis by adding the same volume of fresh media, supplemented with 20 U/ml recombinant IL-2 (PeproTech). For all experiments, the efficiency of transduction was 60–70% for the pRV-IRF2BP2 group and 70–80% for the empty vector controls.

For the cell-transfer experiments, CD90.1 CD4 T cells were transduced with pRV-IRF2BP2 or empty vector using the same protocol described above. At d 2 of the transduction protocol, the cells were put on a 1:1 ratio of GFP+/GFP− immediately before transfer by adding CD90.1 GFP− mock control cells that were subjected to the same treatment as the transduced cells without the addition of the virus for the spin infection. The 1:1 GFP+/GFP− ratio was confirmed by flow cytometry immediately before transfer to the recipients.

Flow cytometry and cytokine analysis

Flow cytometry assays were performed with a Becton Dickinson FACScan and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). All antibodies were purchased from eBioscience (San Diego, CA, USA), with the exception of anti-CD4 and anti-IFN-γ, which were purchased from BD Pharmingen. Surface staining was performed according to the manufacturer’s instructions. Foxp3 labeling was performed after the surface staining using the Foxp3 staining buffer set from eBioscience. For cytokine analysis, the cells were stimulated with 10 nM PMA and 1 μM ionomycin (when different doses were used, they were indicated in the legends) for 6 h at 37°C. A total of 10 μg/ml Brefeldin A was added during the final 2 h of stimulation. At the end of the stimulation period, the cells were fixed with 2% paraformaldehyde for at least 16 h. After fixation, the cells were permeabilized in perm buffer (PBS with 1% BSA and 0.5% saponin) for 5 min at room temperature for intracellular staining, which was accomplished by incubating with the antibodies in perm buffer for 40 min. The antibodies were added at the final concentration suggested by the manufacturers.

Proliferation assays

For the [3H]-thymidine incorporation assays, transduced cells (d 2 of the transduction protocol) were stimulated with 1 μg/ml anti-CD3 and anti-CD28 for 24 h. During the last 8 h of stimulation, the cells were pulsed with 1 μCi [3H]-thymidine. At the end of the pulse, the cells were lysed, and the DNA content was recovered on filter paper using a cell harvester to further analysis of [3H] incorporation in a β-counter. For the CFSE proliferation assays, the Cell Trace kit (Thermo Fisher Scientific) was used to label newly purified CD4 T cells (2 μM CFSE using the manufacturer’s suggested protocol). Labeled cells were stimulated with 1 μg/ml anti-CD3 and anti-CD28 for 24 h. After this period, MSCV retroviral vectors were added for spin infection, performed as described above for the pRV vectors. After 24 h, the CFSE dilution was measured on DsRed-positive cells on a daily basis by flow cytometry.

Proliferation suppression assay

MSCV-IRF2BP2- and MSCV-DsRed-transduced iTregs were obtained by stimulating CD4 T cells in iTreg-differentiating conditions and transducing these cultures 48 h later with MSCV vectors using the same transduction protocol described earlier in this section. At d 2 of transduction protocol, DsRed+ cells were sorted using a S3 Cell Sorter (Bio-Rad Laboratories, Hercules, CA, USA) and cultured for an additional 24 h. After that, just-purified CD4 T cells were labeled with CFSE, as described in the previous issue, activated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml), and cocultured with MSCV-IRF2BP2- or MSCV-DsRed-transduced iTregs at the indicated proportions for 72 h. At this time point, the proliferation rate of the cocultures was analyzed by flow cytometry by comparing percent of proliferating CFSE- labeled CD4 T cells that did not receive iTreg along with activation. For that, the amount of proliferation obtained in the mock culture was considered as 100%, and the percentages of proliferating cells of each coculture were converted into a percentage of this amount.

Cell death analysis

Aptosis was induced in pRV-IRF2BP2 and empty vector control transduced cultures by irradiation with 10 Gy X-rays at d 2 of transduction protocol. Analyses were made 24 h after radiation by staining cells with Annexin-V-allophycocyanin (eBioscience) and 7-AAD (BD Biosciences, San Jose, CA, USA), according to manufacturers’ suggested protocols. DNA fragmentation analyses were made by staining of nuclear content with PI (75 μM) in the presence of Nonidet P-40 (0.1%). After staining, the nuclear content of DNA fragmentation (Sub-G0) was analyzed immediately by flow cytometry.

