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Phenotypical characterization of regulatory T cells in humans and rodents

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Summary

Regulatory T cells (T_{regs}) constitute a fascinating subpopulation of $CD4^+$ T cells due to their ability to limit the immune response against self and non-self antigens. Murine models and antibodies directed against surface and intracellular molecules have allowed elucidation of the mechanisms that govern their development and function. However, these markers used to their classification lack of specificity, as they can be expressed by activated T cells. Similarly, there are slight differences between animal models, in steady state and pathological conditions, anatomical localization and strategy of analysis by flow cytometry. Here, we revised the most common markers utilized for T_{reg} typification by flow cytometry such as CD25, forkhead box protein 3 (FoxP3) and CD127, along with our data obtained in different body compartments of humans, mice and rats. Furthermore, we revised and determined the expression of other molecules important for the phenotypical characterization of T_{reg} cells. We draw attention to the drawbacks of those markers used in chronic states of inflammation. However, until a specific marker for the identification of T_{regs} is discovered, the best combination of markers will depend upon the tissue or the degree of inflammation from which T_{regs} derive.

Keywords: human, regulatory T cells, rodent, spleen and lymph nodes

Introduction

Regulatory $CD4^+$ T cells expressing the transcription factor forkhead box protein 3 (FoxP3) [regulatory T cells (T_{regs})] constitute a fascinating subpopulation of T cells, capable of fine-tuning the immune response raised against autoantigens and foreign antigens. In the 1980s, the introduction of a specific monoclonal antibody directed against the α-chain of the interleukin (IL)-2 receptor (CD25) opened a vast field in clinical and basic immunology that has led to the identification by flow cytometry of 'suppressor' cells critical for the maintenance of self-tolerance [1]. T_{regs} can be divided into two groups according to their site of origin: thymus-derived T_{regs} (tT_{regs}) and peripherally derived T_{regs} (pT_{regs}) [2]. tT_{regs} are selected positively in the thymus through the major histocompatibility complex (MHC) class II-dependent T cell receptor (TCR) interactions resulting in a relatively high-avidity selection [3]. In contrast, pT_{regs} originate in the periphery from conventional T cells (T_{cons}) as a result of TCR stimulation in the presence of the transforming growth factor (TGF)-β and IL-2 [3]. Later, new markers were introduced, allowing a better identification of the T_{regs} population, such as FoxP3 and the low expression or absence of alpha chain of the IL-7R (CD127); however, these markers do not allow accurate delineation of the T_{reg} population or distinguish between tT_{regs} and pT_{regs}. In addition, many subphenotypes of T_{regs} have been identified in human and rodent models based on the expression of different markers, identifying profiles associated with a major specific suppressive function, migration pattern and/or activation, suggestive of their heterogeneous nature.

Although other markers, such as Helios (a transcription factor of the Ikaros family) and Neuropilin-1 (Nrp1, a surface molecule), have been introduced, their use is still controversial [4,5]. Fortunately, tT_{regs} could be distinguished from pT_{regs} through the identification of a non-coding sequence within the *FOXP3* locus named TSDR (T_{reg}-specific demethylated region) that is completely demethylated on tT_{regs}, contrary to pT_{regs} or T_{cons} in which TSDR is methylated. As this strategy requires cell lysis to extract the DNA, it is not possible to use it to isolate viable cells [6]. However, it would be desirable to assess the methylation status of FoxP3, in conjunction with characterization by flow cytometry.

The heterogeneous nature of T_{regs} highlights the different ways to turn off the immune response of T_{cons} and dendritic cells (DCs), such as cell-to-cell contact, metabolic disruption of the microenvironment and release of immunosuppressive cytokines [7]. One of the most relevant contact mechanisms for T_{regs} is the expression of the cytotoxic T lymphocyte associated-antigen 4 (CTLA-4), a member of the CD28 superfamily of immunoreceptors that competes with CD28 for binding to B7 molecules (B7·1 or CD80 and B7·2 or CD86) on antigen-presenting cells (APCs), that in turn inhibit DC-T_{cons} aggregates, DC maturation and T cell activation at the immunological synapse [8–10]. Programmed death-1 (PD-1) is another inhibitory molecule of the CD28 superfamily expressed on T_{regs} that interacts with its ligand PDL-1 on $CD4^+$ and $CD8^+$ T cells, inhibiting their proliferation and release of proinflammatory cytokines [11]. Moreover, the expression of PD-1 on $CD4^+$ T cells seems to mediate their conversion to T_{regs} [12]. Interestingly, PD-1-expressing T_{regs} up-regulate Tim-3, a cell immunoglobulin and mucin domain-containing protein (TIM), which induces selectively the cell death of fully differentiated T helper type 1 (Th1) cells through interaction with its ligand, galectin-9, expressed in the latter cells [13]. Inhibition of maturation and co-stimulatory ability of DCs can also be mediated through contact of its MHC class II with the lymphocyte-activation gene 3 (LAG-3) expressed on T_{regs} [14]. Although other molecules such as GITR [glucocorticoid-induced tumour necrosis factor (TNF) receptor-related protein] and OX-40 (CD134) [TNF receptor superfamily, member 4 (TNF-RSF4)] expressed on T_{regs} mediate contact with other immune cells, their role on T_{reg} function is more controversial; thus, it will be discussed in their corresponding section.

