Calcium-mediated shaping of naive CD4 T-cell phenotype and function

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Abstract

Continuous contact with self-major histocompatibility complex ligands is essential for the survival of naive CD4 T cells. We have previously shown that the resulting tonic TCR signaling also influences their fate upon activation by increasing their ability to differentiate into induced/peripheral regulatory T cells. To decipher the molecular mechanisms governing this process, we here focus on the TCR signaling cascade and demonstrate that a rise in intracellular calcium levels is sufficient to modulate the phenotype of mouse naive CD4 T cells and to increase their sensitivity to regulatory T-cell polarization signals, both processes relying on calcineurin activation. Accordingly, in vivo calcineurin inhibition leads the most self-reactive naive CD4 T cells to adopt the phenotype of their less self-reactive cell-counterparts. Collectively, our findings demonstrate that calcium-mediated activation of the calcineurin pathway acts as a rheostat to shape both the phenotype and effector potential of naive CD4 T cells in the steady-state.

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Introduction

T-cell precursors originate in the bone-marrow and are educated in the thymus through processes called positive and negative selections, which result in MHC-restriction and self-tolerance, respectively (Stritesky et al., 2012). Only those T cells that bear an αβ T-cell receptor (TCR) recognizing self-MHC with a relatively low affinity will differentiate and exit into the systemic circulation as self-MHC restricted T cells. T cells carrying an αβ TCR that reacts with self-MHC with very low affinity die by neglect, whereas those recognizing self-MHC with high affinity are mostly deleted by apoptosis or differentiate into regulatory T cells called ‘natural’ or thymically derived (tTreg) in order to prevent autoimmunity (Bautista et al., 2009; Leung et al., 2009). Therefore, self-MHC and the associated self-reactivity of T cells influence both T-cell production and phenotype in the thymus.

In the periphery, the pre-immune repertoire of T cells is composed of almost 70% of naive T cells. The remaining 30% are divided between recent thymic emigrants with a comparable phenotype, regulatory T cells (Foxp3+) and cells with an activated/memory phenotype. Naive T cells are kept alive through continuous TCR interactions with MHC molecules complexed with various self-peptides. Such TCR/MHC interactions plus contacts with IL-7 cause low-level signaling, which promotes long-term survival of naive T cells in interphase through the synthesis of anti-apoptotic molecules such as Bcl-2 (Martin et al., 2006; Takada and Jameson, 2009).

The degree of TCR self-reactivity of a given T-cell clone has been correlated with its expression of CD5 and Nur77 (Azzam et al., 1998; Moran et al., 2011). We have recently identified the cell surface GPI-anchored protein, Ly-6C, as an additional and complementary sensor of T-cell self-reactivity (Martin et al., 2013). Indeed, we have shown that, in contrast to CD5 and Nur77 which expression directly correlates with self-reactivity, the expression of Ly-6C by peripheral naive CD4 T cells (CD4
T<sub>N</sub> cells) inversely correlates with their ability to interact with self-MHC (Martin et al., 2013). Ly-6C<sup>-</sup> CD4 T<sub>N</sub> cells were therefore identified as more self-reactive than their Ly-6C<sup>+</sup>-cell counterparts.

In the absence of foreign antigen, peripheral naive T cells continuously recirculate between lymphoid organs (Gowans, 1959), in which they migrate along the fibroblastic reticular cells network (Bajénoff et al., 2006) and interact frequently and briefly with dendritic cells (DC) (Bajénoff et al., 2006; Mempel et al., 2004). It is generally accepted that these frequent DC-T-cell interactions increase the probability of contacts between very rare antigen-specific naïve T cells and the few DCs presenting their cognate antigen during the initial course of an infection. Experimental evidences indicate that self-MHC recognition in the periphery is also required to maintain T cells in a state of responsiveness toward foreign antigen (Persaud et al., 2014; Stefanová et al., 2002; Wülfing et al., 2002), suggesting a crucial role for self-MHC mediated ‘education’ and TCR self-reactivity in determining the intrinsic functional attributes of CD4 T<sub>N</sub> cells. Altogether, this steady-state tonic TCR signaling was shown to influence CD4 T<sub>N</sub>-cell effector fate by increasing the magnitude of their response toward their cognate antigens.

Following activation by antigen-presenting cells (APCs) in the periphery, the bulk of CD4 T<sub>N</sub> cells can differentiate into a variety of well documented T-helper (T<sub>H</sub>) cell subsets, such as T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 or peripherally induced regulatory T cells (pTreg cells), characterized by their cytokine production.
profiles, specific effector functions and lineage-specific transcription factors (T-bet for T_{H}1 cells, GATA-3 for T_{H}2 cells, RORγt/RORα for T_{H}17 cells and Foxp3 for pTreg cells) (Abbas et al., 1996; Bilate and Lafaille, 2012; Fontenot et al., 2003; Hori et al., 2003; Ivanov et al., 2006; Liang et al., 2006; Mosmann et al., 1986; Szabo et al., 2000; Ye et al., 2001; Zheng and Flavell, 1997). Among these effector CD4 T cells, pTreg cells produce TGF-β and share phenotypic and functional characteristics with iTreg cells (Bilate and Lafaille, 2012). The immunological context in which CD4 T_{N} cells are immersed at the time of their activation is known to drive lineage commitment. The strength of the activating TCR signals received by a CD4 T_{N} cell also influences its subsequent polarization toward particular differentiation pathways (Corse et al., 2011). Indeed, in weakly polarizing conditions, low TCR signals favor T_{H}1- and T_{H}17-cell differentiation, whereas T_{H}1- and T_{H}17-cell differentiation arises from stronger signals (Gottschalk et al., 2010; Rogers and Croft, 1999; Turner et al., 2009). Most of these data were obtained in vitro by modulating signal strength with graded dose of peptide-MHC ligands of varying potency. However, only relatively high-affinity TCR–MHC interactions were shown to facilitate the induction of persistent Foxp3^+ T cells in vivo (Gottschalk et al., 2010).

Our recent work has reinforced the link between the tonic TCR signaling received by CD4 T_{N} cells in the steady-state and their fate in the effector phase. Indeed, we have demonstrated that TCR/self-MHC interactions not only increase quantitatively but also shape qualitatively the response of CD4 T_{N} cells to their cognate antigens in the effector phase (Martin et al., 2013). More precisely, by taking advantage of our data showing that Ly-6C expression can be considered as a new sensor of CD4 T_{N}-cell self-reactivity, we have demonstrated that CD4 T_{N} cells with the highest avidity for self-MHC (Ly-6C^+) have a biased commitment toward the iTreg/pTreg-cell lineage (Martin et al., 2013).

The binding of antigen/MHC complexes to the TCR triggers the recruitment of a series of signaling molecules and adaptors to the TCR/CD3 complex that ultimately results in the phosphorylation and activation of phospholipase C-γ (PLCγ). PLCγ then cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane to generate diacylglycerol, which activates protein kinase C (PKC) and Ras-dependent pathways, as well as 1,4,5-inositol triphosphate (IP3), which induces the release of calcium (Ca^{2+}) from intracellular stores (the endoplasmic reticulum (ER)). This Ca^{2+} store release only transiently elevates intracellular Ca^{2+} concentrations but this transient rise induces in turn a massive and sustained Ca^{2+} entry from the extracellular space (Hogan et al., 2010).

With the aim of deciphering the molecular mechanisms involved in the tonic TCR-signaling-mediated shaping of the CD4 T_{N}-cell compartment, we have focused on the TCR signaling cascade. By using transcriptomic and phenotypic approaches as well as in vitro and in vivo assays, we have identified the Ca^{2+} signaling pathway as key for the acquisition of both the phenotype of the most self-reactive CD4 T_{N} cells and their enhanced cell-intrinsic ability to commit into regulatory T cells upon activation in vitro (iTreg) and in vivo (pTreg).

**Results**

**Cell-intrinsic enhanced ability of Ly-6C^- CD4 T_{N} cells to commit into iTreg cells**

We have recently shown that CD4 T_{N} cells with the highest avidity for self-MHC (Ly-6C^- CD4 T_{N} cells) have a biased commitment toward the iTreg/pTreg-cell lineage (Martin et al., 2013). As T_{H}1- and T_{H}2-cell-derived cytokines are known to inhibit iTreg-cell induction in vitro (Henderson et al., 2015), we first wondered whether Ly-6C^- and Ly-6C^+ CD4 T_{N} cells had the same ability to produce such cytokines after stimulation. Ly-6C^- and Ly-6C^+ CD4 T_{N} cells were thus stimulated with αCD3- and αCD28-coated antibodies in the presence or absence of TGFβ. Interferon-gamma (IFN-γ) and interleukins (IL) -4, -17 and -10 were assayed in the supernatants collected 24 hr after the beginning of the culture. We found that, whatever the presence or absence of TGFβ in the culture medium, Ly-6C^- and Ly-6C^+ CD4 T_{N} cells produced similar amounts of these cytokines (Figure 1—figure supplement 1A,B). To further characterize the enhanced ability of Ly-6C^- CD4 T_{N} cells to commit into iTregs in vitro, we asked whether this feature was cell-intrinsic. To this end, Ly-6C^- and Ly-6C^+ CD4 T_{N} cells were isolated from peripheral LNs of C57BL/6 Foxp3-GFP mice by flow cytometry sorting, barcoded with CTv or CTv and CTfr proliferation dyes, and stimulated with αCD3- and αCD28-
coated antibodies in the presence of graded doses of TGFβ. These cells were cultured separately or together (Figure 1A,B). The percentages of Foxp3+ cells among the progeny of both naive cell-subsets were assessed on day 4. For suboptimal doses of exogenous TGFβ, Ly-6C+ CD4 T<sub>N</sub> cells gave rise to a twofold higher proportion of iTreg cells than their Ly-6C+ counterparts in both culture conditions (Figure 1C,D). The concentration of TGFβ needed to obtain 50% of the maximal percentage of iTreg cells (effective concentration, EC50) was calculated by fitting the dose-response curves of both CD4 T<sub>N</sub>-cell subsets in the different culture conditions (Figure 1D,E). EC50 values for TGFβ were statistically different between the 2 CD4 T<sub>N</sub>-cell subsets whether they were cultured separately or together. Of note, and in line with their similar ability to produce T<sub>H</sub>1- and T<sub>H</sub>2-cell-derived cytokines, blocking IFN-γ and IL-4 during in vitro iTreg-cell polarization did not abolish the difference in the ability of Ly-6C+ and Ly-6C+ CD4 T<sub>N</sub> cells to differentiate into iTreg cells. (Figure 1—figure supplement 1C–E). These results suggest strongly that the greater sensibility of Ly-6C+ CD4 T<sub>N</sub> cells to iTreg-cell polarization signals is cell-intrinsic.