PBMC transfection and analysis

Human PBMCs were obtained from healthy donors in the blood bank of INCA after written, informed consents. The experiments were approved and conducted according to the Human Ethics Committee from INCA (CET Process No. 158/10). The leukocytes were harvested of white blood cell reduction filters, and PBMCs were isolated by Ficol density gradient centrifugation. hIRF2BP2-A overexpression in these cells was obtained by electroporation using MAXMA Nucleofector Ibb (Lonza, Basel Switzerland), according to a previously described protocol using the homemade buffer set ISM [18]. Cells were stimulated with PMA (10 nM) and ionomycin (1 μM), 24 h after electroporation for an additional 24 h. Next, CD25 surface expression was analyzed by flow cytometry.

Gene-expression analysis

Gene expression was assessed by total RNA extraction using TRIzol (Thermo Fisher Scientific), followed by cDNA synthesis via RT-PCR using Oligo(dT) primers and the ImProm-II RT (Promega, Madison, WI, USA). Relative quantification of hIrf2bp2 transcripts was performed by real-time PCR using the Gene Expression Assay kit (Thermo Fisher Scientific). HPRT was used as the housekeeping gene for mass normalization. All procedures were performed according to the manufacturer’s suggested protocols.

Western blotting

Total protein from transduced primary CD4 T cells was obtained from cell lysates in buffer containing 40 mM Tris (pH 7.5), 60 mM sodium pyrophosphate, 10 mM EDTA, and 5% SDS, followed by incubation at 100°C for 15 min. Total cell lysates were resolved by SDS-PAGE, and the separated proteins were transferred onto a nitrocellulose membrane. The antibodies used for protein identification were as follows: anti-GAPDH (6C5) and anti-IRF2BP2 (L-13; both from Santa Cruz Biotechnology, Dallas, TX, USA); anti-c-Myc (9E10; Thermo Fisher Scientific); and anti-STAT5 (polyclonal) and anti-phosphorylated STAT5 (C11C5; both from Cell Signaling Technology, Danvers, MA, USA). Secondary peroxidase-conjugated anti-mouse or anti-rabbit were used to detect antibodies.
anti-rabbit antibodies were obtained from GE Healthcare (Little Chalfont, United Kingdom). Immunodetection was performed with the ECL Western Blotting Detection Kit (GE Healthcare).

**Statistical analysis**

Statistical analysis of values from the control and treated groups was performed using an unpaired Student’s *t* test for single comparisons or an ANOVA, followed by the Student-Newman-Keuls for multiple comparisons using the program GraphPad Prism (GraphPad Software, San Diego, CA, USA). *P ≤ 0.05* were considered statistically significant.

**RESULTS**

*Irf2bp2* mRNA levels are down-regulated in CD4 T cells upon activation

First, we characterized *Irf2bp2* gene expression in CD4 T cells upon activation. For that, CD4 T cells were purified from peripheral lymph nodes and activated in vitro with anti-CD3 and anti-CD28 for different time periods; *Irf2bp2* mRNA levels were down-regulated from 3 to 48 h upon activation (Fig. 1A). The fact that *Irf2bp2* has been described as a transcriptional repressor in several biologic contexts ([4–9]) and that *Irf2bp2* gene expression is reduced after activation in CD4 T cells suggests that it may also play a role as a repressor in these cells. To evaluate the possible roles for IRF2BP2 in CD4 T cell biology, we overexpressed this protein in activated primary CD4 T cells using a retroviral integrative transduction protocol (Fig. 1B). On d 2 after retroviral transduction, *Irf2bp2* mRNA levels were assessed by quantitative RT-PCR and showed a 7.5-fold induction compared with the controls (Fig. 1C). Exogenous overexpressed protein was detected by Western blotting by staining with an IRF2BP2-specific antibody and an antibody against the c-Myc tag (Fig. 1D). IRF2BP2 endogenous protein levels were not detectable in naive or activated cells (data not shown).