T_{regs} also turn off the immune response through anti-inflammatory cytokine production, such as TGF-β, IL-10 and IL-35, that are important for the control of the immune response in mucosal sites in response to non-self antigens [15]. Another interesting way for T_{regs} to suppress T_{cons} activation is by inducing metabolic alterations in the cellular microenvironment by consuming IL-2 from the medium due their high expression of CD25, affecting T_{cons} survival and proliferation [16]. Expression of ectoenzymes such as CD39 and CD73 catabolizes extracellular ATP generating adenosine that is bound to the A2A adenosine receptor on T cells or DCs, down-regulating the activation and production of cytokines and co-stimulatory molecule expression [17]. Moreover, T_{regs} harbour high levels of cyclic adenosine monophosphate (cAMP), which could be transferred to T_{cons} via gap junctions, thereby inhibiting proliferation and IL-2 production [18]. Remarkably, homing markers that are expressed selectively for infiltrating T_{regs} from peripheral tissues, is required to exert total suppressive action; in particular, the expression of integrin α_E (CD103) allows the migration of T_{regs} from blood to inflamed epithelial tissues or gut by binding to E-cadherin; moreover, its expression on T_{regs} has been associated with an effector/memory phenotype [19].

Currently, the availability of monoclonal antibodies has allowed a better delineation of T_{reg} populations. However, some drawbacks remain, such as the promiscuous nature of expression of several molecules used to identify T_{regs}, technical issues related to clones and fluorochrome conjugates of monoclonal antibodies, the staining and/or permeabilization protocols, the gating strategy for the analysis of flow cytometry to define positive events, the anatomical location of T_{regs} and the immunological status of the host. All these aspects could be the origin of some of the discrepancies observed in both humans and rodent species.

Altogether, these aspects render the characterization of T_{regs} plagued to a certain extent by variations. Hence, we will discuss pros and cons of the use of CD25, FoxP3 and CD127 in humans and rodents along with technical issues concerning to flow cytometry. We also summarize evidence regarding some markers we used to phenotype T_{regs} in humans and rodents, such as CTLA-4, GITR, CD103, PD-1 and OX40, and to compare this information with that obtained in other studies, along with representative gating strategies for T_{reg} identification by flow cytometry in lymphoid tissues from humans and rodents.

Expression of CD25, FoxP3 and CD127 to identify human T_{regs}

In the early 1990s, expression of CD25 was used to identify human T_{regs} on the total circulating $CD4^+$ T cells with values oscillating between 10 and 30% [20,21], comparable to the 15% that we have obtained in healthy individuals (Table 1).

High expression of CD25 in human $CD4^+$ T cells has also been used to define T_{regs}, representing approximately 1–2% of the total $CD4^+$ subset in peripheral blood and exhibiting the greatest suppressive function [22]. However, the definition of 'high' using MFI (mean fluorescence intensity) as the criterion for its characterization [23] is inaccurate as it depends, among others, on the specific configuration of the flow cytometers and on the characteristics of the antibodies. Moreover, CD25 is expressed heterogeneously on T cells; thus, approximately 96% of $CD25^{high}$ T cells express FoxP3, compared to only 35% FoxP3-expressing $CD4^+CD25^{int}$ T cells [24]. Also, the staining shows a smear [25] that hinders the establishing of the minimal level of CD25 expression required to define the T_{reg} population [26]. Moreover, intermediate and high levels of CD25 expression are arbitrary. For this reason, identifying T_{regs} as T cells expressing high levels of CD25 should not be used. Several clones of anti-CD25 antibodies have been reported in the literature; we have used the BC96 clone to identify $CD25^+$ T cells with clear results (Fig. 1). In addition, the use of this antibody, conjugated with the phycoerythrin-cyanin 5 (PE-Cy5) fluorochrome, have allowed a correct discrimination when used alone or in

Table 1. Frequency and phenotype of T_{regs} in healthy humans, mice and rats determined by flow cytometry

	Humans*		Mice†		Rats‡		
Markers for Treg detection	PB°	RM*	LN	Spleen	PB	LN	Spleen
$CD4^+CD25^+$	14·5 ± 2·9	n.d.	14·3 ± 1·3	15·2 ± 0·4	8 ± 2	7·2 ± 1·5	8·5 ± 1·7
$CD4^+FoxP3^+$	6·4 ± 0·9	9·3 ± 4·1	11·2 ± 0·4	13 ± 2·6	7·6 ± 1·5	6·3 ± 0·7	7·2 ± 1·4
$CD4^+FoxP3^+CD25^+$	4·9 ± 1·1	7·5 ± 2·4	9·6 ± 0·3	10·2 ± 1	4·3 ± 1·1	3·6 ± 0·5	4·1 ± 1·2
$CD4^+FoxP3^+CD127^{low/-}$	6·1 ± 1·1	7 ± 2·2	n.d.	n.d.	n.d.	n.d.	n.d.
$CD4^+CD25^+CD127^{low/-}$	6·0 ± 1·5	6·5 ± 3·2	n.d.	n.d.	n.d.	n.d.	n.d.
Phenotype of $CD4^+FoxP3^+$ T_{regs}							
CD25	51·6 ± 9·1	45·4 ± 11·2	84·3 ± 2·5	82·7 ± 0·6	64·7 ± 5·8	62·2 ± 4·3	60·5 ± 5·1
CTLA-4	23 ± 10·4	49·3 ± 13·4	n.d.	n.d.	23·9 ± 11·6	20·2 ± 6·1	18·4 ± 8·6
GITR	10·3 ± 5·5	n.d.	75 ± 14·1	76·7 ± 8·7	n.d.	n.d.	n.d.
CD103	9·3 ± 3·8	21·5 ± 10·7	n.d.	n.d.	39 ± 17·5	38 ± 17·5	30 ± 19·1
PD-1	4·4 ± 3·7	16·8 ± 12	66·3 ± 14·6	65·5 ± 7·1	n.d.	n.d.	n.d.
OX-40	n.d.	n.d.	62·8 ± 10·5	62·3 ± 3·8	n.d.	n.d.	n.d.

