



# CD28<sup>-</sup> and CD28<sup>low</sup>CD8<sup>+</sup> Regulatory T Cells: Of Mice and Men

Yirajen Vuddamalay<sup>1</sup> and Joost P. M. van Meerwijk<sup>2,3,4\*</sup>

<sup>1</sup>School of Health Sciences, University of Technology, Port Louis, Mauritius, <sup>2</sup>Institut National de la Santé et de la Recherche Médicale (INSERM), U1043, Toulouse, France, <sup>3</sup>Centre National de la Recherche Scientifique (CNRS), U5282, Toulouse, France, <sup>4</sup>Université de Toulouse, Université Paul Sabatier, Centre de Physiopathologie de Toulouse Purpan (CPTP), Toulouse, France

Since the rebirth of regulatory (formerly known as suppressor) T cells in the early 1990s, research in the field of immune-regulation by various T cell populations has quickly gained momentum. While T cells expressing the transcription factor Foxp3 are currently in the spotlight, several other T cell populations endowed with potent immunomodulatory capacities have been identified in both the CD8<sup>+</sup> and CD4<sup>+</sup> compartment. The fundamental difference between CD4<sup>+</sup> and CD8<sup>+</sup> T cells in terms of antigen recognition suggests non-redundant, and perhaps complementary, functions of regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cells in immunoregulation. This emphasizes the importance and necessity of continuous research on both subpopulations of regulatory T cells (Tregs) so as to decipher their complex physiological relevance and possible synergy. Two distinct CD8-expressing Treg populations can be distinguished based on expression of the co-stimulatory receptor CD28. Here, we review the literature on these (at least in part) thymus-derived CD28<sup>low</sup> and peripherally induced CD28<sup>-</sup>CD8<sup>+</sup> Tregs.

**Keywords:** tolerance, regulatory T cells, CD8<sup>+</sup> T-lymphocytes, human, mouse, thymus, immunoregulation

## OPEN ACCESS

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### \*Correspondence:

Joost P. M. van Meerwijk  
joost.van-meerwijk@inserm.fr

### Specialty section:

This article was submitted to  
T Cell Biology,  
a section of the journal  
Frontiers in Immunology

**Received:** 14 November 2016

**Accepted:** 09 January 2017

**Published:** 23 January 2017

### Citation:

Vuddamalay Y and van Meerwijk JPM  
(2017) CD28<sup>-</sup> and CD28<sup>low</sup>CD8<sup>+</sup>  
Regulatory T Cells: Of Mice and Men.  
Front. Immunol. 8:31.  
doi: 10.3389/fimmu.2017.00031

## INTRODUCTION

The prerequisite to the prevention of immunopathologies such as autoimmunity and chronic inflammation is the maintenance of an immune homeostasis that relies mainly on intricate mechanisms of tolerance to self and innocuous non-self antigens. Through their multifaceted actions, regulatory T cells (Tregs) play an unparalleled role in modulating both innate and adaptive responses. As such, Tregs prevent autoimmune disorders, control immune reactions at environmental surfaces, modulate anti-infectious responses, and contribute to fetomaternal tolerance [reviewed in Ref. (1–3)].

Historically speaking, the first suppressor population to be described were T cells expressing the CD8 co-receptor. Indeed, the T cell population identified by Cantor et al., which act in an antigen-specific manner to suppress immune reactions, expressed the surface marker Lyt2, now known as CD8 $\alpha$  (4, 5). Since then, the field has had its fair share of whirls on the wheel of scientific (mis)fortune. The concatenation of events from the downfall of suppressor T cells to its rebirth (or

**Abbreviations:** AIRE, autoimmune regulator; APC, antigen-presenting cells; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; APS, autoimmune polyglandular syndrome; EAE, experimental autoimmune encephalomyelitis; Foxp3, forkhead/winged helix transcription factor; GFP, green fluorescent protein; IFN- $\gamma$ , interferon- $\gamma$ ; IPEX, immune dysregulation polyendocrinopathy enteropathy X linked; ILT, immunoglobulin-like transcript; RAG, recombinase activating gene; SCID, severe combined immunodeficiency; TGF- $\beta$ , transforming growth factor  $\beta$ .

rebranding) as Tregs have extensively been reviewed elsewhere (6–8) and will not be discussed here.