Ly-6C+ CD4 T<sub>N</sub>-cell transcriptomic signature reveals both their TCR signaling activity and their bias toward iTreg-cell polarization

To further compare Ly-6C+ and Ly-6C+ CD4 T<sub>N</sub> cells, we obtained Affymetrix gene expression profiles from both CD4 T<sub>N</sub>-cell subsets directly isolated from peripheral LNs of C57BL/6 Foxp3-GFP mice by flow cytometry sorting (Figure 2). Only few genes were significantly differentially expressed between the two types of CD4 T<sub>N</sub> cells (at a 1.3-fold cutoff, 167 genes over-expressed and 164 under-expressed in Ly-6C+ CD4 T<sub>N</sub> cells when compared to Ly-6C+ CD4 T<sub>N</sub> cells; Figure 2A). This set of differentially expressed genes between Ly-6C+ and Ly-6C+ CD4 T<sub>N</sub> cells was compiled into a comprehensive signature that we named 6CSign (Figure 2B,C). The differential expression of several genes by Ly-6C+ and Ly-6C+ CD4 T<sub>N</sub> cells was then validated at the protein level by flow-cytometry (Figure 2—figure supplement 1). In line with our microarray analysis, Ly-6C+ CD4 T<sub>N</sub> cells were expressing higher amounts of CD5, CD73, CD122, CD200, lkr3 and Izumo1r and lower levels of Sca-1 and IL18Rα than their Ly-6C+ CD4 T<sub>N</sub>-cell counterparts (Figure 2—figure supplement 1A,B).

We have previously shown that Ly-6C+ CD4 T<sub>N</sub> cells were more self-reactive than Ly-6C+ CD4 T<sub>N</sub> cells (Martin et al., 2013). Accordingly, among the 6CSign, several genes such as Ctd4, Cd5, Tnfrsf4, Tnfrsf9 and Nr4a1 were previously shown to belong to activation-induced or -repressed gene families (Figure 2C; Wakamatsu et al., 2013). We thus compared more precisely our signature, the 6CSign, with several public Geo Datasets comparing various ‘activated’ CD4 T<sub>N</sub> cells to their non-activated cell counterparts (Figure 3A,B). CD5 expression levels on CD4 T<sub>N</sub> cells are actively maintained by interactions with self-MHC and rapidly decline in their absence (for example in the blood, [Stefanova et al., 2002]). In agreement with a greater self-reactivity of Ly-6C+ CD4 T<sub>N</sub> cells, the 6CSign correlated significantly with the CD5+ versus CD5- CD4 T<sub>N</sub>-cell signature (Richards et al., 2015). Interestingly, whereas the 6CSign genes also correlated with the transcriptional signature of αCD3-activated CD4 T<sub>N</sub> cells (compared to unstimulated cells) (Wakamatsu et al., 2013), there was no significant correlation with the signature of Phorbol 12-Myristate 13-Acetate (PMA)-activated CD4 T<sub>N</sub> cells (Bevington et al., 2016).

Interestingly, the 6CSign contained several genes characteristically expressed in Treg cells such as Ctd4, Izumo1r, Cd200, Lag3 or Il2rb. All these genes were upregulated in Ly-6C+ CD4 T<sub>N</sub> cells when compared to Ly-6C+ CD4 T<sub>N</sub> cells (Figure 2C). By comparing CD4 T-cell effectors with naïve CD4 T cells, Wei et al. (2009) have recently defined the transcriptional signature of the main CD4 T<sub>H</sub>-cell subsets such as in-vitro-induced Treg cells, T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells. Comparison of the 6CSign with these cell signatures revealed that the differences in gene expression observed between Ly-6C+ and Ly-6C+ CD4 T<sub>N</sub> cells correlated significantly with the in-vitro-induced Treg-cell signature, and to a lesser extent with the T<sub>H</sub>17 one but not with the T<sub>H</sub>1 or T<sub>H</sub>2 transcriptional signatures (Figure 3C).

Similarities were also observed between the transcriptional profiles of Ly-6C+ CD4 T<sub>N</sub> cells and ex vivo purified peripheral Treg cells (Figure 3C) (Wei et al., 2009). One common characteristic shared by Ly-6C+ CD4 T<sub>N</sub> cells and CD4 Treg cells is their high degree of self-reactivity. Recent studies have highlighted a continuous requirement of self-MHC recognition and of the associated TCR-mediated signaling for maintaining both the function and transcriptional signature of CD4 Treg cells (Delpoux et al., 2012; Levine et al., 2014; Vahl et al., 2014). Whereas self-deprivation or TCR-ablation did not impair the expression of the transcription factor Foxp3, they induced major transcriptional changes (Delpoux et al., 2012; Levine et al., 2014; Vahl et al., 2014). Interestingly, 6CSign
Figure 1. Cell-intrinsic enhanced ability of Ly-6C⁻ CD4 Tₙ cells to commit into iTreg cells. (A–E) Flow-cytometry sorted Ly-6C⁻ and Ly-6C⁺ CD4 Tₙ cells from C57BL/6 Foxp3-GFP mice were stained with CTv (Ly-6C⁻) or CTv and CTfr (Ly-6C⁺) and stimulated separately or together for 4 days with coated αCD3 and αCD28 Abs (4 μg/mL), in the presence of graded doses of TGFβ1. (A) Diagram illustrating the experimental protocol. (B) Representative CTv/CTfr dot-plots for gated CD4⁺ cells recovered after 4 days of culture. Ly-6C⁻ and Ly-6C⁺ CD4 Tₙ cells were either cultured separately (top left and right panels, respectively) or together (bottom panel) (C) Representative Foxp3/CD4 contour-plots and proportions of Foxp3⁺ cells for gated CD4⁺ cells are shown at a dose of 0.25 ng/mL TGFβ1. (D) Proportions of Foxp3⁺ cells among CD4⁺ cells are shown as a function of TGFβ1 concentration. Mean ± s.e.m of four independent experiments are shown. (E) Concentrations of TGFβ1 needed to obtain 50% of the maximal percentages of iTreg-cell polarization (EC50) were calculated for each CD4 Tₙ cell subset in separated or mixed cultures. Each dot represents an independent experiment. Significance of differences were assessed using a two-tailed paired Student’s t-test. Values of p<0.05 were considered as statistically significant (**p<0.01; ns, not significant).

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The following figure supplement is available for figure 1:

Figure supplement 1. Cytokine-independent enhanced ability of Ly-6C⁻ CD4 Tₙ cells to commit into iTreg cells.

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genes strongly correlated with the transcriptional signature of TCR+ CD4 Treg cells (compared to TCR- CD4 Treg cells, Figure 3D) (Vahl et al., 2014). More precisely, most genes upregulated in Ly-6C- CD4 T$_N$ cells when compared to their Ly-6C+ cell counterparts were positively regulated by steady-state TCR signaling in CD4 Treg cells (such as Cd5, Cd200, Il2rb, Itih5, Maf and Myb; Figure 3E). Conversely, an important proportion of the genes downregulated in Ly-6C- CD4 T$_N$ cells were also down-regulated by steady-state interactions with self-MHC in CD4 Treg cells (Figure 3E). Altogether, these data point to a role for the TCR signaling pathway in the installation and maintenance of the 6CSign.

Figure 2. Transcriptional profiling identifies a set of differentially regulated genes between Ly-6C- and Ly-6C+ CD4 T$_N$-cell subsets. (A–E) Microarray analysis was performed on Ly-6C- and Ly-6C+ CD4 T$_N$ cells sorted from LNs of C57BL/6 Foxp3-GFP mice. (A) ’Volcano plot’ representation (Log2 (ratio) versus Log10 (t test p-value)). Genes expressed >1.3 fold higher or lower in Ly-6C- CD4 T$_N$ cells compared to Ly-6C+ CD4 T$_N$ cells with a p-value of <0.05 are highlighted in red. The number of genes up- or down-regulated (1.3-fold cut-off) for each comparison is indicated. (B) Scheme depicting the selection of genes that were included in the 6CSign (list of the genes differentially expressed between Ly-6C- and Ly-6C+ CD4 T$_N$ cells, at a 1.3-fold cut-off). (C) Heat map of selected differentially expressed genes between Ly-6C- and Ly-6C+ CD4 T$_N$ cells. The scaled expression of each replicate, denoted as the row z-score, is plotted in yellow-blue color scale with yellow indicating high expression and blue indicating low expression. DOI: https://doi.org/10.7554/eLife.27215.005

The following figure supplement is available for figure 2:

Figure supplement 1. Validation of the 6CSign at the protein level.
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Ly-6C–CD4 T<sub>N</sub>-cell phenotype relies on the Ca<sup>2+</sup> signaling pathway in vitro

