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Brief Conclusive Report

Characterization of Schlafen-3 expression in effector and regulatory T cells

Thomas Condamine,*,[1] *Jean-Benoît Le Luduec*,*,[1,2] *Elise Chiffoleau*,* *Gaëlle Bériou*,*,[3] *Cédric Louvet*,*,[4] *Michele Heslan*,* *Gaëlle Tilly*,* *and Maria-Cristina Cuturi*,*[5]

*INSERM, U643, Nantes, France; CHU Nantes, Institut de Transplantation et de Recherche en Transplantation, ITERT, Nantes, France; and Université de Nantes, Faculté de Médecine, Nantes, France

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ABSTRACT

Members of the Slfn protein family have been implicated in the regulation of cell growth, hematopoietic cell differentiation, and T cell development/differentiation in the thymus. Ten members of this family have been described in the mouse, and they have been divided into three subgroups based on the overall sequence homology and the size of the encoded proteins. We have identified Slfn3, a member of Subgroup II, as an overexpressed gene in CD4$^+$ CD25$^+$ T cells in the periphery. Interestingly, we demonstrate that upon activation and proliferation, Slfn3 mRNA is down-regulated in CD4$^+$ CD25$^+$ Tregs and up-regulated in CD4$^+$ CD25$^-$ Teffs. Moreover, TGF-β inhibits the expression of Slfn3 in anti-CD3/CD28-activated CD4+ T cells, and the same conditions induce FoxP3 mRNA. Our results suggest that Slfn3 could have a role in T cell differentiation and activation. *J. Leukoc. Biol.* **87: 451–456; 2010.**

Introduction

Previously, we reported a model of allograft tolerance induced by a short immunosuppressive treatment that is characterized by a specific expansion of CD4$^+$ CD25$^+$ FoxP3$^+$ T cells in the spleen and the graft [1–4]. Using suppressive, subtractive hybridization, we identified Slfn3 as overexpressed gene in vitro donor-stimulated T cells from long-term tolerant animals compared with naïve animals. The Slfn family of proteins was first discovered in mice [5] and currently contains 10 members [6]. This family has been divided into three subgroups based on their overall exon homology [6]. Slfn3, along with Slfn4, Slfn6, and Slfn7, belongs to Subgroup II. Slfn3 and Slfn7 have been described to be up-regulated following T cell activation [5]. These molecules seem to play an important role in the ontogenesis of thymocytes [5, 6], and Slfn1 has been described to be involved in the control of the cell cycle through the inhibition of the induction of cyclin D1 [7, 8]. However, Zhao et al. [9] have reported conflicting data recently, suggesting that Slfn1 and -2 do not inhibit the growth of fibroblasts and myeloid cells. Slfn2 has been shown to be up-regulated in myeloid cells during differentiation or maturation, and this expression is regulated by two transcription factors, AP-1 and NF-κB [10, 11]. Little is known about the function of Slfn3, although it has been shown to have an anti-proliferative effect when ectopically expressed in fibroblasts [5].

In this study, we demonstrate that Slfn3 is overexpressed in natural CD4$^+$ CD25$^+$ Tregs compared with CD4$^+$ CD25$^-$ T cells. Interestingly, Slfn3 is up-regulated in CD4$^+$ CD25$^-$ T cells following polyclonal T cell activation but is down-regulated in CD4$^+$ CD25$^+$ T cells following anti-CD3/CD28 stimulation. Finally, we demonstrate that Slfn3 is negatively regulated by TGF-β in the CD4$^+$ T cell subpopulation. Our results suggest that Slfn3 could be a new marker of T cell activation.

Abbreviations: DC=dendritic cell, FoxP3=forkhead box P3, HPRT=hypoxanthine-guanine phosphoribosyltransferase, Slfn=Schlafen, Teff=effector T cell, Treg=regulatory T cell

MATERIALS AND METHODS

Antibodies

The following anti-rat hybridomas were obtained from the European Collection of Animal Cell Culture (Salisbury, UK) and used for cell depletion in cytofluorometric and cell sorting: OX6 (MHC class II RT1B), OX35 (CD4), OX8 (CD8α), OX42 (CD11b/c), R7/3 (TCRαβ), OX62 (integrin αE2 chain or CD103), OX33 (anti-CD45 present on B cells), OX39 (CD25, IL-2R α-chain), and CD161a (clone 3.2.3). Purified mAb were conjugated to FITC, biotin, PE, allophycocyanin (Bioatlantic, Nantes, France), or Alexa-647 (Molecular Probes, Eugene, OR, USA) as needed. The anti-CD45R (clone His 24) and anti-CD3 (clone KT3) antibodies were purchased from BD PharMingen (San Diego, CA, USA), and the hybridoma JJ319 (anti-rat CD28) was kindly provided by Dr. Thomas Hünig (University of Wurzburg, Germany). A neutralizing murine anti-human TGF-β mAb-producing hybridoma (clone 2G7) [12] was kindly provided by Dr. Cornelius J. M. Melief (Academish Ziekenhuis Leiden, The Netherlands). The human CD16 mAb (clone 3G8) was used as an isotype-matched control.