The advent of molecular immunology in the postsuppressor era unequivocally established the T cell population expressing the forkhead/winged helix transcription factor Foxp3 as a key player in the fine regulation of immune responses [reviewed in Ref. (9)]. Indeed, in mice, invalidating mutations in the *Foxp3* gene or specific ablation of Foxp3<sup>+</sup> T cells lead to the development of a fatal lymphoproliferative disorder (10–13) and humans with mutations in the FOXP3 gene suffer from the lethal immune-dysregulation polyendocrinopathy enteropathy X linked syndrome (14, 15). In parallel, several other regulatory CD4<sup>+</sup> and CD8<sup>+</sup> subsets have been identified and characterized in both mice and humans (16–20). Distinct Treg (sub)populations differ in their origin, development, and mechanisms of action which *in fine* define their physiological role. As such, determining the specific function of a given Treg population mandates extensive research to identify the different molecular and cellular factors that govern its existence. We and others have contributed to unveil some key features of the CD8-expressing Treg population that is characterized by the expression of low levels of the co-stimulatory molecule CD28; CD8<sup>+</sup>CD28<sup>low</sup> Treg.

## CD8<sup>+</sup>CD28<sup>low</sup> TREG IN MICE

The immunosuppressive capacity of CD8<sup>+</sup>CD28<sup>low</sup> was first described in a murine model of multiple sclerosis. Najafian et al. showed that CD8 knockout (CD8 KO) mice were more susceptible to the induction of experimental autoimmune encephalomyelitis (EAE) than wild-type (WT) mice suggesting a protective effect of CD8<sup>+</sup> cells. Adoptive transfer of CD8<sup>+</sup>CD28<sup>low</sup> T cells from WT animals into CD8 KO recipients significantly reduced the severity of the disease. No such decrease was observed with the adoptive transfer of CD8<sup>+</sup>CD28<sup>high</sup> T cells. Furthermore, CD8<sup>+</sup>CD28<sup>low</sup> T cells but not their CD28<sup>high</sup> counterpart could suppress *in vitro* the production of interferon- $\gamma$  by CD4<sup>+</sup> T cells specific for the myelin oligodendrocyte glycoprotein used to induce EAE. The suppressive function of the CD8<sup>+</sup>CD28<sup>low</sup> Treg required an interaction with antigen-presenting cells (APC), which led to the downregulation of CD80, CD86, and CD40 expression on the APC (21). In a similar model, Yang et al. have shown that pretreatment of mice with a group of 15-amino acid-long trichosanthin-derived peptides reduced the clinical score of EAE as compared to untreated animals. Attenuation of the disease was attributed to the expansion and activation of IL10-producing-CD8<sup>+</sup>CD28<sup>low</sup> Treg (22).

Previous work by our team has shown that CD8<sup>+</sup>CD28<sup>low</sup> Treg can prevent intestinal inflammation in a well-established experimental colitis model where pathology is induced by the adoptive transfer of naïve CD4<sup>+</sup>CD45RB<sup>high</sup> T cells into lymphopenic animals [recombinase activating gene 2 (RAG2) deficient or severe combined immunodeficiency mice (23, 24)]. Cotransfer of freshly isolated splenic CD8<sup>+</sup>CD28<sup>low</sup> T cells from WT animals with the colitogenic cells prevented onset of colitis. Similar results were obtained with CD8<sup>+</sup>CD28<sup>low</sup> T cells isolated from the lamina propria of the intestine (25). These CD8 $\alpha\beta$ <sup>+</sup>CD28<sup>low</sup> Treg expressed a large repertoire of the TCR $\alpha\beta$  heterodimer (26). Protection

from colitis was dependent on IL-10 production by the Treg and on the responsiveness of the colitogenic T-cells to transforming growth factor  $\beta$  (TGF- $\beta$ ), underlining the non-redundant functions of these two immunomodulatory cytokines in the control of intestinal inflammation by CD8<sup>+</sup>CD28<sup>low</sup> Treg (25). Importantly, in contrast to CD4<sup>+</sup>CD25<sup>high</sup> Treg, CD8<sup>+</sup>CD28<sup>low</sup> Treg from unmanipulated mice do not express the transcription factor Foxp3. More recently, in mice immunized with ovalbumin and subsequently intranasally challenged with ovalbumin encased in oligomannose-coated liposomes, an expansion of CD8<sup>+</sup>CD28<sup>low</sup> (and CD4<sup>+</sup>Foxp3<sup>+</sup>) Treg was observed. Upon adoptive transfer, the CD8<sup>+</sup>CD28<sup>low</sup> Treg reduced the severity of allergic diarrhea (27).