![Graph 1](image1.png)

**Figure 1. Irf2bp2 regulation and overexpression on CD4 T cells.** (A) Relative quantification of *Irf2bp2* gene expression by real-time PCR on CD4 T cells purified from peripheral lymph nodes from 8-wk-old C57BL/6 mice. The indicated time point refers to the cell activation time in vitro with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) compared with the lower mean obtained for naive expression. (B) Retroviral transduction protocol scheme. (C) *Irf2bp2* mRNA fold induction obtained with the pRV transduction protocol on d 2. (D) IRF2BP2 protein overexpression obtained with the pRV transduction protocol on d 2. Bar graphs represent the means of at least 3 independent experiments ± se. For relative quantification of PCRs, the mass of cDNA used for the reaction was normalized using the HPRT housekeeping gene. *P < 0.05; **P < 0.001* compared with the naive (A) or empty vector (C) group.

IRF2BP2 represses primary CD4 T cell proliferation and sensitizes cells to death induced by apoptotic stimulation in vitro

Upon activation, T cells quickly undergo clonal expansion through intense proliferation. Rapid down-modulation of a transcriptional repressor upon TCR activation may be involved in this early process. To evaluate a possible role for IRF2BP2 in early CD4 T cell proliferation, we expanded our transduced cell cultures by adding recombinant IL-2 (20 U/ml) with fresh culture medium on a daily basis for up to 6 d and followed the GFP expression in these cultures daily. Interestingly, the percentage of GFP expression was very stable on all days analyzed for the empty vector-transduced cells, whereas in the pRV-IRF2BP2-transduced cell cultures, the percentage of GFP-negative cells diminished along with their expansion (Fig. 2A). This observation suggested that IRF2BP2-overexpressing cells displayed a disadvantage in expanding compared with nontransduced GFP-negative cells in the same culture. This disadvantage might be a result of 2 main factors: impaired proliferation capacity of cells overexpressing IRF2BP2 and/or an enhancement in cell death promoted by IRF2BP2 overexpression.

To address these possibilities, we used cultures on d 2 of the cell expansion after retroviral transduction, in which the GFP dilution phenomenon was already observed, but there was still a majority of GFP-positive cells in both cultures. First, we evaluated cell proliferation in transduced cultures using [³H]-thymidine incorporation assays. The results showed a diminished proliferation rate for pRV-IRF2BP2 cell cultures compared with empty vector-transduced controls (Fig. 2B). We also evaluated the role of IRF2BP2 in CD4 T cell proliferation, specifically on transduced cells, using CFSE labeling by overexpressing this protein with the MSCV-DsRed vector. In these assays, transduced DsRed+ cells overexpressing IRF2BP2 displayed a lower proliferation rate compared with control empty vector-transduced cells starting on d 2 of the transduction protocol (Fig. 2C). Moreover, a reduction in the percentage of DsRed+ cells was also observed during MSCV-IRF2BP2-transduced cell culture expansion that was similar to our observations for GFP in the pRV-IRF2BP2 cultures (data not shown).

Next, we evaluated whether cell death might also be involved in the GFP-positive cell loss observed during IRF2BP2-transduced culture expansion. To do this, we followed the content of DNA fragmentation in these cultures on a daily basis after transduction. DNA fragmentation was comparable between pRV-IRF2BP2 and empty vector at d 0 and 1 of the transduction protocol, which probably is a consequence of the basal level of cell death caused by the viral transduction (Fig. 3A). At d 2 and 3,
the levels of DNA fragmentation were insignificant, suggesting that the dilution of GFP+ cells observed in pRV-IRF2BP2-transduced cultures is mainly a result of diminished proliferation (Fig. 3A). To assess whether IRF2BP2 overexpression influences CD4 T cell death in these cultures, we irradiated pRV-IRF2BP2- and empty vector-transduced cell cultures on d 2 of the transduction protocol with 10 Gy X-rays and evaluated the levels of apoptosis induced in these cultures 24 h later. In these assays, pRV-IRF2BP2-irradiated cell cultures displayed an increase in cell death induced by irradiation compared with the empty vector control cultures (Fig. 3B). These data showed that IRF2BP2 overexpression has a deleterious role in cell survival in...
the presence of an apoptotic stimulus by making these cells more prone to die.

**IRF2BP2-overexpressing CD4 T cells display impaired expression of the high-affinity α-chain of IL-2R**

Previous data from our group demonstrated a role for hIRF2BP2 in repressing IL-2 production in lymphocytes [5]. To assess whether murine IRF2BP2 could also repress IL-2 production, we stimulated pRV-IRF2BP2-transduced cells with decreasing doses of PMA and ionomycin on d 2 of transduction protocol and evaluated IL-2 production. In these experiments, no differences were observed between pRV-IRF2BP2- and empty vector-transduced cells in terms of IL-2 production for all of the stimulus doses tested (Fig. 4A).