Data are expressed as mean ± standard deviation. *Data from humans were taken from our previous studies [29,105]. †BALB/c mice and ‡Wistar rats data were taken from our original unpublished results. PB = peripheral blood; RM = rectal mucosae; LN = lymph nodes; n.d. = not determined; FoxP3 = forkhead box protein 3; T_{regs} = regulatory T cells; PD-1 = programmed death-1; GITR = glucocorticoid-induced tumour necrosis factor receptor-related protein; CTLA-4 = cytotoxic T lymphocyte-associated-antigen 4.

combination with other antibodies constituting the T_{reg} panel, as other authors have reported [27].

The crucial role of FoxP3 as a master regulator in the development and function of T_{regs} was demonstrated by a frameshift mutation in the *FOXP3* gene that leads to its impaired expression and to the development of a syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans and scurfy in mice, resulting in a variety of autoimmune disorders that could be incompatible with life [3].

In humans, the percentage of $CD4^+FoxP3^+$ cells is variable, representing approximately 1–10% of the $CD4^+$ population in peripheral blood [28–30]; we have observed approximately 6% in blood (Table 1). Interestingly, lower

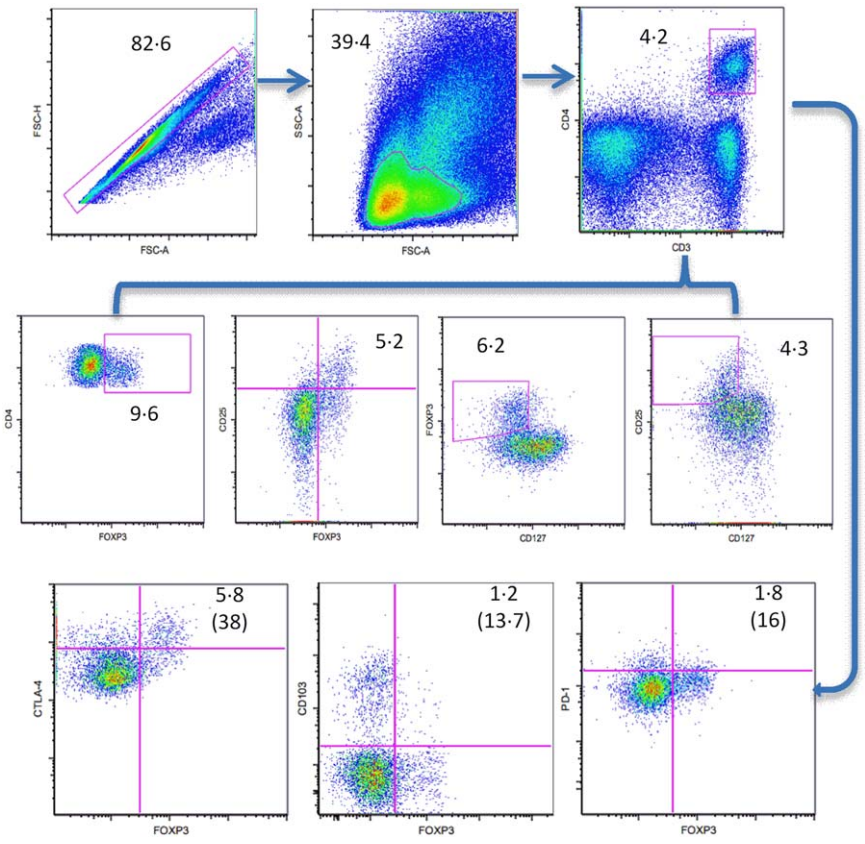

Fig. 1. Representative plots of gating strategy for flow cytometry showing different ways of human T_{reg} characterization. Upper panel shows the forward-scatter area (FSC-A) *versus* forward-scatter height (FSC-H) plot for doublet exclusions, followed by a FSC-A *versus* side-scatter area (SSC-A) plot to delimitate the lymphocytes region. Then, a CD3 *versus* CD4 plot was used to gate $CD4^+$ T cells, and percentages of $CD4^+$forkhead box protein 3 ($FoxP3^+$), $CD25^+FoxP3^+$, $CD127^{-/low}FoxP3^+$ and $CD127^{-/low}$ are shown on plots from rectal biopsies of healthy volunteers. Lower panel shows representative plots and percentages of $FoxP3^+$cytotoxic T lymphocyte antigen-4 ($CTLA\text{-}4^+$) $FoxP3^+CD103^+$ and $FoxP3^+$programmed death-1 ($PD\text{-}1^+$) cells on $CD4^+$ gate. Numbers in brackets represent the % of $CD4^+FoxP3^+$ T cells positive for CTLA-4, CD103 and PD-1. Clone and fluorochrome conjugates from the monoclonal antibodies used are shown in Supporting information, Table S1. Clone of anti-FoxP3 was PCH101.