The transcriptional signature of Ly-6C–CD4 T<sub>N</sub> cells revealed some similarities between these cells and αCD3-stimulated CD4 T<sub>N</sub> cells. We therefore decided to analyze the effect of TCR signaling on...
Ly-6C expression. Ly-6C+ CD4 T+N cells were isolated from peripheral LNcs of C57BL/6 Foxp3-GFP mice by flow cytometry sorting and incubated with various stimulating agents mimicking all or part of TCR-induced signals (Figure 4A). As expected from our transcriptomic analysis and previous work (Martin et al., 2013), Ly-6C expression was clearly downregulated when cells were stimulated with αCD3 and αCD28-coated antibodies for 5 days (Figure 4A,B). To decipher which TCR-induced signals led to Ly-6C down-regulation, we roughly dichotomized the TCR signaling cascade into its two main components, for example the Ca2+ signaling pathway that can be elicited by Thapsigargin (TG) and the PKC and ERK signaling pathways activated by PMA. When combined, PMA and TG, induced complete Ly-6C down-regulation, whereas, when separated, each drug had an opposite effect on Ly-6C expression. Indeed, whereas PMA alone upregulated Ly-6C expression, TG alone induced a near-complete disappearance of Ly-6C protein at the surface of Ly-6C+ CD4 T+N cells. Interestingly, while in all other conditions, CD4 T+N cells were proliferating, this phenotypic conversion of Ly-6C+ CD4 T+N cells into Ly-6C− CD4 T+N cells induced by TG alone occurred without any proliferation (Figure 4A,B). Importantly, to avoid TG-induced cell death, a sub-optimal dose (4 nM) was used in these culture conditions. 4 nM TG induced a reproducible increase in intracellular calcium levels, although to a lesser extent than the classical dose of 200 nM (Figure 4C). Accordingly, by analyzing basal Ca2+ contents at the end of the culture period (5 days), we observed that 4 nM TG treated Ly-6C+ CD4 T+N cells exhibited higher cytoplasmic Ca2+ levels than control cells cultured in IL-7 alone (Figure 4D,E). To further characterize the long-term effect of this low-dose TG, subcellular localization of the nuclear factor of activated T-cell protein 1 (NFAT1) was assessed in Ly-6C+ CD4 T+N cells in the presence or absence of 4 nM TG at various time points along the culture. Indeed, increases in intracellular Ca2+ levels result in the activation of calcineurin that dephosphorylates members of the NFAT family, leading to their translocation into the nucleus. NFAT1 localization was quantified by high-resolution imaging flow-cytometry using the ImageStreamX technology (Figure 4F). In line with the Ca2+ increase induced by 4 nM TG treatment, NFAT was translocated into the nucleus of Ly-6C+ CD4 T+N cells in the presence of TG while it remained cytoplasmic in its absence. NFAT translocation into the nucleus peaked on day 1 and remained significantly higher in TG-treated cells than in control cells throughout the culture.

Finally, in agreement with their resting status, Ly-6C+ CD4 T+N cells cultured in TG alone for 5 days maintained a naive phenotype according to their low forward scatter profile and expression of CD44 and CD62L (Figure 4—figure supplement 1). We then studied the kinetic aspect of the TG-mediated conversion of Ly-6C+ CD4 T+N cells into Ly-6C− CD4 T+N cells and found that it occurred in 3–4 days of culture (Figure 4G).

Altogether, our results suggest that the Ca2+ signaling pathway is sufficient to induce Ly-6C down-regulation on CD4 T+N cells in vitro. We therefore hypothesized that the Ca2+ signaling pathway might be involved as part of the self-mediated tonic TCR signaling in the generation/maintenance of Ly-6C+ CD4 T+N cells in the periphery of a normal mouse in the steady-state. To evaluate the activation status of the Ca2+ signaling pathway within Ly-6C− and Ly-6C+ CD4 T+N cells in vivo in the steady-state, we analyzed NFAT1 subcellular localization in both cell types. To this aim, LN cells were isolated from peripheral LNs of C57BL/6 Foxp3-GFP mice by flow cytometry sorting and incubated with various stimulating agents mimicking all or part of TCR-induced signals (Figure 4A). As expected from our transcriptomic analysis and previous work (Martin et al., 2013), Ly-6C expression was clearly downregulated when cells were stimulated with αCD3 and αCD28-coated antibodies for 5 days (Figure 4A,B). To decipher which TCR-induced signals led to Ly-6C down-regulation, we roughly dichotomized the TCR signaling cascade into its two main components, for example the Ca2+ signaling pathway that can be elicited by Thapsigargin (TG) and the PKC and ERK signaling pathways activated by PMA. When combined, PMA and TG, induced complete Ly-6C down-regulation, whereas, when separated, each drug had an opposite effect on Ly-6C expression. Indeed, whereas PMA alone upregulated Ly-6C expression, TG alone induced a near-complete disappearance of Ly-6C protein at the surface of Ly-6C+ CD4 T+N cells. Interestingly, while in all other conditions, CD4 T+N cells were proliferating, this phenotypic conversion of Ly-6C+ CD4 T+N cells into Ly-6C− CD4 T+N cells induced by TG alone occurred without any proliferation (Figure 4A,B). Importantly, to avoid TG-induced cell death, a sub-optimal dose (4 nM) was used in these culture conditions. 4 nM TG induced a reproducible increase in intracellular calcium levels, although to a lesser extent than the classical dose of 200 nM (Figure 4C). Accordingly, by analyzing basal Ca2+ contents at the end of the culture period (5 days), we observed that 4 nM TG treated Ly-6C+ CD4 T+N cells exhibited higher cytoplasmic Ca2+ levels than control cells cultured in IL-7 alone (Figure 4D,E). To further characterize the long-term effect of this low-dose TG, subcellular localization of the nuclear factor of activated T-cell protein 1 (NFAT1) was assessed in Ly-6C+ CD4 T+N cells in the presence or absence of 4 nM TG at various time points along the culture. Indeed, increases in intracellular Ca2+ levels result in the activation of calcineurin that dephosphorylates members of the NFAT family, leading to their translocation into the nucleus. NFAT1 localization was quantified by high-resolution imaging flow-cytometry using the ImageStreamX technology (Figure 4F). In line with the Ca2+ increase induced by 4 nM TG treatment, NFAT was translocated into the nucleus of Ly-6C+ CD4 T+N cells in the presence of TG while it remained cytoplasmic in its absence. NFAT translocation into the nucleus peaked on day 1 and remained significantly higher in TG-treated cells than in control cells throughout the culture.

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Ly-6C<sup>+</sup> CD4<sup>+</sup> T<sub>N</sub>-cell phenotype relies upon calcium signaling pathway in vitro.  

(A–B) Flow-cytometry sorted Ly-6C<sup>+</sup> CD4<sup>+</sup> T<sub>N</sub> cells from C57BL/6 Foxp3-GFP mice were labelled with CTv and cultured in IL-7 (10 ng/ml) in the presence of either coated αCD3/28 (4 μg/ml), PMA and TG (1.25 ng/ml and 4 nM, respectively), PMA alone (1.25 ng/ml) or TG (4 nM). Cells were recovered and analyzed after 5 days of culture. (A) Representative Ly-6C/CTv contour-plots are shown. (B) Ly-6C Mean fluorescence intensities (MFIs), for gated CD4<sup>+</sup> TCR<sup>+</sup> cells, are shown as means ± s.e.m. for a representative

Figure 4 continued on next page

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The Ca\(^{2+}\)-calcineurin signaling pathway shapes the phenotype of the CD4 T\(_N\)-cell compartment

We have identified several proteins differentially expressed between Ly-6C\(^{-}\) and Ly-6C\(^{+}\) CD4 T\(_N\) cells (Figure 2—figure supplement 1) and have showed that TG induced Ly-6C\(^{-}\) downregulation at the cell surface of Ly-6C\(^{-}\) CD4 T\(_N\) cells. We next studied whether proteins of the 6CSign other than Ly-6C\(^{-}\), were also modulated by an increase in intracellular Ca\(^{2+}\). To go further, we examined in parallel the involvement of the calcineurin phosphatase in these processes. To this aim, Ly-6C\(^{-}\) CD4 T\(_N\) cells were isolated from peripheral LNs of C57BL/6 Foxp3-GFP mice by flow cytometry sorting and cultured in IL-7 in the presence or absence of TG and calcineurin-inhibitors (Cyclosporin A, CsA and Tacrolimus, FK506, FK). Ly-6C\(^{-}\) CD4 T\(_N\) cells cultured in IL-7 were added as control. After 5 days of culture in these conditions, the expression of Ly-6C, CD5, CD73, CD122, CD200 and Izumo1r was analyzed by flow-cytometry (Figure 5A). For all these proteins, TG induced changes in their expression at the cell surface of Ly-6C\(^{-}\) CD4 T\(_N\) cells. More precisely, their level of expression reached those observed in Ly-6C\(^{-}\) CD4 T\(_N\) cells. Blocking calcineurin activation with either CsA or FK506 led to the complete inhibition of this phenotypic conversion of Ly-6C\(^{-}\) (CD5\(^{lo}\), CD73\(^{lo}\), CD122\(^{lo}\), CD200\(^{lo}\), Izumo1r\(^{lo}\)) CD4 T\(_N\) cells into Ly-6C\(^{+}\) (CD5\(^{hi}\), CD73\(^{hi}\), CD122\(^{hi}\), CD200\(^{hi}\), Izumo1r\(^{hi}\)) CD4 T\(_N\) cells. This Ca\(^{2+}\)-induced phenotypic conversion thus depends on the activity of the canonical Ca\(^{2+}\)-calcineurin signaling pathway.