As IL-2 is the main cytokine produced and consumed by T cells during clonal expansion, we also investigated the expression of its high-affinity receptor α-chain (CD25). CD25 is usually expressed on activated T cells undergoing clonal expansion and is a classic activation marker for T cells. Interestingly, IRF2BP2 overexpression in our transduced CD4 T lymphocytes led to a strong down-modulation of CD25 expression on the cell surface (Fig. 4B and C). Moreover, STAT5 phosphorylation was diminished at the pRV-IRF2BP2-transduced cell cultures, suggesting that the IL-2 signaling pathway may still be involved in the impaired proliferation observed in these cells (Fig. 4D).

Then, we addressed whether the hIRF2BP2 protein has the same effect on CD25 regulation by overexpressing hIRF2BP2-A in PBMCs. In these analyses, CD25 expression induced upon activation with PMA plus ionomycin was reduced in lymphocytes overexpressing hIRF2BP2-A compared with the empty vector-transfected cells (Fig. 4E). These data demonstrated a conserved role for IRF2BP2 and the murine IRF2BP2 in the regulation of CD25 expression.

We also evaluated the expression of the CD62L, CD44, and CD69 activation markers on the surface of murine-transduced CD4 T lymphocytes to clarify whether CD25 down-regulation mediated by IRF2BP2 overexpression was related to a general deactivating status induced by this protein or a specific regulation of CD25 protein expression. There was no change in the expression pattern of CD44 and CD62L, but CD69 was also down-regulated by IRF2BP2 overexpression (Supplemental Fig. 1). This result suggests that CD25 down-modulation is not a result of a general impaired activation of the CD4 T cell, induced when IRF2BP2 is overexpressed, but it also shows that IRF2BP2 ectopic expression can down-regulate more than one protein related to CD4 T cell activation.

**Figure 4. IL-2 and IL-2R α-chain expression on transduced CD4 T cells overexpressing IRF2BP2.**

(A) Transduced CD4 T cells were stimulated with decreasing doses of PMA and ionomycin, as indicated in the figure on d 2 of the transduction protocol. IL-2 production by transduced cells was assessed by flow cytometry. Cells are gated as GFP+ for analysis, and the percentages shown refer to the gates in which they are displayed. (B) Transduced CD4 T cells were analyzed for surface expression of CD25 by flow cytometry on d 2 of the retroviral transduction protocol. (C) Percentage of CD25+transduced CD4 T cells on d 2 of the transduction protocol. Cells were gated as GFP+ for analysis; the data shown represent the means of 3 independent experiments ± se. *P < 0.05 compared with the empty vector group. (D) Western blotting analysis for total and phosphorylated STAT5 (p-STAT5) of pRV-IRF2BP2- and empty vector-transduced CD4 T cell cultures on d 2 of transduction protocol. (E) CD25 analyses of transfected PBMC from 2 independent donors overexpressing hIRF2BP2-A compared with control empty vector-transfected cells. The cells were stimulated with PMA (10 nM) and ionomycin (1 μM) 24 h post-transfection and analyzed 24 h after stimulus. Lymphocytes were selected by side- and forward-scatter, and transfected cells were gated as GFP+ for analysis.
IRF2BP2 overexpression impairs CD4 T cell expansion in vivo

To determine whether the IRF2BP2-induced expansion impairment was a physiologic event also observed in vivo, we transferred IRF2BP2-overexpressing CD4 T cells to sublethally irradiated C57/BL6 mice and followed their expansion in vivo. For that, CD90.1, GFP+ CD4 T cells transduced with pRV-IRF2BP2 or empty vector were mixed with GFP- mock control cells on a 1:1 ratio and then transferred to sublethally irradiated recipients, as depicted in Fig. 5A. Next, we followed the expansion of the transferred cells in vivo by evaluating the proportion of pRV-transduced (GFP+) and not-transduced (GFP-) CD90.1 CD4 T cells in blood samples collected 7 and 15 d after transfer (Fig. 5B) and in the spleens and peripheral lymph nodes 30 after transfer (Fig. 5C–E).