percentages of $CD4^+FoxP3^+$ T_{regs} have been observed in frozen samples [30], which have been demonstrated previously to reduce the pool of $CD4^+CD25^+FoxP3^+$, specifically in cells from HIV-infected individuals [31]. However, some authors have reported comparable values of T_{regs} in frozen samples and fresh samples, suggesting that these variations could be related to differences between cryopreservation and thawing protocols. To detect FoxP3 by flow cytometry, clone PCH101 (e-Bioscience, San Diego, CA, USA) has been used widely with the highest detection of FoxP3; however, other clones such as 259D/C7, 236A/E7 and 206D have detected FoxP3 correctly, whereas clones 150d and 3G3 yielded the lowest values [32]. In addition, detection of FoxP3 is linked to the fixation and permeabilization protocols used. In our experience, which is supported by other studies, the combination of clone PCH101 and the e-Bioscience permeabilization buffer gave optimal results [32] in comparison to other clones and permeabilization buffers. We have also demonstrated good resolution between $FoxP3^+$ and $FoxP3^-$ events when anti-FoxP3 is coupled to the fluorescein isothiocyanate (FITC) fluorochrome. However, other authors have reported that Alexa647 showed a better separation to detect $FoxP3^+$ events than the R-PE or FITC fluorochromes [32], and between PE and FITC, PE could be more effective in the detection of $FoxP3^+$ events [33,34].

We have observed the highest proportion of T_{regs} in samples from rectal mucosae compared to peripheral blood (approximately 9%, Table 1, Fig. 1 [29]), which could be related to a major local tolerance for the continuous exposition to exogenous antigens from the microbiota or diet [35]. However, other authors have found similar percentages of T_{regs} in rectal samples and peripheral blood [36], which could be related to aggressive procedures used to obtain rectal mononuclear cells, affecting the expression of those markers.

Determination of FoxP3 and CD25 could share some disadvantages, as these molecules can be up-regulated following *in-vitro* activation in T_{cons} [21,37–39]; however, it has also been suggested that up-regulation of FoxP3 in T_{cons} under these conditions is due to TGF-β present in the serum of culture media, without conferring a suppressive function [40]. In addition, expression of FoxP3 can be down-regulated in inflammatory microenvironments, where T_{regs} are called 'ex-T_{regs}' [41]. Moreover, as we have reported, CD25 can also be down-regulated during choric immune activation such as HIV infection [29], where a reduction in IL-2 levels is observed [42]. Another drawback of the use of FoxP3 is related to the necessity of fixation/permeabilization protocols preventing the isolation of viable cells for functional experiments.

In the early 2000s, the addition of FoxP3 to CD25 efficiently improved the identification of T_{regs}, and this strategy has been used widely by us (Fig. 1) and others [28]. However, such a combination of CD25/FoxP3 is not perfect, as the expression of these markers can change depending on the inflammatory status, as mentioned above. In July 2006, two groups introduced a new marker for the identification of T_{regs} [43,44]; they found that low or lack of expression of CD127 in $CD25^+$ T cells identify human T_{regs} with a potent suppressive function [45]. In fact, this strategy has been used commonly for isolation of T_{regs} to assess their functional capacity. However, this strategy may not correspond exactly with $CD4^+CD25^+FoxP3^+$ T_{regs}, as approximately 15% of $CD25^+CD127^{low/-}$ do not express FoxP3 [43,44] and $< 10\%$ of $CD25^+FoxP3^+$ cells retained high expression levels of CD127 [43]. Thus, as other markers to identify T_{reg} cells, CD127 expression has been show to be variable even in healthy individuals [46], and it can be also down-regulated in T_{cons} during chronic activation (HIV infection) or after *in-vitro* activation [47,48].

Similar to other studies [44,49,50], we have shown that in peripheral blood of healthy individuals, the percentage of $CD4^+CD25^+CD127^{-/low}$ cells varies from 6 to 8% of $CD4^+$ T cells (Table 1). However, other authors have found higher values ranging from 7·2 to 30·4% (mean 17·6%) [46]; such a high frequency of $CD25^+CD127^{low/-}$ T_{regs} observed in these healthy donors could be due to decreased expression of CD127 molecules after cryopreservation [24]. In agreement with other researchers [43], we have found that peripheral $CD4^+$ $FoxP3^+$ T_{regs} in healthy individuals are highly comparable to those $CD25^+$ $CD127^{-/low}$ T_{regs}, as a high correlation between both strategies to analyse T_{reg} populations has been observed ($r = 0{\cdot}75$, $P = 0{\cdot}0008$). However, when the $CD25^+FoxP3^+$ combination was used, the strength of the correlation with $CD4^+FoxP3^+$ T_{regs} decreased ($r = 0{\cdot}51$, $P = 0.03$). We have reported that in chronic inflammation, as in HIV infection, there is a high correlation between the $CD4^+FoxP3^+$ and $CD127^{-/low}FoxP3^+$ protocols for T_{reg} identification [51]. Conversely, the $CD25^+FoxP3^+$ combination correlates strongly with $CD4^+FoxP3^+$ in rectal mucosae of healthy humans ($r = 0{\cdot}70$, $P = 0{\cdot}02$), rather than with the $CD127^{-/low}FoxP3^+$ combination, probably reflecting the fact that in mucosal tissues there is a particular regulation of CD127 expression on T_{regs}.

Considering the issues regarding the activation status that can modulate the expression of these markers, the use of a T_{reg} basic panel could be constituted by CD3, CD4, CD25, CD127 and FoxP3 which, in conjunction, may facilitate T_{reg} identification.