We then investigated whether this in vitro observation could be mimicked in vivo. We first confirmed that the Ca\(^{2+}\)-calcineurin signaling cascade was active in vivo in Ly-6C\(^{-}\) CD4 T\(_N\) cells by showing that blocking calcineurin activation for 18 hr with FK506 was sufficient to abrogate the nuclear localization of NFAT in these cells (Figure 5—figure supplement 1). We then wondered whether a longer treatment with this calcineurin inhibitor would affect the phenotype of CD4 T\(_N\) cells in vivo. To this aim, C57BL/6 Foxp3-GFP mice were injected daily with FK506 or PBS for 2 weeks (Figure 5B). After 14 days, CD4 T\(_N\) cells from peripheral LNs and the spleen were analyzed for their expression of Ly-6C-, Izumo1r and CD200. In line with our in vitro experiments, both the percentage of Ly-6C\(^{-}\) cells among CD4 T\(_N\) cells and the MFI of Ly-6C\(^{-}\) at the cell surface of Ly-6C\(^{-}\) CD4 T\(_N\) cells increased in treated mice when compared to control mice (Figure 5C–E). Moreover, FK506 induced significant decreases of Izumo1r and CD200 surface levels in CD4 T\(_N\) cells (Figure 5C,F). Such changes in the...
Figure 5. The calcium-calcineurin pathway shapes the phenotype of the CD4 T<sub>N</sub>-cell compartment in vivo. (A) Flow-cytometry sorted Ly-6C<sup>+</sup> CD4 T<sub>N</sub> cells from C57BL/6 Foxp3-GFP mice were cultured in IL-7 (10 ng/mL) alone or in the presence of either TG (4 nM), TG and Cyclosporin A (CsA; 50 nM) or TG and FK506 (FK; 200 nM). Flow-cytometry sorted Ly-6C<sup>-</sup> CD4 T<sub>N</sub> cells rested in IL-7 were used as control. After 5 days, cells were analyzed for their expression of Ly-6C, CD5, CD73, CD122, CD200 and Izumo1r. Representative contour-plots of cell surface markers are shown for gated CD4 T<sub>N</sub> cells.

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(CD4+ TCRβ+ CD44lo CD25lo Foxp3-GFP) as a function of culture condition. (B–F) C57BL/6 Foxp3-GFP mice were daily injected intraperitoneally with Prograf (FK506; 2.5 mg/kg) or diluent (PBS). Two weeks after treatment LNs (pooled pLNs and mLNs) and spleen were recovered and CD4 T cells were analyzed. (B) Diagram illustrating the experimental procedure. (C) Ly-6C and Izumo1r fluorescence histograms for gated CD4 T cells (CD4+ TCRβ+ CD44lo CD25lo Foxp3-GFP) recovered from LNs of PBS (white) and FK506 (grey) treated mice. (D) Percentage of Ly-6C+ cells among CD4 TTN (CD4+ TCRβ+ CD44lo CD25lo Foxp3-GFP) are shown for LNs and spleens of PBS (white) and FK506 (grey) treated mice. (E) Ly-6C Mean fluorescence intensities (MFIs), for gated Ly-6C+ TTN (Ly-6C+ TCRβ+ CD44lo CD25lo Foxp3-GFP) cells recovered from LNs of PBS (white) and FK506 (grey) treated mice, are shown as means ± s.e.m. for two independent experiments with three mice per group. (F) Izumo1r and CD200 mean fluorescence intensities (MFIs), for gated Ly-6C+ TTN (Ly-6C+ TCRβ+ CD44lo CD25lo Foxp3-GFP) cells recovered from LNs of PBS (white) and FK506 (grey) treated mice, are shown as means ± s.e.m. for a representative experiment with three mice per group. DOI: https://doi.org/10.7554/eLife.27215.011

**Figure 5 continued.**

**Figure 5** continued

**Figure 5G**. Ly-6C+ TTN cells recovered from both FK506 and PBS-treated mice (Figure 5G). 10^6 Ly-6C+ CD4 TTN cells purified from LNs of CD45.1+ Foxp3-GFP donor mice were adoptively transferred into CD45.2+ C57BL/6 Foxp3-GFP recipient mice daily injected intraperitoneally with Prograf (FK506, 2.5 mg/kg) or diluent (PBS). Two weeks after transfer and treatment, LNs (pooled pLNs and mLNs) and spleen were recovered and donor-derived CD4 T cells were analyzed. (G) Diagram illustrating the experimental model. (H) Absolute numbers of donor-derived CD4 TTN (CD45.1+ CD44lo CD25lo Foxp3-GFP) cells recovered from LNs and spleen of recipient mice are shown as means ± s.e.m. for two independent experiments with three mice per group. (I) Percentage of Ly-6C+ among donor-derived CD4 TTN (CD45.1+ CD44lo CD25lo Foxp3-GFP) cells recovered from LNs and spleen of recipient mice are shown as means ± s.e.m. for two independent experiments with three mice per group. (D, F, H, I) Each dot represents an individual mouse. (D–F, H, I) Significance of differences were assessed using a two-tailed unpaired Student’s t-test. Values of p<0.05 were considered as statistically significant (**p<0.01; ***p<0.001; ns, not significant). DOI: https://doi.org/10.7554/eLife.27215.012

The following figure supplement is available for figure 5:

**Figure supplement 1.** The calcium-calcineurin cascade drives NFAT nuclear translocation in CD4 TTN cells in vivo. DOI: https://doi.org/10.7554/eLife.27215.011

The phenotype of the bulk of CD4 TTN cells could result from either the conversion of Ly-6C+ CD4 TTN cells into Ly-6C+ CD4 TTN cells or the disappearance of the Ly-6C- cell subset. We therefore decided to compare the behavior of adoptively transferred Ly-6C+ CD4 TTN cells in FK506 or PBS-treated mice (Figure 5G). 10^6 Ly-6C+ CD4 TTN cells purified from LNs of CD45.1+ Foxp3-GFP donor mice were adoptively transferred into CD45.2+ Foxp3-GFP recipient mice. Host mice were then daily injected with FK506 or PBS for 2 weeks (Figure 5G). After 14 days, donor-derived CD4 TTN cells from peripheral LNs and the spleen were analyzed. Although similar numbers of donor-derived CD4 TTN cells were recovered from both FK506 and PBS-treated mice (Figure 5H), these cells were still greatly enriched in Ly-6C-expressing cells in FK506-treated recipients (Figure 5I).

Altogether, our data demonstrate that the activation of the Ca^{2+}-calcineurin signaling pathway drives the phenotypic conversion of Ly-6C+ CD4 TTN cells into Ly-6C+ CD4 TTN cells both in vitro and in vivo.

**Ca^{2+}-mediated shaping of the CD4 TTN-cell iTreg-cell differentiation potential**

As a rise in intracellular Ca^{2+} level converts phenotypically Ly-6C+ CD4 TTN cells into Ly-6C+ CD4 TTN cells, we then tested whether the in vitro iTreg-cell polarization potential of these ex-Ly-6C+ CD4 TTN cells (referred thereafter as ‘Ca^{2+}-converted’ Ly-6C+ CD4 TTN cells) was also modified. Ly-6C- and Ly-6C+ CD4 TTN cells were recovered from C57BL/6 Foxp3-GFP mice and cultured in vitro with or without TG. After 5 days of culture, viable cells were FACS-sorted and stimulated with αCD3- and αCD28-coated antibodies in the presence of graded doses of TGFβ for 4 days (Figure 6A). Of note, even after 5 days of resting in the presence of IL-7, Ly-6C- CD4 TTN cells were keeping a greater sensitivity to iTreg-cell polarization signals, than Ly-6C+ CD4 TTN cells cultured in the same conditions. Importantly, the iTreg-cell polarization potential of Ly-6C+ CD4 TTN cells rose up when these cells were pre-incubated in the presence of TG and became similar to the one observed for Ly-6C+ CD4 TTN cells (Figure 6B,C). In agreement with the fact that calcineurin inhibitors blocked the TG-mediated phenotypic conversion of Ly-6C+ CD4 TTN cells into Ly-6C+ CD4 TTN cells (Figure 5A), adding CsA at the time of TG pre-incubation also abrogated the sensitization of Ly-6C+ CD4 TTN cells to iTreg-cell polarization signals (Figure 6B). EC50 values for TGFβ were calculated in these conditions and were statistically different between the 2 CD4 TTN-cell subsets when cells were pre-incubated in IL-7 alone but dropped to similar levels when TG was added in the pre-culture medium (Figure 6C). Of note, pre-incubating Ly-6C- CD4 TTN cells with TG and CsA further limit their ability to commit
into iTreg cells as reflected by a significant increase in EC50 (Figure 6C). Altogether, our data demonstrate that an increase in intracellular Ca\textsuperscript{2+} levels not only shapes the phenotype of the CD4 T\textsubscript{N} cell compartment but also sensitizes in vitro these cells to iTreg-cell polarization signals, both processes occurring through a calcineurin-dependent pathway.

To confirm these data in vivo, we used the well-known model of antigen-specific pTreg-cell development induced by oral tolerance (Coombes et al., 2007; Sun et al., 2007). This protocol studies the behavior of CD4 T\textsubscript{N} cells from ovalbumin-specific TCR transgenic OT-II mice adoptively transferred into wild-type mice fed with ovalbumin (OVA). Indeed, in these conditions, a significant proportion of OT-II cells rapidly differentiate into pTreg cells in the mesenteric lymph nodes and Peyer Patches of recipient mice. Most OT-II CD4 T\textsubscript{N} cells expressed Ly-6C ex vivo (Figure 7—figure supplement 1A). FACS-sorted CD45.1/2\textsuperscript{+} OT-II CD4 T\textsubscript{N} cells were first cultured in IL-7 in the presence or absence of TG (Figure 7A). After 5 days of culture, TG led to a marked downregulation of Ly-6C (Figure 7—figure supplement 1B). Living cells were then FACS-sorted and 0.5–1.10\textsuperscript{6} cells were adoptively transferred into CD45.1 Foxp3-GFP mice. Finally, recipient mice were fed or not for 7 days.