In all the analyses, for the animals that received the empty vector-transduced lymphocytes, the proportion of GFP+ and GFP- cells remained close to 1:1, showing that the transduction protocol did not influence the capacity of these cells to expand in vivo (Fig. 5B–E). In contrast, in the animals that received pRV-IRF2BP2-transduced lymphocytes, GFP+ cells expanded less than GFP- -transferred cells, showing that IRF2BP2 overexpression promoted an impairment of CD4 T cell expansion in vivo (Fig. 5B–E). Moreover, the GFP+ cells that remained in the animals transferred with pRV-IRF2BP2-transduced cells displayed a lower GFP MFI compared with the transferred empty vector-transduced cells on d 30 (MFI = 415.42 ± 13.39 s.d and 328.15 ± 16.82 s.d for empty vector-transduced cells and 222.91 ± 27.84 s.d and 227.31 ± 18.64 s.d for pRV-IRF2BP2-transduced cells at lymph nodes and spleens, respectively), suggesting that the cells that were maintained in the periphery of the recipients for long periods were the ones expressing lower levels of exogenous IRF2BP2. Taken together, these data reveal a role for IRF2BP2 in restraining CD4 T cell expansion in vivo, possibly caused by reduced cell proliferation and/or survival.

Irf2bp2 expression in differentiated Th cell cultures

In addition to proliferation, another important event in the course of the CD4 T cell response is differentiation. To evaluate the possible roles for IRF2BP2 in this process, we differentiated CD4 T cells into Th1, Th2, Th17, and iTreg cultures in vitro (Supplemental Fig. 2A). The differentiation of the cultures was confirmed by their cytokine profiles (Supplemental Fig. 2B). First, we characterized Irf2bp2 gene expression in terminally differentiated CD4 T cell cultures (Fig. 6A). No significant differences were observed by comparing Th1 with Th2 cultures; nevertheless, there was a significant induction in Th17 cultures when compared with iTregs (Fig. 6A). IRF2BP2 endogenous protein expression was not detectable in any of the differentiated cell cultures (data not shown). We also evaluated Irf2bp2 gene expression in differentiated Th cell cultures restimulated with anti-CD3 and anti-CD28. Unlike our observations for naive T cells (Fig. 1A), activation of Th2 and Th17 did not lead to modulation of Irf2bp2 gene expression (Supplemental Fig. 2C). On the other hand, Th1 cultures displayed Irf2bp2 down-modulation upon restimulation, whereas iTregs showed an increase shortly after restimulation, followed by rapid down-regulation reaching back to normal levels at 48 h (Supplemental Fig. 2C).

Figure 5. Transduced CD4 T cells impaired expansion in vivo. (A) Scheme of the transduced CD4 T cell transfer protocol. In vivo expansion of transduced cells was evaluated by transferring CD90.1 CD4 T cells mixed at a 1:1 ratio of transduced (GFP+) to mock control (GFP-) cells into sublethally irradiated recipients, followed by flow cytometry analysis of the GFP ratio in transferred cells at different time points after transfer. (B) Blood GFP analysis on d 7 and 15 after transfer. (C) Dot plots of representative spleens and peripheral lymph node analyses on d 30 after transfer. (D) Spleen and (E) peripheral lymph node GFP analyses on d 30. For all of the GFP analyses, the cells were gated as CD90.1+. Bar graphs represent the means of results obtained for the analysis of recipients (n = 5/group) ± s.e. *P < 0.0001 comparing the percentages of either GFP- or GFP+ cells of each organ and time point group with its respective empty vector control group.

www.jleukbio.org  Volume 100, November 2016  Journal of Leukocyte Biology 7
To assess whether any of the differentiated patterns would be favored by IRF2BP2 overexpression, we expanded our transduced cell cultures in default conditions for longer periods and verified their cytokine profiles. However, regardless of IRF2BP2 overexpression, the cells differentiated preferentially toward the Th1 profile producing mainly IFN-γ in all circumstances under our transduced culture conditions (data not shown).