Expression of CD25, FoxP3 and CD127 to identify T_{regs} in mice

In mice, the identification of T_{regs} as $CD4^+CD25^+$ T cells has been used widely, representing approximately 5–10% of $CD4^+$ T cells in the periphery [28]. We have observed higher percentages of T_{regs} in lymphoid tissues, such as spleen (15·2%) and lymph nodes (14·3%) of healthy BALB/c mice (Table 1, Fig. 2), which corresponds with values reported previously in other studies [52]. Slight differences in the levels of these cells could

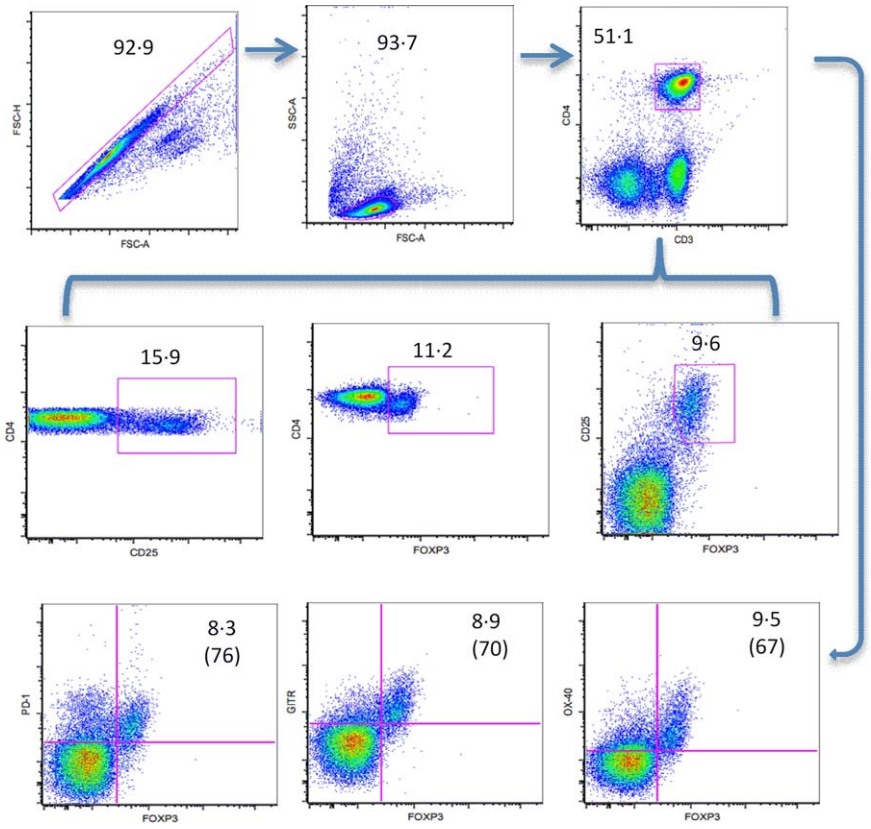

Fig. 2. Representative plots of gating strategy for flow cytometry showing different ways of characterizing mouse T_{regs}. Upper panel shows the forward-scatter height (FSC-H) *versus* forward-scatter area (FSC-A) plot for doublet exclusions, followed by a FSC-A *versus* side-scatter area (SSC-A) plot to delimitate the lymphocyte region. Then, a CD3 *versus* CD4 plot was used to gate $CD4^+$ T cells, and percentages of $CD4^+CD25^+$, $CD4^+$forkhead box protein 3 ($FoxP3^+$) and $CD25^+FoxP3$ cells are shown on plots from lymph nodes of healthy BALB/c mice. Lower panel shows representative plots and percentages of $FoxP3^+$programmed death-1 ($PD\text{-}1^+$), $FoxP3^+$glucocorticoid-induced tumour necrosis factor (TNF) receptor-related protein (GITR) and $FoxP3^+OX\text{-}40^+$ cells on $CD4^+$ gate. Numbers in brackets represent the % of $CD4^+FoxP3^+$ T cells positive for PD-1, GITR and OX-40. Clone and fluorochrome conjugates from the monoclonal antibodies used are shown in Supporting information, Table S1. Clone of anti-FoxP3 was FJK-16s.

be due to the microorganism-free environment in which mice are maintained, to their age or strain [53,54]. Similarly, gating strategy excluding cells expressing low levels of CD25 could be excluding T_{regs} receiving deficient co-stimulator signals (CD28 or IL-2) [55,56]. Although the use of CD25 as surrogate marker of functional T_{regs} was suggested previously, CD25 can also be expressed in T_{cons} after activation [16], as it can be lost in T_{regs} from aged mice even though they maintain their suppressive function [57]. With regard to the anti-CD25 clone, we have used PC61, which is used frequently.

Regarding FoxP3 expression, we have found 13 and 11% $CD4^+$ T cells expressing FoxP3 in spleen and lymph nodes, respectively (Table 1, Fig. 2); similar values have been reported by others [58]. As well as in humans, the co-expression of CD25 and FoxP3 for the characterization of T_{regs} in rodents is accepted widely. Analyses using both markers have shown a decreased frequency of this subpopulation when compared to expression of total FoxP3 alone (Table 1). This could be explained by the transient loss of CD25 expression in $CD4^+FoxP3^+$ T cells after chronic activation by internalization of the IL-2/IL-2R complex [59], or pT_{regs} with an effector memory phenotype that in steady-state conditions has a low level of CD25 [60]. Nevertheless, after homeostatic proliferation or activation, CD25 expression could be restored, constituting a T_{reg} reservoir [61]. We have found a high correlation between the $CD25^+FoxP3^+$ and $CD4^+FoxP3^+$ protocols for T_{reg} identification in spleen ($r = 0{\cdot}82$, $P = 0{\cdot}03$) of healthy mice. In addition, the use of the anti-Foxp3 FJK-16s clone conjugated with PE, in combination with permeabilization buffer from e-Bioscience, allows a clearer separation of $FoxP3^+$ cells compared to the MF23 clone [62].