**Figure 6.** Ly-6C\textsuperscript{−} CD4 T\textsubscript{N}-cell sensitization to iTreg-cell polarization signals relies upon calcium signaling pathway in vitro. Flow-cytometry sorted Ly-6C\textsuperscript{−} and Ly-6C\textsuperscript{+} CD4 T\textsubscript{N} cells from C57BL/6 Foxp3-GFP mice were cultured in IL-7 (10 ng/mL) with or without TG (4 nM) and CsA (50 nM). After 5 days, live cells were flow-cytometry sorted and stimulated with coated αCD3 and αCD28 Abs (4 μg/ml) in the presence of graded doses of TGFβ1. Cells were analyzed after 4 days of stimulation. (A) Diagram illustrating the experimental procedure. (B) Representative Foxp3/CD4 contour-plots and proportions of Foxp3\textsuperscript{+} cells for gated CD4\textsuperscript{+} cells are shown at a dose of 0.25 ng/mL TGFβ1, as a function of pre-culture condition. (C) Concentrations of TGFβ1 needed to obtain 50% of the maximal percentage of iTreg-cell polarization (EC50) were calculated for each CD4 T\textsubscript{N}-cell subset and each pre-culture condition. Each dot represents an independent experiment. Significance of differences were assessed using a two-tailed paired Student’s t-test. Values of p<0.05 were considered as statistically significant (*p<0.05; **p<0.01; ***p<0.001; ns, not significant).

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days with OVA in their drinking water. As expected, OVA administration led to the activation of OT-II cells, as reflected by a significant CD44 upregulation at their cell surface (Figure 7—figure supplement 1C). Similar numbers of OT-II CD4 T cells were recovered from the secondary lymphoid organs of OVA-fed mice whether they were initially injected with ‘Ca\(^{2+}\)-converted’ or not OT-II CD4 T\(N\) cells (Figure 7B). In all secondary lymphoid organs, Ca\(^{2+}\)-converted OT-II CD4 T\(N\) cells gave rise to greater proportions and absolute numbers of Foxp3-expressing cells than OT-II CD4 T\(N\) cells cultured with IL-7 alone prior to injection (Figure 7C–E). Specifically, a total of 1.16 ± 0.22 \times 10^5 pTreg (Foxp3-expressing) cells were recovered from the whole periphery of recipient mice injected with Ly-6C\(^+\) OT-II CD4 T\(N\) cells compared to 2.26 ± 0.32 \times 10^5 pTreg cells when mice were injected with Ca\(^{2+}\)-converted OT-II CD4 T\(N\) cells (p<0.05).

These latter results were confirmed by using a second protocol of oral administration of OVA. In this setting, ‘Ca\(^{2+}\)-converted’ OT-II CD4 T\(N\) cells were co-transferred with OT-II CD4 T\(N\) cells cultured in IL-7 alone in order to compare their ability to convert into pTreg in the same recipient mice. FACS-sorted CD45.2\(^+\) and CD45.1/2\(^+\) OT-II CD4 T\(N\) cells were first cultured in IL-7 in the absence or presence of TG, respectively (Figure 7—figure supplement 1A). After 5 days of culture, living cells were FACS-sorted, mixed at a 1:1 ratio and 1.10\(^6\) cells were adoptively transferred into CD45.1 Foxp3-GFP mice. Finally, recipient mice were fed with OVA by gavage (4 and 24 hr after the transfer of OT-II cells). Nine days later, secondary lymphoid organs were recovered and the phenotype of donor-derived OT-II T cells was analyzed. In this setting, ‘Ca\(^{2+}\)-converted’ OT-II CD4 T\(N\) cells were also giving rise to greater absolute numbers of Foxp3-expressing cells than OT-II CD4 T\(N\) cells cultured with IL-7 alone prior to injection (Figure 7—figure supplement 1B,C). More precisely, more than three quarters of the Foxp3\(^+\) OT-II cells arising in these conditions derived from ‘Ca\(^{2+}\)-converted’ cells (Figure 7—figure supplement 1D).

These latter results validate our in vitro data showing that a rise in intracellular Ca\(^{2+}\) leads to an enhanced sensitivity of CD4 T\(N\) cells to iTreg-cell polarization signals.

Discussion

In the steady-state, naive T cells continually recirculate between the blood, lymph and secondary lymphoid organs, scanning dendritic cells (DCs) for the presence of foreign antigens. In the course of their journey, naive T cells also make weak, but functional, interactions with self-peptides presented by self-MHC molecules (self-MHC). Such contacts with self-MHC are required for the long-term survival of peripheral naive T cells (Martin et al., 2006, 2003; Stritesky et al., 2012; Tanchot et al., 1997). The signals derived from the recognition of self-MHC by TCRs also allow maintaining naive T cells in a state of greater sensitivity for responses to foreign antigens (Dorfman et al., 2000; Stefanová et al., 2002). The seminal work of Štefanová et al. showed a rapid decline in the ability of CD4 T\(N\) cells to respond to their cognate antigen once contacts with self-MHC were disrupted (Štefanová et al., 2002). These findings were confirmed by several groups using various elegant experimental models (Hochweller et al., 2010; Lo et al., 2009; Mandl et al., 2013; Persaud et al., 2014). Beside these works, we have recently demonstrated that CD4 T\(N\)-cell self-reactivity not only increases quantitatively but also shapes qualitatively their response toward their cognate antigens in the effector phase by increasing their ability to commit toward the iTreg/pTreg-cell lineage (Martin et al., 2013). In the present paper, we first wondered whether the enhanced ability of the most self-reactive CD4 T\(N\) cells to convert into iTreg/pTreg cells upon appropriate stimulation was a cell-intrinsic property. The unchanged ability of both Ly-6C\(^-\) and Ly-6C\(^+\) CD4 T\(N\) cells to polarize into Foxp3-expressing iTreg cells in vitro whether they were cultured together or separately demonstrate that the biased commitment of the most self-reactive CD4 T\(N\) cells toward the iTreg-cell lineage is cell-intrinsic.

We have recently described the cell surface GPI-anchored protein, Ly-6C, as an additional and complementary sensor of T-cell self-reactivity (Martin et al., 2013). However, significant differences may be noticed between CD5 and Ly-6C. First, whereas CD5 surface levels directly correlate with self-reactivity, Ly-6C expression by peripheral CD4 T\(N\) cells inversely correlates with their ability to interact with self-MHC. Second, in contrast to CD5, Ly-6C expression at the cell surface of CD4 T\(N\) cells is stable over time in homeostatic conditions as its up-regulation after self-MHC deprivation takes several days (Martin et al., 2013). Notwithstanding these differences, Ly-6C\(^+\) CD4 T\(N\) cells express higher protein and mRNA levels of CD5 than their Ly-6C\(^-\)-cell counterparts. Two recent
Calcium-mediated shaping of the CD4 T~N~ cell pTreg-cell differentiation potential in vivo. Purified CD4 T cells from CD45.1+/2+ C57BL/6 Foxp3-GFP OT-II mice were cultured in IL-7 (10 ng/ml) with or without TG (4 nM). After 5 days live CD4~N~ (CD44lo CD25lo CD8b- CD11b- CD11c- NK1.1- TCRgd- Foxp3-GFP-) cells were flow-cytometry sorted and injected intravenously (0.5–1 x 10^6 cells) into sex-matched CD45.1+ C57BL/6 Foxp3-GFP recipient mice fed with Ovalbumin (OVA; 1.5% w/v) in the drinking water. One week after transfer, peripheral and mesenteric LNs (pLNs and mLNs, respectively), Peyer’s Patches (PPs) and spleen were recovered separately and donor-derived CD4 T cells were analyzed. (A) Diagram illustrating the experimental model. (B) Absolute numbers of donor-derived OT-II CD4 T (CD45.1+ CD45.2+ CD4+ TCRb+ Vb5+) cells recovered from pLNs, mLNs, PPs and spleen of recipient mice are shown as means ± s.e.m. for three independent experiments with two or three mice per group. (C) Representative Foxp3/Vb5 contour-plots and proportions of Foxp3-GFP+ cells for gated donor-derived OT-II CD4 T (CD45.1+ CD45.2+ CD4+ TCRb+ Vb5+) cells recovered from mLNs are shown. (D–E) Percentages (D) and absolute numbers (E) of Foxp3-GFP+ among donor-derived OT-II CD4 T (CD45.1+ CD45.2+ CD4+ TCRb+ Vb5+) cells recovered from pLNs, mLNs, PPs and spleen of recipient mice are shown as means ± s.e.m. for three independent experiments with two or three mice per group. (B, D, E) Significance of differences were assessed using a two-tailed unpaired Student’s t-test. Values of p<0.05 were considered as statistically significant (*p<0.05; ns, not significant). FIGURE SUPPLEMENT LEGENDS.