CD25 is important for the expansion of Th1 cells (which is our default culture), and its repressor Ifh2bp2 is down-modulated upon activation at default conditions (Fig. 1A). Based on these observations, we next speculated that in the presence of Th17-driving cytokines, Ifh2bp2 down-modulation would not be observed upon activation once the IL-2 signaling pathway is inhibited by the Th17 differentiation program [19, 20], and CD25 is very important in this pathway [21]. However, down-modulation of Ifh2bp2 gene expression was observed in CD4 T cells activated in the presence of IL-6 and IL-1β, which are related to Th17 but not Th1, Th2, or iTreg differentiation, in a similar way observed for the default conditions (Fig. 6B).

To investigate whether IRF2BP2 had a role in CD4 T cell effector functions in terms of cytokine response, we analyzed the “signature cytokine” production in the effector profiles of terminally differentiated transduced cell cultures. For that, Th1, Th2, or Th17 cell cultures on d 6 of the differentiation protocol (Supplemental Fig. 2A) were restimulated with anti-CD3 and anti-CD28 for 24 h and then transduced with pRV vectors by spin infection. After 48 h of transduction, cytokine production was assessed by PMA and ionomycin stimulation, followed by intracellular staining. In these experiments, we did not observe any differences in the production of IFN-γ or IL-17 in the Th1 and Th17 cultures, respectively (Fig. 6C). In the Th2 cultures, we obtained a very discreet yet reproducible reduction in IL-4-producing cells, which was in agreement with previous observations that showed a role for hIRF2BP2 as a repressor of IL-4 [5].

**Diminished LAP and CD25 expression induced by IRF2BP2 overexpression in iTreg cultures**

As the IL-2 signaling pathway is crucial for iTreg differentiation [19, 20], and CD25 is very important in this pathway [21], we induced IRF2BP2 overexpression during the course of iTreg differentiation by transducing CD4 T cells activated under iTreg-driving conditions with pRV vectors. For these experiments, the cells were activated in the presence of cytokines and blocking antibodies used for iTreg differentiation and then transduced 48 h later. Three days after cell transduction, iTreg generation was assessed by Foxp3 expression. We did not observe significant differences in the percent of Foxp3+ cells generated in pRV-IRF2BP2- or empty vector-transduced cell cultures (Fig. 7A). Additionally, there was no change in the levels of Foxp3 expression (Fig. 7B). Interestingly, CD25 expression, which is usually very high on Treg, was also down-modulated in Foxp3+ cells by IRF2BP2 overexpression (Fig. 7B), suggesting a very strong role for IRF2BP2 in the down-regulation of CD25.

To evaluate further the iTreg differentiation, we analyzed the expression of the LAP (also known as pro-TGF-β) on the surface of pRV-IRF2BP2 or empty vector-transduced Foxp3+ cells. In fact, we observed a diminished expression of LAP on
DISCUSSION

The signaling pathways involved in CD4 T cell activation and differentiation are complex and depend on diverse signals received concomitantly by these cells at the moment of activation given by TCR signaling, costimulatory molecules, and the cytokine milieu [23]. Diverse regulatory mechanisms have been reported to be important in the regulation of this process; however, the entire scenario concerning CD4 T cell activation and differentiation is not fully understood.

Furthermore, naïve and antigen-experienced T cells have different kinetics of response and different stimulation requirements for activation. Indeed, these cells have been reported to activate different signaling pathways in response to TCR stimulation with anti-CD3 antibodies [24]. Herein, we describe a role for the transcriptional repressor IRF2BP2 as a restrainer of the induction of early activation markers upon TCR activation and of cell proliferation during CD4 T lymphocyte clonal expansion.

Interestingly, we observed a down-modulation of Irf2bp2 expression upon naïve CD4 T cell activation (Fig. 1A) but not in most terminally differentiated CD4 T cells subjected to the same stimuli (Supplemental Fig. 2C), suggesting that IRF2BP2 most probably plays its main role during the early stages of naïve activation signaling pathways in CD4 T cells.