The use of CD127 to identify mice T_{regs} has also been suggested, because their expression levels are lower than in $CD4^+CD25^-$ cells [63], and the depletion of T_{regs} through anti-CD25 antibodies leads to a 90% reduction of $CD4^+CD25^{high}CD127^{low}$ cells [64]. Similar to what occurs in humans, the use of this marker could also be controversial, as there is a subpopulation of activated $CD4^+FoxP3^+$ T_{regs} with high expression of CD127; in particular, T_{regs} expressing CD103 (*Integrin* α_E) and the inducible co-stimulatory molecule (ICOS) [65]. It is thought that the variation of CD127 expression in T_{regs} is mediated by their anatomical localization rather than by representing an intrinsic characteristic of T_{regs} [65]. Consequently, the identification of mice T_{regs} requires at least the use of CD3, CD4, CD25 and FoxP3.

Expression of CD25, FoxP3 and CD127 to identify T_{regs} in rats

Characterization of T_{regs} in rats is less common, due mainly to lower availability of genetically modified animals and rat-specific monoclonal antibodies. In general, the frequency of $CD4^+CD25^+$ T cells in Wistar rats is approximately 8% in peripheral and lymphoid tissue (Table 1, Fig. 3) and is supported by other studies in CD-IGS and Dark Agouti rats

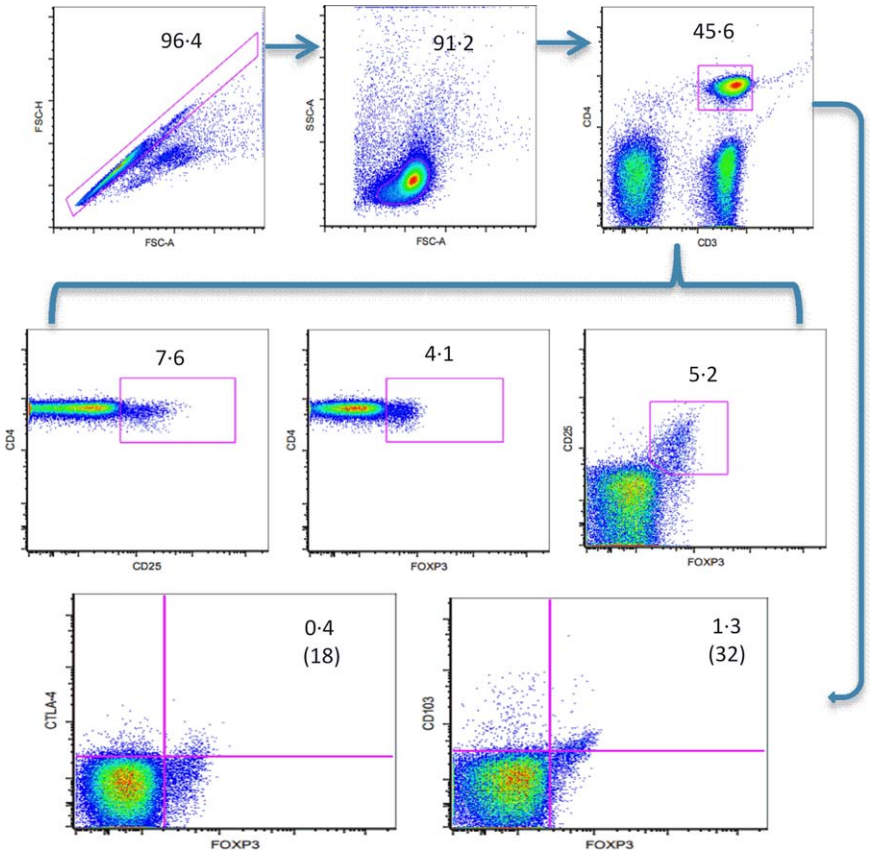

Fig. 3. Representative plots of gating strategy for flow cytometry showing different ways for rat regulatory T cell (T_{reg}) characterization. Upper panel shows the forward-scatter height (FSC-H) *versus* forward-scatter area (FSC-A) plot for doublets exclusions followed by a FSC-A *versus* side-scatter area (SSC-A) plot to delimitate the lymphocyte region. Then, a CD3 *versus* CD4 plot was used to gate $CD4^+$ T cells, and percentages of $CD4^+CD25^+$, $CD4^+$forkhead box protein 3 ($FoxP3^+$) and $CD25^+$FoxP3 cells are shown on plots from lymph nodes of healthy Wistar rats. Lower panel shows representative plots and percentages of $FoxP3^+$ cells, cytotoxic T lymphocyte antigen-4 ($CTLA\text{-}4^+$) and $FoxP3^+CD103^+$ cells on the $CD4^+$ gate. Numbers in brackets represent the % of $CD4^+FoxP3^+$ T cells positive for CTLA-4 and CD103. Clone and fluorochrome conjugates from the monoclonal antibodies used are shown in Supporting information, Table S1. Clone of anti-FoxP3 was FJK-16s.