1. These authors contributed equally to this work.
2. Current address: INSERM U851, 21 Avenue Tony Garnier, 69365 Lyon, France.
3. Current address: Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.
4. Current address: Diabetes Center, Department of Medicine, University of California, San Francisco, CA 94143-0540, USA.
5. Correspondence: INSERM U643, 30 Boulevard Jean Monnet, 44093 Nantes Cedex 1, France. E-mail: Maria-Cristina.Cuturi@univ-nantes.fr

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Cell separations

DCs, B cells, NK cells, monocytes, T cells, Tregs, and Teffs have been purified as described previously [13–15].

MLR and anti-CD3 and anti-CD28 stimulation

Cells were cultured in complete RPMI 1640 (Gibco, Carlsbad, CA, USA), supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate (Gibco), 1% nonessential amino acids (Gibco), 100 U/ml penicillin (Gibco), 0.1 mg/ml streptomycin (Gibco), and 10% heat-inactivated FCS (Gibco). The cultures were incubated at 37°C in 5% CO_2. The cells were stimulated for the indicated times.

Total T cells, $CD4^+$ $CD25^+$ T cells, or $CD4^+$ $CD25^-$ T cells from naïve rats (5×10^4 cells/well) were stimulated with soluble anti-CD28 (1 ng/ml) in a final volume of 200 μl in 96-well flat-bottom plates (Nunc, Merck, Eurolab, France) that had been precoated with an anti-CD3 antibody (1 μg/ml). When indicated, rTGF-β1 (2 ng/mL; R&D Systems, Minneapolis, MN, USA), an anti-TGF-β antibody (clone 2G7; 1 μg/mL) or an antibody control (clone 3G8; 1 μg/mL) was added.

The cultures were incubated at 37°C in 5% (v/v) CO_2 and pulsed for the last 8 h with 0.5 mCi [^{3}H]TdR (Amersham, Les Ulis, France). The cells were harvested on glass fiber filters, and the [^{3}H]TdR incorporation was measured using standard scintillation procedures (Packard Institute, Meriden, CT, USA).

Real-time quantitative RT-PCR

Total RNA from tissues or cells was isolated using the TRIzol extraction kit (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed as described previously [16] using a GenAmp 7700 sequence detection system and the SYBR Green PCR Master Mix (Applied Biosystems, Applera, Courtaboeuf, France). The following oligonucleotides were used in this study: Slfn3 forward, AGCATGTTTCTCCAAGCAAGAT, Slfn3 reverse, TTTAGGAAGCGAGTCCAACTGT; HPRT forward, CCTTGGTCAAGCAGTACAGCC, HPRT reverse, TTCGCTGATGACACAAACATGA; 18S forward, AGTTCCGACCATAAACGATGC, 18S reverse, CCCTTCCGTCAATTCCTTTAA; FoxP3 forward, CCCAGGAAAGACAGCAACCTT, FoxP3 reverse, CTGCTTGGCAGTGCTTGAGAA.

HPRT or 18S rRNA was used as endogenous control genes to normalize for variations in the starting amount of RNA. The relative expression was calculated using the $2^{-\Delta\Delta\text{comparative threshold}}$ method [14, 17] and is expressed in arbitrary units.

Statistical analysis

Real-time quantitative PCR data were analyzed using the nonparametric Mann-Whitney test. P values <0.05 were considered significant.