IRF2BP2 ectopic expression on recently activated CD4 T cells leads to a down-modulation of the CD25 and CD69 activation markers (Fig. 4B and C and Supplemental Fig. 1). CD69 is one of the first proteins to be up-regulated on the T cell surface after activation and is a classic T cell activation marker [25]. However, very little is known about the function of this protein in T cell biology. Actually, apart from its very early induction upon activation, no significant differences have been observed in early proliferation or cytokine response in CD69−/− CD4 T cells [26]. CD25 expression is also rapidly induced on the T cell surface upon activation, which enhances the binding affinity of the IL-2 cytokine for its receptor by ~10- to 100-fold, thereby promoting an important increase in signaling of this pathway [21, 27]. In the present work, the diminished CD25 expression induced by ectopic expression of IRF2BP2 was correlated with decreased STAT5 phosphorylation (Fig. 4B–D), suggesting an impairment in the IL-2 signaling pathway that probably contributes to the reduced proliferation rate observed in these cells during clonal expansion (Fig. 2B and C). Furthermore, the hIrf2bp2-A was also capable of regulating CD25 expression in lymphocytes upon activation, suggesting a conserved role for this protein.

Previously, we have shown that hIrf2bp2 represses the NFAT1-dependent transactivation of IL-2 promoter and IL-2 production in CD4 T cells [5]. However, with the present work, we did not see any differences in IL-2 production in cells overexpressing murine Irf2bp2 when compared with empty (Fig. 4A). The observed differences might be explained by the fact that the cited work used the human protein isoform B, whereas in the present work, we used the murine protein. Of note, murine cells present only 1 Irf2bp2 isoform, which

possible roles of IRF2BP2 in Treg generation, stability, and function in vivo.
exhibits a structural similarity to human isoform A. These data might be related to differential properties of IRF2BP2 protein isoforms or a nonconserved role for mice and human proteins in some specific gene regulation. Additional experiments are needed to further clarify this issue. However, it is important to note that in both systems, the IL-2 signaling was compromised, demonstrating that the IRF2BP2 protein plays a key role in the IL-2 pathway. Recently, a role for IRF2BP2 has been demonstrated in erythroid differentiation by corepressing the expression of erythroid lineage-specific genes at a primed state that allow their rapid activation when required. In the same work, it was demonstrated that IRF2BP2 is an important player of a transcriptional repressor complex that plays a major role in erythroid development by holding the expression of the erythroid lineage-specific gene program until differentiation is induced [9]. Collectively, our data suggest a very similar role for IRF2BP2 in the gene regulation of CD4 T cells in the context of first activation.

In addition to the reduced proliferation and survival of IRF2BP2-overexpressing cells in vitro (Figs. 2B and C and 3B), these cells displayed an impaired expansion capacity in vivo (Fig. 5B-E). For these analyses, IRF2BP2-overexpressing cells were transferred into sublethally irradiated recipients and allowed to expand together with nontransduced control cells. In these assays, for the animals that received IRF2BP2-overexpressing lymphocytes, transduced cells expanded less compared with nontransduced cotransferred cells, showing that IRF2BP2 ectopic expression resulted in a disadvantage in CD4 T cell expansion and/or survival in vivo. Recently, a gain-of-function mutation in IRF2BP2 was associated with a genetic familial immunodeficiency disorder, where family members carrying the mutation displayed an improper antibody response showing a role for this protein in the immune system [15].

In the differentiation context, we could not find a correlation between IRF2BP2 overexpression and a bias for differentiation toward one of the studied profiles (data not shown). However, this question is not fully addressed, as there is an intrinsic limitation in our model, as the time taken to induce ectopic expression by retroviral transduction is probably too long to modulate the initial signaling pathways that determine differentiation upon activation. Interestingly, Ifh2bp2 gene expression was found to be higher in terminally differentiated Th17 than in iTreg cultures (Fig. 6A), suggesting a possible role for IRF2BP2 in the balance between these 2 profiles. Actually, IRF2BP2 was identified as one of the genes with altered expression in a screening study performed to identify novel factors involved in the Th17 differentiation program for a patent petition using human CD4 T cells. In this study, Th17-differentiated cell cultures expressed 2.1-fold more IRF2BP2 compared with default control cultures [28]. Thus, we might suppose that a reduction in CD25 expression at an early stage of CD4 T cell activation would favor Th17 differentiation once the IL-2 signaling pathway is inhibitory of the Th17 differentiation program shifting cells toward iTreg differentiation through STAT5 signaling [19]. Nevertheless, in the present work, we did not observe any changes in the Ifh2bp2 regulation upon activation in the presence of distinct Th17 driving inflammatory cytokines (Fig. 6B).

As a counterpart of the Th17 inflammatory profile, Treg are deeply dependent on IL-2 signaling and express high levels of CD25. The importance of this pathway for in vivo tolerance could be inferred by the observation that IL-2- and CD25-deficient animals develop an intense peripheral lymphoproliferation associated with autoimmune disorders [29, 30].