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The following figure supplements are available for figure 7:

*Figure 7 continued on next page*
papers by the group of Daniel Hawiger have highlighted a crucial role of CD5 in promoting the conversion of CD4 T\textsubscript{N} cells into iTreg/pTreg cells (Henderson et al., 2015; Jones et al., 2016). CD5 would block the activation of the mammalian target of rapamycin (mTOR) and would allow activated CD4 T\textsubscript{N} cells to resist to the inhibition of iTreg-cell induction induced by T\textsubscript{hi} and T\textsubscript{lo} cell-derived cytokines. Accordingly, in the absence of effector-differentiating cytokines, CD5\textsuperscript{hi} and CD5\textsuperscript{lo} CD4 T\textsubscript{N} cells were shown to differentiate with a similar efficiency into iTreg/pTreg cells (Henderson et al., 2015). Such a phenomenon is unlikely to account for the greater ability of Ly-6C\textsuperscript{hi} CD4 T\textsubscript{N} cells to commit to the iTreg/pTreg-cell lineage. Indeed, Ly-6C\textsuperscript{hi} and Ly-6C\textsuperscript{lo} CD4 T\textsubscript{N} cells produced similar amounts of these cytokines after stimulation and Ly-6C\textsuperscript{hi} CD4 T\textsubscript{N} cells still differentiated more efficiently than their Ly-6C\textsuperscript{lo} counterparts into iTreg cells in vitro in the presence of anti-cytokine (IL-4 and IFN-\(\gamma\)) blocking antibodies (Figure 1—figure supplement 1C–E). Moreover, whereas rapamycin drastically diminished the difference in the ability of CD5\textsuperscript{hi} and CD5\textsuperscript{lo} CD4 T\textsubscript{N} cells to convert to iTreg/pTreg cells in the presence of cytokines known as restraining this effector fate (Henderson et al., 2015), this mTOR inhibitor similarly enhanced the generation of iTreg cells from both Ly-6C\textsuperscript{hi} and Ly-6C\textsuperscript{lo} CD4 T\textsubscript{N} cells and thus preserved the difference between these two cell subsets (data not shown).

In the present study, we have identified the Ca\textsuperscript{2+} signaling pathway as sufficient to induce Ly-6C down-regulation at the cell surface of CD4 T\textsubscript{N} cells in vitro. Indeed, incubation of Ly-6C\textsuperscript{hi} CD4 T\textsubscript{N} cells with the sarco/endoplasmic reticulum calcium ATPase inhibitor, thapsigargin, led to multiple phenotypic changes including not only Ly-6C down-regulation but also variations in the expression of many other genes of the 6CSign (such as CD5, CD73, CD122, CD200 and Izumo1r). This phenotypic conversion of Ly-6C\textsuperscript{hi} CD4 T\textsubscript{N} cells into Ly-6C\textsuperscript{lo} CD4 T\textsubscript{N} cells takes four days to occur in vitro and relies on the activity of Calcineurin, as shown by its complete blocking in the presence of Cyclosporin A or FK506. Interestingly, calcium- and PKC/Ras-dependent signaling pathways had divergent effects on the expression of Ly-6C. Indeed, whereas TG induced Ly-6C down-regulation, PMA led to its upregulation (Figure 4A). In line with this observation, the 6CSign does not correlate with the changes in gene expression induced by PMA (Figure 3B). These opposite effects of TG and PMA may reflect the well-documented and complex interplay between the PKC and Ca\textsuperscript{2+} signaling pathways. For example, PKC translocation to the plasma membrane is strictly Ca\textsuperscript{2+}-dependent (Reith et al., 2006) and calcineurin is phosphorylated and inhibited by PKC (Hashimoto and Soderling, 1989).

Altogether, our results suggest that interactions with self-MHC in the steady-state result in a dominant Ca\textsuperscript{2+} signaling (when compared to PKC and Ras-dependent pathways) leading to down-regulation of Ly-6C expression. This hypothesis is consistent with our results showing that in vivo Calcineurin inhibition leads to an increase in Ly-6C expression at even higher levels than those observed at the cell surface of Ly-6C\textsuperscript{hi} CD4 T\textsubscript{N} cells from untreated mice. This hypothesis is reinforced by the work of Dong et al. (Dong TX et al., co-published with the present article) showing that Ca\textsuperscript{2+} fluxes can be measured in mouse total lymph node T cells in the steady-state and that anti-MHC blocking antibodies significantly reduced their frequency. Continuous interactions with self-MHC in the steady-state may thus induce calcium waves that shape both the phenotype of CD4 T\textsubscript{N} cells and their behavior in the effector phase by favoring their differentiation into pTreg cells.

tTreg and pTreg cells have complementary roles in immune-mediated tolerance (Haribhai et al., 2011). An attractive hypothesis would be that tTreg cells would be responsible for tolerance to self-antigens, whereas pTreg cells would be in charge of restraining deleterious immune responses to non-self-antigens. In particular, pTreg cells are involved in the control of the responses to non-self-antigens leading to allergy and asthma (Josefowicz et al., 2012) as well as to commensal organisms (Lathrop et al., 2011) and food-derived antigens (Mucida et al., 2005) in the gut. Foetus-derived and allograft-derived antigens represent other obvious examples of acute exposure to non-self-antigens arising in the adults and requiring the establishment of a tolerance. In both cases, pTreg cells...
are generated against non-self antigens (either conceptus-male-derived [Samstein et al., 2012] or allograft-derived [Francis et al., 2011; Wood et al., 2012]). These cells are needed to establish an efficient tolerance toward the foetus (Samstein et al., 2012). However, there is still a lack of evidence to definitely implicate pTreg cells in the induction of an efficient tolerance toward allograft, in part because of the difficulties to achieve such a state. We have previously demonstrated that self-reactivity in the steady-state increases the ability of CD4 T\textsubscript{N} cells to differentiate into iTreg/pTreg cells (Martin et al., 2013). Accordingly, the most self-reactive CD4 T\textsubscript{N} cells (i.e. Ly-6C\textsuperscript{−} CD4 T\textsubscript{N} cells) should contribute predominantly to the pTreg-cell pool generated under physiologic and pathologic conditions. In the present study, our data suggest strongly that this tonic TCR-signaling-mediated shaping of the CD4 T\textsubscript{N}-cell compartment is calcineurin-dependent. In particular, chronic treatment with a calcineurin inhibitor leads to the disappearance of Ly-6C\textsuperscript{−} CD4 T\textsubscript{N} cells. Cyclosporin A and Tacrolimus treatments could thus interfere with the neoconversion of CD4 T\textsubscript{N} cells into pTreg cells and limit the development of tolerance in transplant patients. This may explain the difficulty to safely interrupt these immunosuppressive therapies even after years. Thus, besides their obvious clinical utility, calcineurin inhibitors may have potentially harmful side effects that should be studied to better assess and adapt their use.

### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers |
|-----------------------------------|-------------|---------------------|-------------|
| Strain, strain background (Mus musculus) | B6.Cg-Foxp3tm1Mal/J | Wang et al. (2008) (PMID: 18209052) | IMSR Cat#: JAX:018628, RRID:IMSR_JAX:018628 |
| Strain, strain background (Mus musculus) | B6.Cg-Tg(TcraTcrb)425Cbn/J | Barnden et al., 1998 (PMID: 9553774) | IMSR Cat#: JAX:004194, RRID:IMSR_JAX:004194 |
| Antibody | Alexa Fluor 700-conjugated anti CD45.2 (104) | BD Biosciences | Cat#: 560693 |
| Antibody | Allophycocyanin (APC)-conjugated anti-CD25 (PC61) | BD Biosciences | Cat#: 561048 |
| Antibody | Allophycocyanin (APC)-conjugated anti-CD44 (IM7) | BD Biosciences | Cat#: 561862 |
| Antibody | Brilliant Violet (BV) 421-conjugated anti Ly-6C (AL-21) | BD Biosciences | Cat#: 562727 |
| Antibody | BV 510-conjugated anti-CD4 (RM4-5) | BD Biosciences | Cat#: 563106 |
| Antibody | BV 786-conjugated anti-CD25 (PC61) | BD Biosciences | Cat#: 564023 |
| Antibody | Phycoerythrin (PE)-conjugated anti-CD25 (PC61) | BD Biosciences | Cat#: 561065 |
| Antibody | Phycoerythrin (PE)-conjugated anti-CD69 (H1.2F3) | BD Biosciences | Cat#: 553237 |
| Antibody | Phycoerythrin (PE)-conjugated anti-Izumo1r (TH6) | BD Biosciences | Cat#: 560320 |
| Antibody | Phycoerythrin (PE)-conjugated anti-TCRgd (GL3) | BD Biosciences | Cat#: 553178 |
| Antibody | Phycoerythrin (PE)-conjugated anti-Vb5.1/5.2 (MR9-4) | BD Biosciences | Cat#: 553190 |
| Antibody | PE-Cy7-conjugated anti-CD44 (IM7) | BD Biosciences | Cat#: 560569 |
| Antibody | PE-Cy7-conjugated anti-CD45.1 (A20) | BD Biosciences | Cat#: 560578 |
| Antibody | Biotinylated anti-CDS (53-7,3) | BD Biosciences | Cat#: 553019 |
| Antibody | Biotinylated anti-CD62L (MEL14) | BD Biosciences | Cat#: 553149 |
| Antibody | Biotinylated anti-Ly-6C (AL-21) | BD Biosciences | Cat#: 557359 |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers |
|----------------------------------|-------------|---------------------|-------------|
| Antibody                        | Biotinylated anti-Sca1 (E13-161.7) | BD Biosciences | Cat# 553334 |
| Antibody                        | Alexa Fluor 647-conjugated anti-IL18ra (BG/IL18ra) | BioLegend | Cat# 132903 |
| Antibody                        | APC-conjugated streptavidin | BioLegend | Cat# 405207 |
| Antibody                        | BV 421-conjugated anti-Ly-6C (HK1.4) | BioLegend | Cat# 128032 |
| Antibody                        | PE-conjugated anti-Ly-6C (HK1.4) | BioLegend | Cat# 128008 |
| Antibody                        | Alexa 448-conjugated anti-NFAT2 (7A6) | BioLegend | Cat# 649603 |
| Antibody                        | Alexa 448-conjugated anti-NFAT1 (D43B1) | Cell Signaling | Cat# 14324 |
| Antibody                        | PE-conjugated anti-CD200 (OX-90) | eBioscience | Cat# 12-5200-82 |
| Antibody                        | PE-conjugated anti-Ikzf3 (BB2) | eBioscience | Cat# 12-5789-80 |
| Antibody                        | PE-conjugated anti-Nur77 (12.14) | eBioscience | Cat# 12-5965-82 |
| Antibody                        | PerCP-Cy5.5-conjugated anti-TCRb (H57-597) | eBioscience | Cat# 45-5961-82 |
| Antibody                        | Biotinylated anti-CD73 (eBioTY/11.8) | eBioscience | Cat# 14-0731-82 |
| Antibody                        | APC-conjugated anti-Foxp3 (FJK-165) | eBioscience | Cat# 12-5773-82 |
| Antibody                        | PE-conjugated anti-Foxp3 (FJK-165) | eBioscience | Cat# 17-5773-82 |
| Antibody                        | Pacific Blue-conjugated streptavidin | Invitrogen | Cat# S11222 |
| Antibody                        | APC-Vio770-conjugated anti-CD8a (53–6.7) | Miltenyi Biotec | Cat# 130-102-305 |
| Antibody                        | PE-conjugated anti-CD122 (TM-b1) | Miltenyi Biotec | Cat# 130-102-569 |
| Chemical compound, drug         | Phorbol 12-myristate 13-acetate (PMA) | Calbiochem | CAS 16561-29-8 |
| Chemical compound, drug         | FK506 (tacrolimus) | Sigma Aldrich | CAS 109581-93-3 |
| Chemical compound, drug         | CellTrace Violet | Invitrogen | Cat# C34557 |
| Chemical compound, drug         | CellTrace Far Red | Invitrogen | Cat# C34564 |
| Chemical compound, drug         | Recombinant Mouse IL-7 | R and D Systems | Cat# 407 ML-025 |
| Chemical compound, drug         | Thapsigargin | Calbiochem | CAS 67526-95-8 |
| Chemical compound, drug         | TGFβ1 | Invitrogen | Cat# PHG9204 |
| Chemical compound, drug         | DRAQ5 | Cell Signaling | Cat# 4084 |
| Chemical compound, drug         | Indo-1, AM | Invitrogen | Cat# 11223 |
| Software, algorithm             | Illustrator CS5 | Adobe Systems Inc. | http://www.graphpad.com |
| Software, algorithm             | GeneChip Scanner 3000 7G | Affymetrix | N/A |
| Software, algorithm             | Expression Console | Affymetrix | https://imagej.nih.gov/ij/ |
| Software, algorithm             | DIVA8.0.1 | BD Biosciences | N/A |
| Software, algorithm             | R | Bioconductor | N/A |
| Software, algorithm             | Prism 7 | GraphPad | N/A |
| Software, algorithm             | ImageJ | NIH | https://www.bioconductor.org/ |
| Software, algorithm             | Partek Genomics Suite | Partek | N/A |
| Deposited data                  | GSE14308 | Wei et al. (2009), PMID: 19144320 | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14308 |