RESULTS AND DISCUSSION

Slfn3 is expressed preferentially in $CD4^+$ $CD25^+$ Tregs

After screening the subtraction library, we identified Slfn3 as an overexpressed gene in in vitro donor-activated T splenocytes isolated from long-term-tolerant recipients compared with naïve in vitro donor-activated T splenocytes. We have demonstrated previously that $CD4^+$ $CD25^+$ $FoxP3^+$ T cells are highly expanded (in percentage and absolute numbers) in T splenocytes from tolerant recipients [4]. We hypothesized that the increased expression of Slfn3 in long-term-tolerant recipients could be related to the increase in the $CD4^+$ $CD25^+$ T cell population (Tregs). To determine whether the increased expression of Slfn3 was related to the $CD4^+$ $CD25^+$ T cell subpopulation, we then examined the expression of Slfn3 mRNA in purified $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells from naïve animals. As seen in **Figure 1A**, the expression of Slfn3 in $CD4^+$ $CD25^+$ T cells is three times greater than the expression in $CD4^+$ $CD25^-$ T cells ($P<0.05$). We also analyzed the expression of FoxP3 mRNA (a marker of Tregs [18]) in the same experiments. As shown in Figure 1B, FoxP3 is highly expressed in $CD4^+$ $CD25^+$ T cells compared with $CD25^-$ T cells. These results demonstrated that Slfn3 mRNA expression is higher in the $CD4^+$ $CD25^+$ T cell population and suggest that the increased expression of Slfn3 in T cell splenocytes from long-term-tolerant recipients could be a result of the expanded $CD4^+$ $CD25^+$ T cell subpopulation that has been described previously in the spleens of tolerant recipients [2].

Slfn3 mRNA distribution in rat tissues

To analyze the tissue distribution of Slfn3, Slfn3 mRNA expression was examined in rat tissues by real-time quantitative PCR (**Fig. 2A**) and compared with the expression of FoxP3 mRNA (a marker of Tregs [18]; Fig. 2B). Our results demonstrate that Slfn3 and FoxP3 mRNA have a similar tissue distribution. In fact, both mRNAs were highly detected in the lymph nodes, peripheral blood, bone marrow, spleen, and to a lesser extent, in the aorta and thymus. Therefore Slfn3 and FoxP3 mRNA appear to have the same overall tissue distribution. We demon-

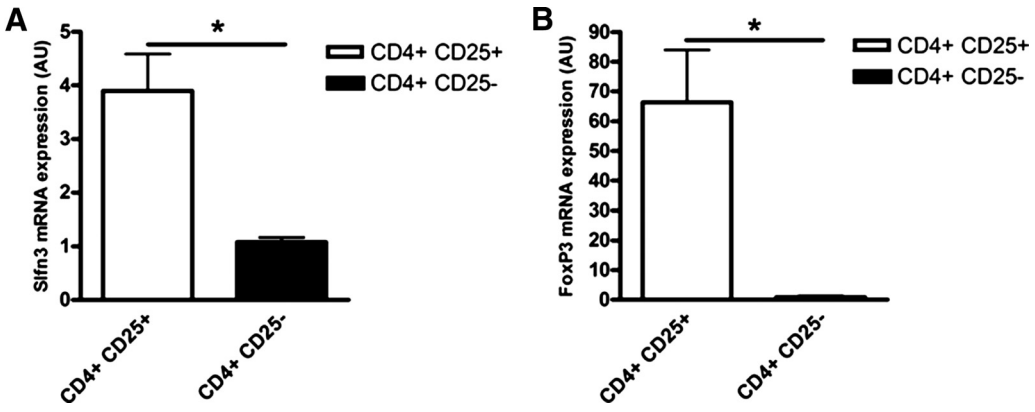


Figure 1. Slfn3 and FoxP3 mRNA expression in Tregs and Teffs. The expression of Slfn3 (A) and FoxP3 (B) mRNA in $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ $CD4^+$ T cells from naïve recipients ($CD4^+$ $CD25^+$, open bars; $CD4^+$ $CD25^-$, closed bars) was assessed using real-time quantitative PCR. Each group is expressed in arbitrary units (AU) as the mean ± SD of three independent experiments for each cell subset. The mRNA levels were normalized to HPRT expression and are expressed in arbitrary units. Significant differences are indicated (*, $P<0.05$).