One of the mechanisms by which the IL-2/STAT5 signaling pathway collaborates for tolerance maintenance is by participating in iTreg generation in the periphery [19]. We also investigated a possible role for IRF2BP2 in iTreg generation by transducing CD4 T cells activated under iTreg driving conditions with pRV vectors (Fig. 7A). However, the percentages of Foxp3+ iTreg generated in pRV-IRF2BP2- or empty vector-transduced cells were comparable (Fig. 7A). We also attempted to induce cells to overexpress IRF2BP2 before initiating the differentiation conditions by addition of recombinant cytokines and blocking antibodies; nevertheless, cells subjected to the retroviral transduction protocol and then induced to differentiate into iTreg failed to differentiate. No Foxp3+ cells were generated in cultures transduced with pRV-IRF2BP2 or the empty vector control, suggesting that the time point after retroviral transduction is too late to enter with the differentiation conditions (data not shown).

Although it is well established that the IL-2 signaling pathway plays a role in tolerance through Treg homeostasis, it is not clear whether this signaling plays a role in the suppressor function of Treg in the periphery [21]. Actually, it has been suggested that IL-2 signaling in Treg is more important for the maintenance of these populations in the periphery than to its suppressive function per se [31]. To exploit a possible role for IRF2BP2 in iTreg function, we first analyzed LAP expression on our IRF2BP2-overexpressing iTreg (Fig. 7B). TGF-β complexed to LAP is expressed on activated Treg surfaces and plays an important role, not only in the suppression of the proliferation of activated T cells but also in the generation of de novo Foxp3+ Treg from naive precursors [32]. Interestingly, IRF2BP2 overexpression in iTreg led to a reduction in LAP expression on the cell surface (Fig. 7B). However, the IRF2BP2 overexpression in iTreg did not affect the Treg-suppressive function (Fig. 7C). Nevertheless, more experiments are necessary to evaluate the possible roles for IRF2BP2 on Treg homeostasis in vivo.

Taken together, our data suggest a role for IRF2BP2 in the control of different CD4 T cell activation parameters (i.e., the expression of CD25 and CD69), in addition to proliferation. In this context, the diminished expression of Ifh2bp2 observed upon initial activation suggests that this protein could be a repressor that controls cell activation in naïve CD4 T lymphocytes. Moreover, we have shown a role for IRF2BP2 in CD4 T cell expansion in vivo, suggesting a function for this protein in the control of lymphocyte expansion and/or survival with possible long-term physiologic implications related to its modulation.

**AUTHORSHIP**

C.S. performed most of the experiments, analyzed the data, contributed to experimental design, and wrote the manuscript. D.V.F. participated in the discussions and helped with the primary CD4 T cell viral transduction and vector construction. S.C.H. and P.S.A-S. participated in the discussions and helped
Acknowledgements

This work was supported by grants to J.P.B.V. from the National Council for Scientific and Technological Development (CNPq: 307296/2011-3 and 476314/2012-7), Filho de Amparo’s Pesquisa Institute of Coordination for the Improvement of Higher Education Personnel (CAPES) fellowship, and M.S.C. was supported by a Coordination for the Improvement of Higher Education Personnel (CAPES) fellowship, and M.S.C. was supported by a Brazilian Ministry of Health fellowship. B6a mice were a kind gift from Dr. Rita Fuchs from UFF (Rio de Janeiro, Brazil); pEF.V5. IRF2BP2A was a kind gift from Dr. Steve Goodbourn (University of London, United Kingdom); and pR vectors were a kind gift from Dr. Anjana Rao (LJAI, San Diego, CA, USA). The authors thank Dr. Miriam B. F. Werneck for critical reading of this manuscript.

Disclosures

The authors declare no conflicts of interest.

References

5. IRF2BP2A was a kind gift from Dr. Steve Goodbourn (University of London, United Kingdom); and pR vectors were a kind gift from Dr. Anjana Rao (LJAI, San Diego, CA, USA). The authors thank Dr. Miriam B. F. Werneck for critical reading of this manuscript.


Key words: lymphocyte proliferation - survival - CD25.

www.jleukbio.org

Volume 100, November 2016 11

Journal of Leukocyte Biology