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Mice
C57BL/6 mice (CD45.2) were obtained from Charles River Laboratories. C57BL/6 CD45.1 mice were maintained in our own animal facilities, under specific pathogen-free conditions. C57BL/6 Foxp3-GFP CD45.2 mice (Wang et al., 2008), initially obtained from Dr Bernard Malissen, Centre d’Immunologie de Marseille-Luminy, France, were crossed with C57BL/6 CD45.1 mice to generate C57BL/6 Foxp3-GFP CD45.1 and CD45.1/.2 mice. C57BL/6 OT-II mice were obtained from Charles River Laboratories and crossed with C57BL/6 Foxp3-GFP CD45.1/.2 (or CD45.2) OT-II mice. Four- to 12-week-old mice were used for all experiments. Experiments were carried out in accordance with the guidelines of the French Veterinary Department. All procedures performed were approved by the Paris-Descartes Ethical Committee for Animal Experimentation (decision CEEA34.CA.080.12). Sample sizes were chosen to ensure the reproducibility of the experiments and according to the 3Rs of animal ethics regulation.

Cell suspensions
Peripheral Lymph Nodes (pLNs), mesenteric Lymph Nodes (mLNs), Peyer’s patches, spleen and thymus were homogenized and passed through a nylon cell strainer (BD Falcon) in PBS supplemented with 10% FCS (Biochrom) for adoptive transfer or cell culture (LNs only), or in 5% FCS and 0.1% NaN₃ (Merck-Sigma-Aldrich, Lyon, France) in PBS for flow cytometry.

Adoptive transfer of Ly-6C⁻ CD4 T₊ cells
CD4 T cells were purified from LNs (pooled superficial cervical, axillary, brachial, inguinal and mLNs) of C57BL/6 Foxp3-GFP CD45.1 mice by incubating cell suspensions on ice for 15 min with a mixture of anti-CD8 (53–6.7), anti-CD19 (1D3) and anti-Ter-119 antibodies (Abs) obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat immunoglobulins (Invitrogen, Cergy-Pontoise, France). Ly-6C⁻ CD4 T₊ cells were sorted as Foxp3-GFP Lineage (CD25, TCRγδ, CD8β, CD11b, CD11c)-PE-CD44⁻/lo Ly-6C⁻ cells using a FACS-ARIA3 flow cytometer (BD Biosciences, Le Pont de Claix, France) and injected intravenously into sex-matched recipient mice whose then were injected intraperitoneally every day for two weeks with 2.5 mg/kg of Prograf (Tacrolimus; Astellas Pharma Inc., Tokyo, Japan).

Adoptive transfer of OT-II CD4 T₊ cells
CD4 T cells were purified from LNs of C57BL/6 Foxp3-GFP OT-II CD45.2 or CD45.1/.2 mice by using Dynabeads Untouched Mouse CD4 Cells Kit (Invitrogen) and cultivated with recombinant mouse IL-7 (10 ng/ml; R and D Systems, Minneapolis, MN) with or without Thapsigargin (4 nM; Merck-Sigma-Aldrich) into 96-well round-bottom treated cell culture microplate (Corning; 1 × 10⁶ cells per well). After 5 days of culture, cells were recovered and labelled with PE-conjugated anti-TCRγδ (GL3), anti-CD8β2 (53–5.8), anti-NK-1.1 (PK136) and APC-conjugated anti-CD44 (IM7), all from BD Biosciences. OT-II CD4 T₊ cells were sorted as GFP Lineage-PE-CD44⁻/lo cells using a FACS-ARIA3 flow cytometer (BD Biosciences) and 0.5 to 1 × 10⁶ cells were injected intravenously into sex-matched C57BL/6 Foxp3-GFP CD45.1 mice. Recipient mice were then continuously fed with Albumin from chicken egg white (OVA; 1.5% w/v; Merck-Sigma-Aldrich) in the drinking water or not. LNs and spleens were

| Reagent type (species) or resource | Designation | Source or reference | Identifiers |
|-----------------------------------|-------------|--------------------|-------------|
| Deposited data                    | GSE42276    | Wakamatsu et al. (2013), PMID: 23277554 | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42276 |
| Deposited data                    | GSE67464    | Bevington et al., 2016, PMID: 26796577 | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67464 |
| Deposited data                    | GSE70154    | Richards et al. (2015), PMID: 26195815 | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70154 |
| Deposited data                    | GSE62532    | Vahl et al. (2014), PMID: 25464853 | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62532 |
| Deposited data                    | GSE97477    | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97477 |
collected at day seven and CD45.2+ CD4 T cells analyzed. In a second protocol, sorted CD4 T\(_N\) cells from CD45.2+ and CD45.1/2+ C57BL/6 Foxp3-GFP OT-II mice were cultured in IL-7 (10 ng/ml) without or with TG (4 nM), respectively. After 5 days live CD4 T\(_N\) (CD44\(^b\)/CD25\(^b\)/CD8\(^b\)/CD11b\(^c\)/CD11c\(^c\)/NK1.1\(^c\)/TCR\(_{\alpha\beta}\)/Foxp3\(^{\text{GFP}}\)) cells were flow-cytometry sorted, mixed at a 1:1 ratio and injected intravenously (0.5–1 × 10\(^6\) cells) into sex-matched CD45.1+ C57BL/6 Foxp3-GFP recipient mice gavaged with Ovalbumin (OVA; 50 mg) 4 and 24 hr later. LNs and spleens were collected at day 10 and donor-derived CD4 T cells were analyzed.

### Cell surface staining and flow cytometry

Cell suspensions were collected and dispersed into 96-well round-bottom microtiter plates (Greiner Bioscience; 6 × 10\(^6\) cells per well). Surface staining was performed by incubating the cells on ice, for 15 min per step, with Abs in 5% FCS and 0.1% NaN\(_3\) with the membrane-permeable fluorescent Ca\(^2+\) markers and kept on ice. Before acquisition, cell aliquots were allowed to equilibrate to 37 °C for 1 h. Cells were stained either in HBSS (for ex-vivo-purified CD4 T cells) or directly in the culture medium (cultured cells). Thereafter, ex-vivo-purified CD4 T cells were stained for surface markers and kept on ice. Before acquisition, cell aliquots were allowed to equilibrate to 37 °C for 5 min and then were analyzed by flow cytometry. After acquisition of background intracellular Ca\(^2+\) concentrations for 2 min, cells were stimulated with Thapsigargin (at a concentration of 4 or 200 nM)

### Intracellular calcium measurement

Ex vivo purified CD4 T cells or cells recovered after 5 days of culture were loaded for 30 min at 37°C with the membrane-permeable fluorescent Ca\(^2+\) indicator dye Indo-1 AM (Invitrogen) at a concentration of 1 μM. Cells were stained either in HBSS (for ex-vivo-purified CD4 T cells) or directly in the culture medium (cultured cells). Thereafter, ex-vivo-purified CD4 T cells were stained for surface markers and kept on ice. Before acquisition, cell aliquots were allowed to equilibrate to 37°C for 5 min and then were analyzed by flow cytometry. After acquisition of background intracellular Ca\(^2+\) concentrations for 2 min, cells were stimulated with Thapsigargin (at a concentration of 4 or 200 nM)