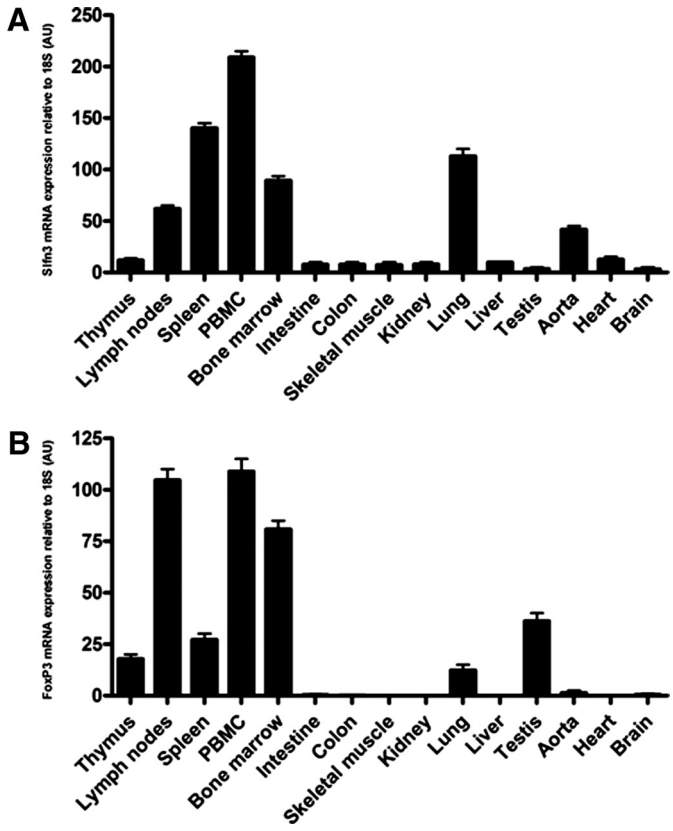


Figure 2. Expression of Slfn3 and FoxP3 mRNA in rat tissues. Slfn3 and FoxP3 mRNA expression was assessed by real-time quantitative PCR. (A) Expression of Slfn3 mRNA in various rat tissues. Slfn3 mRNA levels were normalized to the expression of the 18S rRNA and are expressed in arbitrary units. (B) The expression of FoxP3 mRNA in various rat tissues. FoxP3 mRNA levels were normalized to the expression of the 18S rRNA and are expressed in arbitrary units. The results are expressed as the mean ± SD of two independent experiments.

strated that Slfn3, similar to FoxP3, is highly and preferentially expressed in lymphoid organs, suggesting a role for this molecule in lymphoid function and differentiation.

Slfn3 mRNA distribution in naïve rat leukocytes

We next analyzed the expression of Slfn3 (**Fig. 3A**) and FoxP3 (Fig. 3B) in different populations of freshly isolated leukocytes. As has been described previously, the expression of the natural Treg transcription factor, FoxP3, was limited to the T cell compartment, and the strongest expression was detected in the CD4$^+$ T cell subset (which contains natural Tregs). In contrast, Slfn3 mRNA expression was observed in many subsets of leukocytes. The strongest expression of Slfn3 was observed in the monocyte compartment, and Slfn3 was expressed at an intermediate level in T cells (CD4$^+$ and CD8$^+$ subsets), NK cells, and peritoneal macrophages. Low levels of Slfn3 mRNA were observed in resting DCs and B cells. In summary, in contrast to the restricted expression of FoxP3 (expressed only in T cells), Slfn3 is expressed in many leukocyte subsets and is not restricted to the lymphoid compartment. The expression of the Slfn family of molecules has been described mainly in T cells [5, 6] and in myeloid cells (e.g., Slfn2 expression in macrophages) [11]. In the present study, we demonstrate that Slfn3 is largely expressed in leukocytes, including T cells, NK cells, monocytes, and macrophages.

Slfn3 is highly induced by T cell activation

Slfn3 has been shown to be expressed weakly in total activated T cells in mice, and the ectopic expression of Slfn3 in fibroblasts has an anti-proliferative effect [5]. To determine whether Slfn3 mRNA is modulated upon T cell activation in rats, purified T cells were activated using anti-CD3 and anti-CD28 mAb and analyzed for Slfn3 and FoxP3 mRNA expression (unstimulated and after 48, 72, and 96 h of stimulation). As shown in **Figure 4A**, we observed a strong induction of Slfn3 mRNA following T cell stimulation, with a peak induction at 72 h (a 30-fold increase compared with resting cells). We also measured proliferation in these samples and observed that the kinetics of proliferation was similar to the kinetics of Slfn3 mRNA expression (Fig. 4C). In contrast, FoxP3 expression was decreased strongly following activation and remained low even after 96 h of stimulation (Fig. 4B). This suggests that Slfn3 is strongly induced following T cell activation, and the