[66,67]. In addition, we have found approximately 7·6% and 6·3% of $CD4^+FoxP3^+$ T_{regs} in peripheral blood and lymph nodes, respectively, which seems to be similar to previously reported percentages [68]. Similar to humans and mice, not all $CD4^+FoxP3^+$ T_{regs} express CD25; only approximately 60% are $CD25^+$ (Table 1). In addition, we have found approximately 4% of $CD4^+$ T cells co-expressing CD25 and FoxP3 in the periphery and lymphoid tissues of Wistar rats (Table 1, Fig. 3), similar to reports in Lewis rats [69] and Sprague–Dawley rats [70].

In addition, we have observed that the levels of $CD4^+FoxP3^+CD25^+$ T_{regs} in rats are comparable with those in humans (Table 1), as also shown by Jacobo *et al.* [71]. Because the $CD25^+FoxP3^+$ combination correlates strongly with the $CD4^+FoxP3^+$ protocol in spleen ($r = 0{\cdot}85$, $P < 0{\cdot}0001$), peripheral blood ($r = 0{\cdot}72$, $P = 0{\cdot}003$) and lymph nodes ($r = 0{\cdot}78$, $P = 0{\cdot}0008$) of rats, and to a lesser extent of CD127 to characterize rat T_{regs}, $CD25^+FoxP3^+$ is the best way to identify them.

Phenotypical characterization of T_{regs} in humans, mice and rats

Although several phenotypical markers have been used to characterize T_{regs}, we have focused on some classical molecules for which there is a large body of evidence in the literature concerning the expression of these molecules in T_{regs}, along with our own experience using them in T_{regs} in humans and rodents.

CTLA-4

CTLA-4 expression in T_{regs} is not only important for their suppressive function, but also to regulate their homeostasis and frequency in steady-state conditions [72].

In humans, several authors have reported that $CD4^+CD25^{+/high}$ T cells express intracellular CTLA-4 constitutively [21,22], which correlates with a high suppressive activity, even in the $CD4^+CD25^+FoxP3^-$ subset [73]. However, the percentages of T_{regs} expressing intracellular CTLA-4 are variable, and seem to depend upon the tissues recovered and on interindividual variations. For instance, some researchers have reported that between 50 and 90% of the peripheral $FoxP3^+$ T_{regs} express CTLA-4 in healthy human adults and neonates [74,75]; however, in our population we have found approximately 23% T_{regs} expressing CTLA-4 in peripheral blood (Table 1), similar to that reported by Toussirot *et al.* [76]. In our experience in rectal mucosae, approximately 50% $FoxP3^+$ T_{regs} express CTLA-4 (Table 1, Fig. 1).

In mice, the expression of CTLA-4 by T_{reg} cells can vary depending on the tissue evaluated; in lymphoid tissue approximately 50% of T_{regs} express CTLA-4 [77], whereas

in peripheral blood it is approximately 25% [78]; moreover, these values can increase with age [77].

In rats, T_{reg} phenotypes have been less studied; we have observed that approximately 20% of $CD4^+FoxP3^+$ T_{regs} from blood, spleen and lymph nodes express CTLA-4 (Table 1, Fig. 3); also, in purified splenic $CD4^+CD25^+$ T_{regs} from Wistar rats the expression has been reported to be approximately 40% [79], whereas in Sprague–Dawley rats a lower frequency (approximately 1%) of splenic CTLA-4^+ T_{regs} was observed [80].

Integrin α_E (CD103)

It has been reported that $CD4^+CD25^+CD103^+$ T_{regs} produce high amounts of IL-10 and exhibit the strongest suppressor capacity [81]. In humans it was reported that CD103 is expressed by fewer than 5% of T_{regs} in the blood [75,82]. We have also found approximately 9% of $CD103^+$ T_{regs} in blood and approximately 20% in biopsies from rectal mucosae (Table 1, Fig. 1). The high frequency of these cells in rectal mucosae could be associated with their memory phenotype and with a TGF-β-rich microenvironment provided by $CD103^+$ DCs [83].

Most of the evidence for the role of CD103 in T_{regs} has been reported in mice; in steady state approximately 24% of lymphoid (non-mesenteric lymph nodes) and splenic T_{regs} can express this molecule [78,84,85]; however, in gut tissue the proportions of T_{regs} expressing CD103 are increased, especially in the lamina propria, with percentages of approximately 80% [85].

We have found that approximately 39% of the peripheral blood and lymph nodes $FoxP3^+$ T_{regs} express CD103 in Wistar rats, whereas in spleen approximately 30% express CD103 (Table 1, Fig. 3). In Sprague–Dawley rats it has been observed that approximately 10% of splenic $Foxp3^+$ T_{reg} express CD103 [80].

Programmed death-1 (PD-1)

In healthy individuals, we have observed approximately 4 and 17% of human $CD4^+FoxP3^+$ T_{regs} expressing PD-1 in peripheral blood and rectal mucosae, respectively (Table 1, Fig. 1 [74]). However, under certain conditions, such as chronic viral infection, their expression could be up-regulated [11]. Conversely, compartmentalization of PD-1 expression has been reported, as approximately 90% of the $CD4^+FoxP3^+$ in healthy mice express PD-1 intracellularly in lymph nodes and spleen [86], while we have found that more than 50% of mouse $CD4^+FoxP3^+$ T_{regs} in lymphoid tissue express PD-1 on the surface (Table 1, Fig. 2), which might correspond to a effector/memory phenotype. So far, there are no reports about PD-1 expression in rats.