### Cell culture and in vitro polarization assays

Flow-cytometry sorted Ly-6C\(^+\) and Ly-6C\(^-\) CD4 T\(_N\) cells from LNs of C57BL/6 Foxp3-GFP mice were stained with CellTrace Violet (CTv; 5 μM; Life Technologies) and cultured with IL-7 (10 ng/ml) alone or in combination with Thapsigargin (TG; 4 nM), Phorbol 12-myristate 13-acetate (PMA; 1.25 ng/ml), PMA +TG (1.25 ng/ml and 4 nM, respectively) and immobilized anti-CD3 (clone 145.2C11; 4 μg/ml; obtained from hybridoma supernatants) and anti-CD28 (clone 37.51; 1 μg/ml; eBioscience; 4 μg/ml) Abs. For in vitro polarization assays Ly-6C\(^+\) CD4 T\(_N\) cells were additionally stained with CellTrace Far Red (CTfr; 1.25 μM; Life Technologies). Cells were then stimulated separately or together for 4 days with coated anti-CD3 and anti-CD28 Abs, in the presence of graded concentrations of exogenous recombimant human TGFB\(_\gamma\) (Invitrogen). In some experiments, anti-IFN-γ (clone R4-6A2; 10 μg/mL) and anti-IL-4 (clone 11B11; 10 μg/mL) blocking antibodies were added in the culture.

The concentration of TGFB\(_\gamma\) needed to obtain 50% of the maximal percentage of iTreg cells (Effective Concentration, EC50) was calculated by fitting the dose-response curves of CD4 T\(_N\)-cell subsets in the different culture conditions. To this end, the means of 3 to 5 independent experiments were
used to build dose response curves using nonlinear least-squares regression to the Hill equation. The model used for this function was \( Y = \frac{B + (T - B)}{1 + 10^{(\log_{10} EC_{50} - X) \times \text{HillSlope}}} \), where ‘Y’ represents Foxp3+ cells as a percentage among CD4+ cells, ‘T’ and ‘B’ represent the plateaus at the beginning and end of the curve, respectively, and ‘X’ represents the concentration of TGFβ added at the beginning of the culture. The absolute EC50 was calculated to interpolate X at 50% with 95% confidence intervals.

Cytokine multiplex assay

Flow-cytometry sorted Ly-6C- and Ly-6C+ CD4 T\(N\) cells from LNs of C57BL/6 Foxp3-GFP mice were stimulated as described above with immobilized anti-CD3 and anti-CD28 Abs in the presence or absence of exogenous recombinant human TGFβ1 (Invitrogen, 4 μg/mL). Supernatants were recovered 24 hr later and cytokines were quantified by MSD multi-array U-PLEX assays (IFN-γ, IL-4, IL-17A/F and IL-10; Meso Scale Discovery, Rockville, MD) according to the manufacturer’s instructions.

Imaging flow cytometry

LNs cells of C57BL/6 mice were harvested and fixed in 4% paraformaldehyde, immediately or after 30 min of resting or stimulation with 200 nM of Thapsigargin in RPMI 1640 Glutamax (Gibco). Cells were washed in 1% FCS and 0.1% Na\(NO_3\) in PBS and incubated in glycine (0.1M) for 10 min. Cell surface was stained with biotinylated anti-Ly-6C (AL-21), BV 510-conjugated anti-CD4 (RM4-5), PE-conjugated anti-CD25 (PC61), anti-TCR \(\alpha\) (GL3), anti-CD8.\(b\) (53–5.8), anti-NK-1.1 (PK136), anti-CD11b (M1/70), PE-Cy7-conjugated anti-CD44 (IM7) and PerCP-Cy5.5-conjugated streptavidin, all from BD Biosciences. Intracellular stainings were performed using Foxp3 Staining kit (eBioscience) and Alexa 448-conjugated anti-NFAT1 (D43B1; Cell Signaling, Leiden, The Netherlands) or anti-NFAT2 (7A6; BioLegend) and APC-conjugated anti-Foxp3 (FJK-165; eBioscience) Abs were used. Ly-6C- and Ly-6C+ CD4 T\(N\) cells were sorted as CD4-BV510+ Lineage-PE-CD44-\(lo\)-Foxp3-APC- Ly-6C+/− cells using a FACS-ARIA3 flow cytometer (BD Biosciences). After sort, DRAQ5 (Cell Signaling) was used to stain nuclei. Cells were acquired with ImageStreamX (Amnis; EMD Millipore) and analyzed with IDEAS software. NFAT1 and NFAT2 nuclear localization was calculated as the similarity score between NFAT and DRAQ5 intensities.

Microarray

CD4 T cells from LNs of C57BL/6 Foxp3-GFP mice were enriched as described above. Then, Ly-6C- and Ly-6C+ CD4 T\(N\) cells were flow-cytometry sorted as CD4+ CD8α− TCR\(\beta\)+ GFP+ CD25+ CD444−/lo cells using a FACS-ARIA3 flow cytometer. Total RNA was extracted using the RNeasy Mini kit (QIAGEN, Courtaboeuf, France). RNA quality was validated with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit). Experimental and analytical part of the microarray analysis was performed according to the MIAME standards. Amplified, fragmented and biotinylated sense-strand DNA targets were synthesized from 50 ng total RNA according to the manufacturer’s protocol (Ovation PicoSL WTA System V2 and Encore Biotin Module kit (Nugen, Leek, The Netherlands)) and hybridized to a mouse gene 2.0 ST array (Affymetrix, Paris, France). The stained chips were read and analysed with a GeneChip Scanner 3000 7G and Expression Console software (Affymetrix). Raw data (.cel files) were then processed and normalized using the quantile normalization method in RMA with R package (Biocductor). Statistical analysis was then performed with Partek Genomics Suite software (Partek). Gene expression was Z-transformed, for visualization, using the following formula: \( z = (X - \mu) / \tau \), with X = normalized intensity, \(\mu\) = mean of the normalized intensity across replicates and \(\tau = \text{s.d. of mean of the normalized intensity across replicates} \). Experimental and analytical part of the microarray was performed on the Cochin Genomic facility. Raw and processed data microarray data are provided in the Gene Expression Omnibus (GEO) under accession number GSE97477.

Comparison with public GEO datasets

Normalized microarray datasets (GSE14308, GSE42276, GSE67464, GSE70154 and GSE62532) were recovered from NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). For each datasets the values mean of Probset with the same Gene-id was performed to generate a file (.xlsx) with a unique value per Gene-id for each sample. These files were then statistical analyzed as described above. The newly created public GEO Datasets were then aligned with our microarray...
data by keeping only the commons Gene-id. Finally, these alignment files were filtered on our data for a p-value<0.05 and a fold change >1.3 and the differential expression of genes was compared between our and public GEO microarray.

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Vincent Guichard, Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review and editing; Nelly Bonilla, Aurélie Durand, Alexandra Audemard-Vergaer, Investigation, Methodology; Thomas Guilbert, Software, Investigation; Bruno Martin, Funding acquisition, Validation, Investigation; Bruno Lucas, Conceptualization, Data curation, Supervision, Funding acquisition, Project administration, Writing—review and editing; Cédric Auffray, Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Ethics
Animal experimentation: Experiments were carried out in accordance with the guidelines of the French Veterinary Department. All procedures performed were approved by the Paris-Descartes Ethical Committee for Animal Experimentation (decision CEEA34.CA.080.12). Sample sizes were chosen to ensure the reproducibility of the experiments and according to the 3Rs of animal ethics regulation.
Additional files

Supplementary files

- Transparent reporting form
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Major datasets

The following dataset was generated:

| Author(s) | Year | Dataset title | Dataset URL | Database, license, and accessibility information |
|-----------|------|---------------|-------------|--------------------------------------------------|
| Guichard V, Bonilla N, Durand A, Audemand-Verger A, Guilbert T, Martin B, Lucas B, Auffray C | 2017 | Calcium-mediated shaping of naive CD4 T cell phenotype and function | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97477 | Publicly available at the NCBI Gene Expression Omnibus (accession no. GSE97477) |

The following previously published datasets were used:

| Author(s) | Year | Dataset title | Dataset URL | Database, license, and accessibility information |
|-----------|------|---------------|-------------|--------------------------------------------------|
| Wei L, Wei G, Zhu J, Hu-Li J, O'Shea JJ, Zhao K | 2009 | Epigenetic Mechanisms Underlie T Cell Plasticity | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14308 | Publicly available at the NCBI Gene Expression Omnibus (accession no. GSE14308) |
| Wakamatsu E, Mathis D, Benoist C | 2012 | Gene expression profile of conventional T cells (Tconv) and regulatory T cells (Treg) stimulated with anti-costimulatory molecule antibodies | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42276 | Publicly available at the NCBI Gene Expression Omnibus (accession no. GSE42276) |
| Bevington S, Cau-chy P, Jason P, Elizabeth B, Nav-een L, Ott S, Bon-nier C, Cockerill P | 2016 | Defining the molecular mechanisms underlying immunological memory in T cells (expression) | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67464 | Publicly available at the NCBI Gene Expression Omnibus (accession no. GSE67464) |
| Richards DM, Hofer A, Feuerer M | 2015 | The contained self-reactive peripheral T cell repertoire: size, diversity and cellular composition | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70154 | Publicly available at the NCBI Gene Expression Omnibus (accession no. GSE70154) |
| Vahl JC, Schallen-berg S, Buch T, Kretschmer K, Schmidt-Supprian M | 2014 | Continuous T cell receptor signals maintain a functional regulatory T cell pool | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62532 | GSE62532 |

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