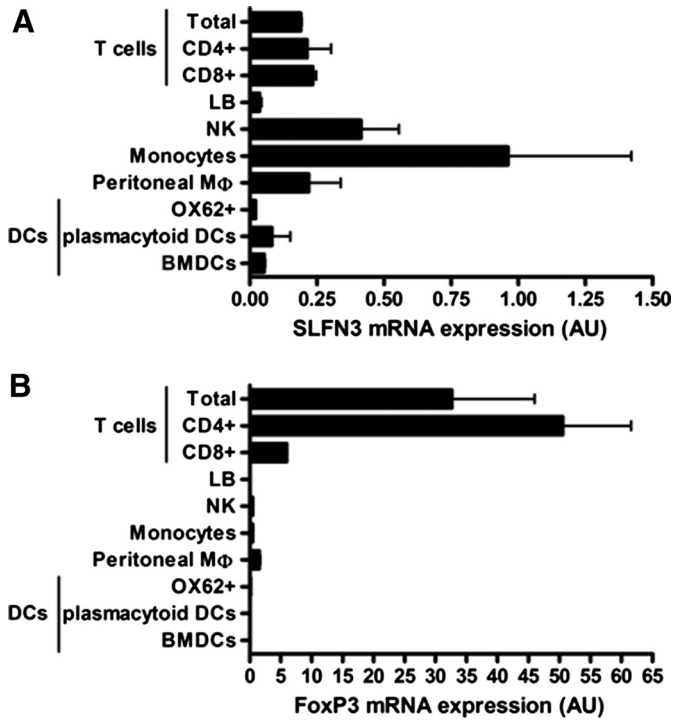


Figure 3. Expression of Slfn3 and FoxP3 mRNA in immune cells. The expression of Slfn3 and FoxP3 mRNA in cDNA, generated from mRNA extracts from freshly isolated leukocytes, excluding bone marrow-derived DCs (BMDCs), was assessed by real-time quantitative PCR and normalized to HPRT mRNA expression. The results are expressed as the mean ± SD of two independent experiments in each cell subset. (A) The expression of Slfn3 mRNA in purified immune cells. (B) The expression of FoxP3 mRNA in purified immune cells. LB, Lymphocyte B; Mϕ, macrophage.