## AUTOIMMUNE REGULATOR (AIRE) AND THE DEVELOPMENT OF CD8<sup>+</sup>CD28<sup>low</sup> TREG

The transcription factor AIRE is primarily expressed by medullary epithelial cells of the thymus (mTEC) where it controls cellular maturation and the ectopic expression of thousands of tissue-specific antigens (28, 29). Presentation of these peripheral antigens by mTEC leads to the negative selection of auto-specific conventional T cells (30–32). Furthermore, AIRE modulates the production of chemokines by mTEC, involved in the migration of thymocytes and dendritic cells from the cortex to the medulla in the thymus (33, 34). As such, AIRE is a key regulator of central tolerance. Indeed, loss-of-function mutations in the AIRE gene lead to the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome also known as APS for autoimmune polyglandular syndrome (35, 36). While chronic mucocutaneous candidiasis, hypoparathyroidism, and hypoadrenalism are considered to be the classic triad hallmarks of this autoimmune syndrome (37), about 25% of APECED patients are also affected by gastrointestinal diseases ranging from chronic diarrhea and obstipation (38). In children suffering from APECED, these intestinal ailments can lead to malabsorption, various deficiencies, growth impairment, and even death (39, 40). Importantly, some do even consider gastrointestinal symptoms to be the first manifestation of APECED (38). Mice deficient for AIRE also exhibit (though to a lesser extent) autoimmune symptoms such as presence of autoantibodies and cellular infiltration in various organs (41). Since CD8<sup>+</sup>CD28<sup>low</sup> Treg can efficiently prevent intestinal inflammation, a prominent symptom in APECED, the potential role of AIRE in the development of this Treg population was evaluated.

Our comparative study of CD8<sup>+</sup>CD28<sup>low</sup> Treg from WT and AIRE-deficient (AIRE KO) mice revealed that while both Treg populations were present in similar proportions and exhibited comparable immunosuppressive activity *in vitro*, Treg from AIRE KO animals failed to prevent intestinal inflammation in the colitis model (26). Gene expression patterns, cell-surface marker expression, IL-10 production, and *in vitro* suppressive capacity of WT and AIRE KO CD8<sup>+</sup>CD28<sup>low</sup> Treg were indistinguishable. However, a small difference was found between the T-cell receptor (TCR) repertoires expressed by WT vs. KO

Treg. Based on these observations, we concluded that AIRE is involved in shaping the TCR-repertoire of CD8<sup>+</sup>CD28<sup>low</sup> Treg. To our knowledge, this was the first definite demonstration that a deficiency in AIRE leads to the functional defect of a Treg population. This pioneer study is in line with more recent studies that have provided molecular evidence, through TCR repertoire analysis, that AIRE is essential for the thymic development of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg with unique individual TCRs (42–44). Taken together, these studies have established that AIRE not only drives the negative selection of conventional T cells but is also involved in the differentiation of CD8<sup>+</sup> and CD4<sup>+</sup> Treg populations.