GITR

GITR (CD357) is a member of the TNF receptor superfamily (TNF-RSF) expressed constitutively in T_{regs} and up-regulated in T_{cons} after activation [78]. It has been demonstrated to be crucial for their thymic development, enhancing sensitivity to IL-2 [87]. The role of GITR on T_{regs} is controversial in the periphery, as GITR engagement favours the expansion of competent functional T_{regs} preferentially [88]; however, other reports have suggested that signalling mediated by GITR in T_{regs} leads to down-regulation of different pathways that control their suppressive function [89]. Moreover, it was proposed recently that GITR can inhibit FoxP3 cell induction, and in turn induce polarization to $Th9^+$ cells mediated by epigenetic changes [90].

In humans, we have observed approximately 10% of $CD4^+FoxP3^+$ or $CD4^+CD25^+FoxP3^+$ expressing GITR in peripheral blood (Table 1), in agreement with the results of Li *et al.*, who found approximately 15% of $CD4^+CD25^+GITR^+$ cells in healthy donors, whereas only 5·2% of the $CD4^+CD25^-$ cells expressed this marker [91]. In contrast, in lamina propria of colonic mucosae approximately 40% of $CD4^+CD25^+$ cells can express GITR [92].

In mice, high expression levels of GITR have been observed in different tissues. We observed that more than 75% of T_{regs} in lymphoid tissues from BALB/c mice express this molecule (Table 1, Fig. 2). Previous studies in BALB/c and C57BL/6 mice have also reported that more than 80% of $FoxP3^+$ T_{regs} express GITR in the spleen, lymphoid nodes, peripheral blood and thymus [52,77]. The high expression of GITR in T_{regs} from lymphoid tissues could be due the high proportion of memory T_{regs} found in these compartments compared to blood [93].

So far, there are no reports about GITR expression in rats.

OX40 (CD134)

OX40 is a co-stimulatory molecule that belongs to the TNF-RSF family. It is expressed constitutively in T_{regs}, although it is also up-regulated in T cells following activation [78]. As well as GITR, OX40 triggering on T_{cons} and $CD8^+$ T cells promotes their proliferation and survival [94], but their function on T_{regs} is controversial. Some researchers have demonstrated that the OX40 signal promotes proliferation and survival of $CD4^+FoxP3^+$ T_{regs} *in vitro* and *in vivo* [95,96]; however, some evidence suggests that it did not appear to affect T_{reg} homeostasis [97,98]. Moreover, OX40 can also down-regulate FoxP3 expression and impair the suppressive capacity of T_{regs} [98,99]. Probably, a dual effect as observed in T cell subpopulations could be attributed to the ligation strength of OX40, as high concentrations of the anti-hOX40 antibody decreases T_{reg} suppressive function and cell numbers preferentially but maintains the proliferation of naive and memory T cells that become resistant to suppression by T_{regs} [100]. In addition, ligation of OX40 from T_{regs} with OX40L on T_{cons} partially boosts the T_{reg} survival capacity in low inflammation conditions, where other factors such as TNF and

plasmacytoid DCs could act to help T_{reg} activation in such conditions [101]. Furthermore, in highly inflammatory conditions OX40 seems to mediate T_{reg} accumulation in the colon and peripheral lymphoid organs [102].

In humans, it has been reported that OX40 expression in $CD25^+CD127^{-/low}FoxP3^+$ T_{regs} is ∼10%; on the contrary, in young and aged BALB/c mice its expression is approximately 60% in splenic $CD4^+FoxP3^+$ T_{regs} and values of approximately 80% in colonic T_{regs} has been observed [102,103]. We have observed similar percentages of OX40 in BALB/c mice in $CD4^+FoxP3^+$ T_{regs} from secondary lymphoid tissues (Table 1, Fig. 2). Conversely, the percentages of OX40 in splenic $CD4^+CD25^+$ T_{regs} are approximately 17% in Lewis rats [104].

Conclusions

The establishment of different experimental scenarios generated through polyclonal or antigen-specific activation or through *in-vivo* models mimicking steady state or low-/high-grade inflammation conditions have allowed delineation of the biology of T_{regs}. Several antibodies have been used to identify and characterize the T_{reg} populations, using the differential expression of markers, which could be mediating functional advantages. However, as these molecules can be expressed by other cells, it is necessary to select the combination of markers with the lowest variability, being particularly cautious when inflammatory conditions could be present. Similarly, the frequency and phenotype of T_{regs} can differ according to evaluated tissue or species. Finally, among the key points regarding T_{reg} typification that need to be considered are the following:

- Use viability stain, especially when handling cryopreserved samples.
- A proper selection of the antibody clones and fluorochromes. Information about the antibodies that we have used is shown in Supporting information, Table S1.
- Titration of antibodies and, where possible, using the same batch of antibodies.
- To define positive events it is recommended to use fluorescence minus one (FMO) rather than isotype controls. In some cases, negative populations that do not express the specific marker can also help to identify positive populations.
- Avoid discrimination of the T_{reg} population using high or intermediate expression levels of markers composing the T_{reg} panel.
- The minimum panel to characterize T_{reg} cells from all species should be CD3, CD4, CD25, and FoxP3.
- On flow cytometry, collect at least 100 000 gated lymphocytes for each sample.

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Disclosure

The authors declare that there are no disclosures regarding the publication of this paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Antibodies used for flow cytometry