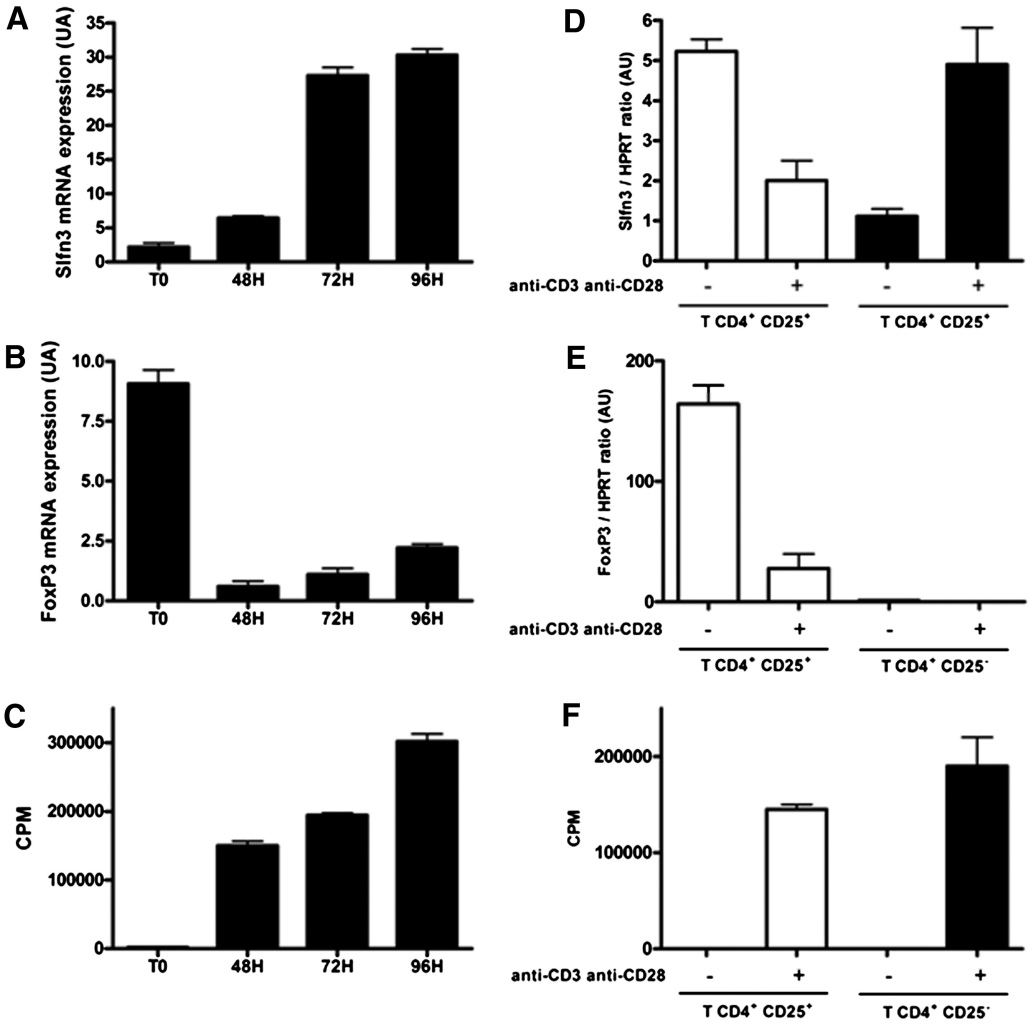


Figure 4. Slfn3 and FoxP3 mRNA expression in activated total T cells and $CD4^+CD25^+$ or $CD4^+$ $CD25^-$ T cells. The expression of Slfn3 and FoxP3 mRNA in cells activated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) was assessed by real-time quantitative PCR. The mRNA levels were normalized to HPRT expression and are expressed in arbitrary units. The kinetics of Slfn3 (A) and FoxP3 (B) mRNA expression following T cell activation with anti-CD3/CD28. (C) The proliferation of these cells was measured by thymidine incorporation, and the results are presented in CPM. The results are the mean of three independent experiments. The expression of Slfn3 (D) and FoxP3 (E) mRNA in $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ activated T cells 48 h following anti-CD3/CD28 stimulation. (F) The proliferation of these cells was measured by thymidine incorporation, and the results are presented in CPM. The results represent the mean ± SD of three independent experiments.

expression of FoxP3 was strongly decreased. The up-regulation of Slfn3 expression following activation in naïve T cells was confirmed at the protein level by Western blot analysis (**Fig. 5, A** and **B**).

In conclusion, we observed a strong time-dependent induction of Slfn3 expression at the mRNA and protein level following T cell stimulation. In addition, we examined the expression of Slfn3 in T cells using immunofluoresence microscopy. Interestingly, the expression of Slfn3 was intracellular and restricted to a single point in the cytoplasm of the cells (Fig. 5C).

Differential expression and regulation of Slfn3 mRNA expression in $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells

We next investigated the expression of Slfn3 mRNA specifically in $CD4^+$ $CD25^+$ T cells. As shown in Figure 4D, Slfn3 mRNA is expressed strongly in purified $CD4^+$ $CD25^+$ T cells compared with the one observed in $CD4^+$ $CD25^-$ T cells from naïve animals. As seen in Figure 1A, the expression of Slfn3 in $CD4^+$ $CD25^+$ T cells is three times greater than the expression in $CD4^+$ $CD25^-$ T cells ($P<0.05$).

To study the regulation of Slfn3, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells were purified and stimulated with the anti-CD3 and anti-CD28 mAb. We analyzed the expression of Slfn3 mRNA in unstimulated cells and in cells stimulated for 48 h. As shown in Figure 4D, the mRNA expression level of Slfn3 in $CD4^+$ $CD25^+$ T cells was strongly decreased following 48 h of stimulation. Interestingly, a similar pattern of expression was observed for FoxP3 present in $CD4^+$ $CD25^+$ T cells following stimulation (Fig. 4E). In contrast, the mRNA expression level of Slfn3 was increased significantly in $CD4^+$ $CD25^-$ T cells under the same conditions. As expected, FoxP3 expression remained low in the $CD4^+$ $CD25^-$ T cell population (Fig. 4E). Interestingly, although we observed a differential regulation of Slfn3 expression in the $CD4^+$ $CD25^-$ (increased expression) and $CD4^+$ $CD25^+$ (decreased expression) T cells following stimulation, the stimulation induced comparable levels of proliferation in both cells types (Fig. 4F). These results suggest that Slfn3 could have a role in T cell activation/differentiation, but Slfn3 does not have a direct role in proliferation, as has been suggested by Schwarz et al. [5], for others members of the Slfn family. However, even if Slfn3 is preferentially expressed in natural Tregs, this molecule is not specific for Tregs, as Slfn3 is up-regulated strongly following T cell activation.