## ORIGIN OF CD8<sup>+</sup>CD28<sup>low</sup> TREG

Based on our current understanding of the development of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg, it is commonly accepted that Treg in general can have two distinct origins: intrathymic development of “tTreg” from hematopoietic precursors and extrathymic (or peripheral) differentiation of “pTreg” from conventional T cells given appropriate environmental cues [reviewed in Ref. (45, 46)]. Since data from the literature have attributed distinct singular functions to tTreg and pTreg (47–49), the identification of the origin of CD8<sup>+</sup>CD28<sup>low</sup> Treg was an important milestone in the quest to better characterize this population. Our observation that AIRE, which is primarily expressed in the thymus, is involved in the development of the CD8<sup>+</sup>CD28<sup>low</sup> Treg repertoire suggested a thymic origin for CD8<sup>+</sup>CD28<sup>low</sup> Treg. However, expression of AIRE has also been reported in both hematopoietic and stromal lineages outside of the thymus (50–52). Importantly, these extrathymic AIRE-expressing cells have tolerogenic properties (53) and thus in theory may induce differentiation of conventional T cells into Tregs. We recently demonstrated that mature CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>high</sup> thymocytes expressing low levels of CD28, isolated from WT mice, can efficiently suppress the *in vitro* proliferation of CD4<sup>+</sup> T cells (54). However, since T cells including Tregs can recirculate from the periphery back to the thymus (55, 56), their presence in this primary lymphoid organ was not sufficient to confirm their origin. Definite proof of the thymic origin of CD8<sup>+</sup>CD28<sup>low</sup> Treg came from the analysis of transgenic mice expressing the green fluorescent protein (GFP) under the control of the RAG2 promoter [RAG–GFP mice, Ref. (57)]. In the thymus, thymocytes express RAG2 at the early stages of their development and then terminate its expression after positive selection (58). As such, in RAG–GFP animals, the GFP protein whose expression parallels that of RAG2 and has a half-life of 56 h serves as a molecular marker for lymphocyte aging in the thymus allowing for the discrimination between “freshly” developed mature T cells that express GFP and recirculating T cells that do not (59). Analysis of RAG–GFP mice revealed that while approximately 20% of mature thymic CD8<sup>+</sup>CD28<sup>low</sup> T cells are deprived of GFP expression (i.e., recirculating or long-term thymus resident cells), the major proportion of this T cell population are newly developed cells. Importantly, the GFP<sup>+</sup> compartment of the mature thymic CD8<sup>+</sup>CD28<sup>low</sup> T cells demonstrated immunosuppressive activity *in vitro* hence firmly

establishing the thymic origin of CD8<sup>+</sup>CD28<sup>low</sup> Treg in mice (54). However, the interesting possibility that the pool of circulating CD8<sup>+</sup>CD28<sup>low</sup> Treg may be composed of both tTreg and pTreg must also be considered. Indeed, in an experimental model of myasthenia gravis (MG), exposure to specific antigens (the dual-altered peptide) led to the emergence of CD8<sup>+</sup>CD28<sup>low</sup> Treg (60). While it can be argued that the emergence of Treg could be due to the expansion of preexisting tTreg, the alternate hypothesis of an induction of *bona fide* pTreg cannot be excluded (Figure 1).

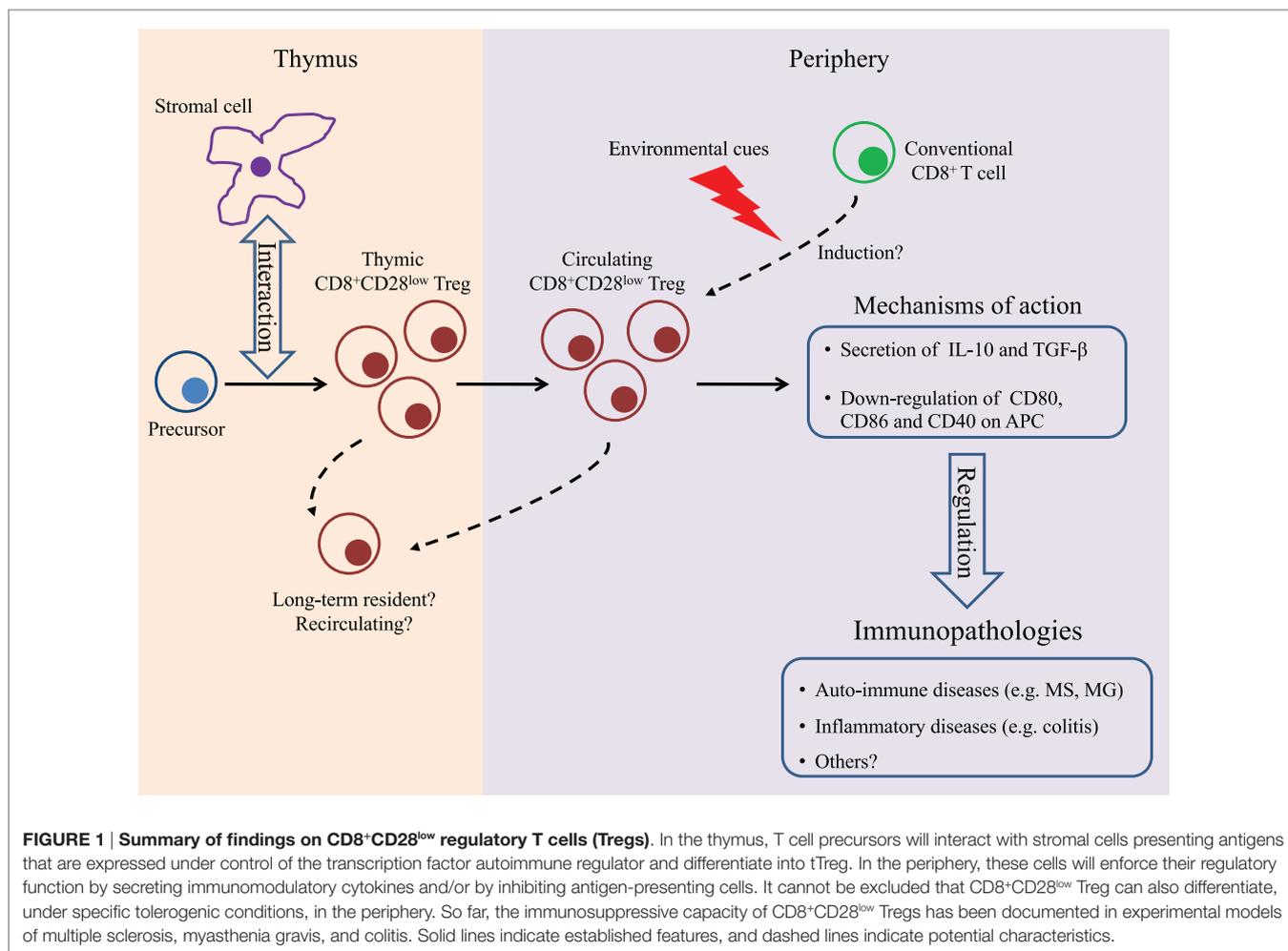
## CD8<sup>+</sup>CD28<sup>low</sup> TREG IN HUMANS