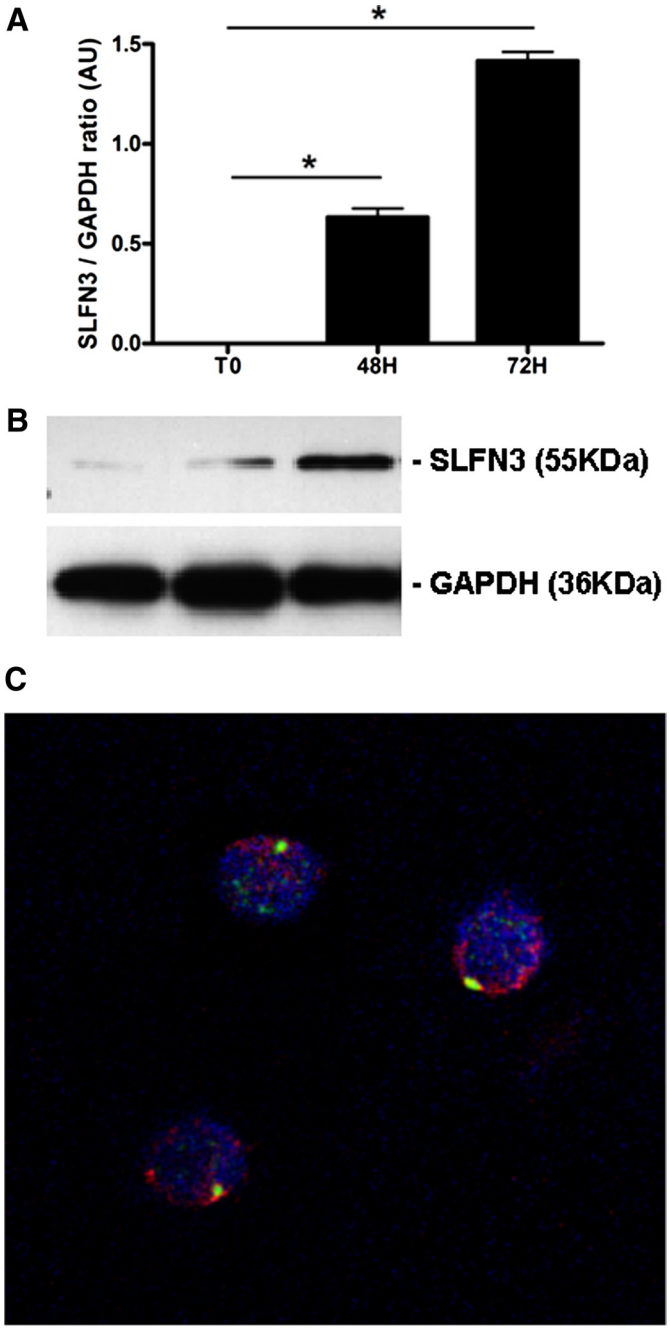


Figure 5. Slfn3 protein expression in naïve and activated T cells. (A and B) The expression of Slfn3 protein in cells activated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) was assessed by Western blot (n=3). (C) The localization of Slfn3 in naïve T cells. Immunofluorescence microscopy was used on naïve T cells using a polyclonal anti-Slfn3 antibody (green staining), an anti-TCR antibody (red staining), and the molecule Draq5 (blue staining), which stains DNA. *, $P < 0.05$.

TGF-β regulates Slfn3 mRNA expression during T cell stimulation

TGF-β has been shown to play an important role in the regulation of T cell activation/differentiation [19–21] and to have a regulatory effect on certain T cell functions (e.g., proliferation, apoptosis) [22–24]. Therefore, we tested the effect of TGF-β on CD4$^+$ T cell activation by anti-CD3 and anti-CD28 mAb after 48, 72, or 96 h of stimulation. As seen in **Figure 6A**, TGF-β treatment inhibited the expression of Slfn3. This effect was specific to TGF-β, as the addition of a neutralizing anti-TGF-β mAb reversed the effect. This demonstrates that TGF-β down-regulates the expression of Slfn3 in activated T cells and