A population of CD8<sup>+</sup>CD28<sup>low</sup> T cell exhibiting similar immunosuppressive characteristics as its murine homolog has recently been identified in humans. Analysis of peripheral blood mononuclear cells (PBMCs) has revealed a substantial percentage (between 10 and 13%) of CD28<sup>low</sup>-expressing cells among naive CD8<sup>+</sup> T cells. Importantly, following *in vitro* activation, these cells produce the same cytokines (i.e., IL-10 and TGF-β), which confer CD8<sup>+</sup>CD28<sup>low</sup> Treg their immunomodulatory ability in experimental mouse models. Similar results were obtained when human thymii isolated from children aged from 0 to 10 years were analyzed (54). Taken together, these results from human studies strongly suggest that, similar to mouse, CD8<sup>+</sup>CD28<sup>low</sup> T cell endowed with immunosuppressive capacity are present in human PBMCs and that they develop in the human thymus.

## CD8<sup>+</sup>CD28<sup>-</sup> TREG IN HUMANS AND MICE

Based on CD28 expression, another Treg population has previously been described in humans. Cyclic stimulations of PBMCs with allogenic APC induced CD8<sup>+</sup> T cells deprived of CD28 expression, which inhibited cellular proliferation in these *in vitro* cultures (61). Since then, several groups have tried to develop, with more or less success, their own strategies to induce CD8<sup>+</sup>CD28<sup>-</sup> Treg *in vitro* by stimulating PBMCs with cocktails of cytokines in the presence or absence of antigens (62, 63), with phorbol12-myristate 13-acetate/ionomycin or phytohemagglutinin (64) or with a recombinant immunoglobulin-like transcript 3 (ILT3)-Fc fusion protein (65, 66). CD8<sup>+</sup>CD28<sup>-</sup> Tregs express GITR, CD25, CD103, CD62L, and 4-1BB and are MHC class I restricted (67). They exert their immunomodulatory activity by inducing the expression of ILT3 and ILT4 on dendritic cells, thus rendering them tolerogenic (66). Intriguingly, human mesenchymal stromal cells have recently been shown to enhance the immunomodulatory function of CD8<sup>+</sup>CD28<sup>-</sup> Treg by reducing their rate of apoptosis (68).

CD8<sup>+</sup>CD28<sup>-</sup> T cells with a regulatory phenotype have been observed in patients having undergone successful organ transplantation (69–71), alloenergized HLA-mismatched bone marrow graft (72), and allogenic platelet transfusion (73) or suffering from autoimmune diseases (74–76), pregnancy complications (77), and cancers (78–80). Importantly, CD8<sup>+</sup>CD28<sup>-</sup> T cells isolated from healthy donors are not immunosuppressive (69). Hence, it would seem that CD8<sup>+</sup>CD28<sup>-</sup> Treg are induced in the periphery following disturbances of the immune homeostasis.



A mouse homolog of human CD8<sup>+</sup>CD28<sup>-</sup> pTreg may also exist. Ben-David et al. showed that in an experimental model of MG where pathology is triggered by immunization with a myasthenogenic peptide, injection of a dual-altered peptide induces the emergence of CD8<sup>+</sup>CD28<sup>-</sup> Treg that efficiently suppress the autoimmune response. Flow cytometry analysis of these cells suggested that these Tregs may express low levels of Foxp3 (60).

## CD28<sup>-</sup> VS. CD28<sup>low</sup>CD8<sup>+</sup> TREG IN HUMANS AND MICE

Najafian et al. initially showed that total CD8<sup>+</sup> T cells isolated from CD28-deficient mice (i.e., CD8<sup>+</sup>CD28<sup>-</sup> cells) exhibited immunosuppressive activity *in vitro* and decreased the severity of EAE in adoptive transfer experiments. However, the CD8<sup>+</sup> T cells isolated from WT mice that inhibited severity of EAE clearly expressed low levels of CD28 (21). In our initial report on the prevention of experimental colitis in the mouse, the CD8<sup>+</sup> Treg, which we inaccurately termed CD28<sup>-</sup>, also clearly expressed low but detectable levels of CD28. In unmanipulated specific pathogen-free WT mice, we only observed subsets of CD8<sup>+</sup> T cells expressing low or high levels of CD28 but none that are deprived

of expression of this co-stimulatory molecule (25, 26, 54). In humans, their low but readily detectable level of expression of CD28, their presence in the thymus, and their naive phenotype clearly distinguish CD8<sup>+</sup>CD28<sup>low</sup> Treg from CD8<sup>+</sup>CD28<sup>-</sup> Treg that do not express CD28 at levels exceeding background, are not found in the thymus, and have an activated phenotype (54). We therefore conclude that the co-stimulatory molecule CD28 allows for the identification of two distinct CD8<sup>+</sup> subsets: CD28<sup>low</sup> tTreg and CD28<sup>-</sup> p Treg.

## CONCLUDING REMARKS

While the various studies discussed here have helped to decipher key features of CD8<sup>+</sup>CD28<sup>low</sup> T cells and in parallel establish them as a potent Treg population in both mice and humans, several burning questions concerning these Treg remain unanswered, the most important one being perhaps their biological function(s) under homeostatic and pathologic conditions. We believe that the identification of other, more discriminative, markers of CD8<sup>+</sup>CD28<sup>low</sup> Treg will greatly help in achieving this goal. Currently, this Treg population can only be characterized by their low levels of expression of CD28 allowing for only a minimal

estimation of their proportions by flow cytometry analysis (25, 26, 54). Furthermore, the absence of a better marker is hindering a panoply of key experiments such as specific localization in tissues and lymphoid organs, antibody-specific depletion, germline, and/or conditional knockout strategies.

Up till now, research on CD8<sup>+</sup>CD28<sup>low</sup> Treg had been confined to murine studies (21, 25, 26). Even though the potent immunoregulatory capacity of CD8<sup>+</sup>CD28<sup>low</sup> has been documented in these experimental models of inflammation, its relevance in human diseases remains unknown. In parallel, defects in various CD4<sup>+</sup> and CD8<sup>+</sup> Treg populations have been reported in human autoimmune diseases and immune-mediated inflammatory pathologies (81–87). The identification of CD8<sup>+</sup>CD28<sup>low</sup> Treg in humans is hence paving the way to further studies so as to gain insight into the physiological

function of this Treg population and its potential involvement in human pathologies.

## AUTHOR CONTRIBUTIONS

YV and JPMvM designed the outline and wrote the manuscript.

## ACKNOWLEDGMENTS

We thank M. Rolland, J. Series, R. T. K. Ujoodha, and M. Putteeraj for their support. This work was financially supported by grants from the “Association François Aupetit” (2013, 2014), the “Ligue Contre le Cancer” (to YV), and the “Fondation ARC pour la Recherche sur le Cancer” (SFI20101201917, PJA 20131200290).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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