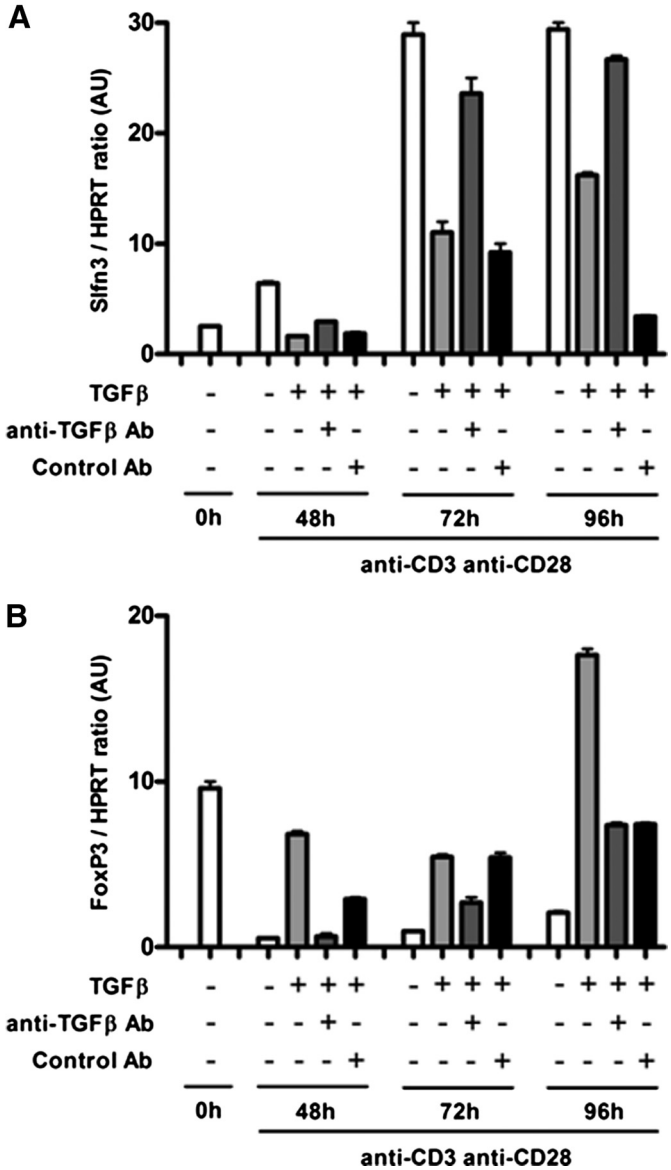


Figure 6. Effect of TGF-β on Slfn3 and FoxP3 mRNA expression in activated T cells. The mRNA expression was assessed by real-time quantitative PCR. Purified CD4$^+$ T cells were activated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) in the presence of 2 ng/mL TGF-β, 1 μg/mL anti-TGF-β, or 1 μg/mL of a control antibody. The cells were collected 48, 72, or 96 h following activation. The mRNA levels were normalized to HPRT expression and are expressed in arbitrary units. (A) The expression of Slfn3 mRNA. The results are representative of three independent experiments. (B) The expression of FoxP3 mRNA. The results are representative of three independent experiments.

that this regulation is the same in total T cells, $CD4^+$ T cells, or $CD8^+$ T cells (data not shown). We also analyzed the expression of FoxP3 mRNA in the same experiments. As shown in Figure 6B, FoxP3 mRNA expression is increased by TGF-β in anti-CD3/CD28-activated $CD4^+$ T cells. These results suggest that TGF-β differentially regulates the mRNA expression levels of Slfn3 (negative regulation) and FoxP3 (positive regulation) in activated T cells.

Our results demonstrate that Slfn3 expression increases during the activation and proliferation of naïve $CD4^+$ T cells. However, our results suggest that this increase does not depend on the regulation of cell proliferation, as suggested for Slfn2 (i.e., the inhibition of cyclin D1) [7]. In fact, we observed the down-regulation of Slfn3 in proliferating Tregs. This suggests that the anti-proliferative effect of TGF-β and the decrease of Slfn3 mRNA expression, which we observed, could reflect two independent mechanisms. In the same experiments, we demonstrated that TGF-β treatment in the presence of anti-CD3 and anti-CD28 stimulation increased FoxP3 mRNA expression, confirming the role of TGF-β in Treg differentiation, as has been demonstrated previously [19]. We have demonstrated that Slfn3 is overexpressed during the activation of naïve $CD4^+$ and $CD8^+$ T cells and that this increase is negatively regulated by TGF-β.

Interestingly, Sohn et al. [11] reported recently that Slfn2 transcription is controlled by two transcription factors, NF-κB and AP-1, which are involved in T cell activation [25, 26]. Similar to Slfn2, Slfn3 transcription could be controlled by NF-κB and AP-1. It has been shown that TGF-β inhibits the activation of AP-1 [27] and therefore, could negatively regulate Slfn3 expression.

In conclusion, we have demonstrated in this study that Slfn3 is preferentially expressed by resting $CD4^+$ $CD25^+$ T cells in the periphery and that the expression of this molecule is decreased in this population upon stimulation. We have also demonstrated that Slfn3 is up-regulated upon T cell activation. Moreover, the expression of Slfn3 in anti-CD3/CD28 mAb-activated T cells is inhibited by TGF-β, and the expression of Foxp3 mRNA is increased under the same conditions. This suggests that Slfn3 could have a role in T cell differentiation and could be a new marker of T cell activation.

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KEY WORDS:
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