

ORIGINAL ARTICLE

Promiscuous Foxp3-cre activity reveals a differential requirement for CD28 in Foxp3⁺ and Foxp3⁻ T cells

Dean Franckaert^{1,2}, James Dooley^{1,2}, Evelyne Roos^{1,2}, Stefan Floess³, Jochen Huehn³, Herve Luche^{4,5,6}, Hans Joerg Fehling⁷, Adrian Liston^{1,2}, Michelle A Linterman^{8,9} and Susan M Schlenner^{1,2,9}

Costimulatory signals by CD28 are critical for thymic regulatory T-cell (Treg) development. To determine the functional relevance of CD28 for peripheral Treg post thymic selection, we crossed the widely used Forkhead box protein 3 (Foxp3)-CreYFP mice to mice bearing a conditional *Cd28* allele. Treg-specific CD28 deficiency provoked a severe autoimmune syndrome as a result of a strong disadvantage in competitive fitness and proliferation of CD28-deficient Tregs. By contrast, Treg survival and lineage integrity were not affected by the lack of CD28. This data demonstrate that, even after the initial induction requirement, Treg maintain a higher dependency on CD28 signalling than conventional T cells for homeostasis. In addition, we found the Foxp3-CreYFP allele to be a hypomorph, with reduced Foxp3 protein levels. Furthermore, we report here the stochastic activity of the Foxp3-CreYFP allele in non-Tregs, sufficient to recombine some conditional alleles (including *Cd28*) but not others (including *R26-RFP*). This hypomorphism and 'leaky' expression of the Foxp3-CreYFP allele should be considered when analysing the conditionally mutated Treg.

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A major challenge for the immune system is to prevent pathogens from causing disease, although ensuring the tolerance to self is maintained. Within the T-cell compartment, tolerance is established in the thymus, where T cells develop and undergo selection in an attempt to limit the number of self-reactive T-cell clones that exit the thymus as mature, naïve T cells. Those self-reactive T-cell clones that escape central tolerance in the thymus are censored by peripheral tolerance mechanisms, including anergy, clonal deletion and control by regulatory T cells (Treg). The pathology of both mice and humans with defective Forkhead box protein 3 (Foxp3), the key transcription factor for Treg, demonstrate the central role for Treg in maintaining tolerance and preventing aberrant inflammation.^{1–5}

Multiple molecules have been described to be important for Treg formation, maintenance and function. Indeed, one of the first to be implicated in Treg control of autoimmunity was the receptor CD28, as CD28-deficient non-obese diabetic mice have exacerbated autoimmune diabetes, which can be prevented by the transfer of Treg.⁶ In helper T cells CD28 is the major costimulatory molecule that supports T-cell activation and prevents anergy induction.⁷ CD28 signalling induces T-cell proliferation, interleukin 2 (IL-2) secretion and allows the stabilisation of the anti-apoptotic molecule Bcl-xL.^{8–10} By contrast,

in Treg CD28 signalling is critical for development through the stabilisation of Foxp3 mRNA in thymic Treg precursors.^{11,12} In addition to its essential role in Treg development, CD28 is also required in the periphery for Treg homeostasis.¹³ Disentangling the Treg cell-intrinsic effects from the requirement of CD28 signalling on effector T cells to produce IL-2 that is essential for Treg survival¹⁴ is problematic in studies using CD28-deficient mice or blocking the CD28 ligands CD80 and CD86.

To assess the Treg intrinsic requirement for CD28 signalling we, and others,¹⁵ set out to generate mice where the loss of CD28 is restricted to the Treg lineage (*Foxp3^{cre} Cd28^{fl/fl}*). Surprisingly, this strain of mice revealed that CD28 was lost on conventional CD4 cells, in addition to Foxp3⁺ Treg, demonstrating the promiscuous expression of *Foxp3^{cre}*. Despite the loss of CD28 on a proportion of conventional CD4⁺ T cells we observed a lymphoproliferative disorder in *Foxp3^{cre} Cd28^{fl/fl}* mice. CD28-deficient Treg were at a competitive disadvantage compared with control cells. This is due to reduced proliferation in the absence of CD28, as neither Treg lineage stability nor survival was affected by the loss of CD28 on Treg cells. Taken together, this demonstrates that CD28 expression on Tregs is important for Treg proliferation and the suppression of aberrant lymphocyte expansion.

¹Autoimmune Genetics Laboratory, VIB, Leuven, Belgium; ²Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium; ³Department of Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany; ⁴Centre d'Immunologie de Marseille-Luminy (CIML), UM2 Aix-Marseille Université, Marseille Cedex 9, France; ⁵INSERM U1104, Marseille Cedex 9, France; ⁶CNRS UMR7280, Marseille Cedex 9, France; ⁷Institute of Immunology, University Clinics Ulm, Ulm, Germany and ⁸Lymphocyte Signalling and Development, Babraham Institute, Babraham Research Campus, Cambridge, UK

⁹These authors contributed equally to this work.

Correspondence: Dr SM Schlenner, Department of Microbiology and Immunology, University of Leuven, Herestraat 49, Campus Gasthuisberg, CDG8, 3000 Leuven, Belgium.

E-mail: susan.schlenner@vib-kuleuven.be

or Dr M Linterman, Lymphocyte Signalling and Development, Babraham Institute, Babraham Research Campus, Cambridge CB22 3AT, UK

E-mail: michelle.linterman@babraham.ac.uk

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RESULTS

***Foxp3^{Cre}Cd28^{fl/fl}* mice reveal promiscuous Foxp3 expression**

In order to determine the post-selection functions for CD28 in Foxp3⁺ Treg, we crossed the previously described Foxp3-CreYFP knockin mice¹⁶ (hereafter referred to as *Foxp3^{Cre}*) to mice carrying a conditional CD28 allele (*Cd28^{fl/fl}*) derived from EUCOMM embryonic stem cells.¹⁷ CD28 excision was monitored by surface staining for CD28, which validated Cre activity within Treg, with efficient deletion of CD28 within YFP⁺ Treg (Figures 1a and b). Unexpectedly, and in contrast to previous studies published using the *Foxp3^{Cre}* allele,^{15,16,18} we also observed the deletion of CD28 from conventional CD4⁺

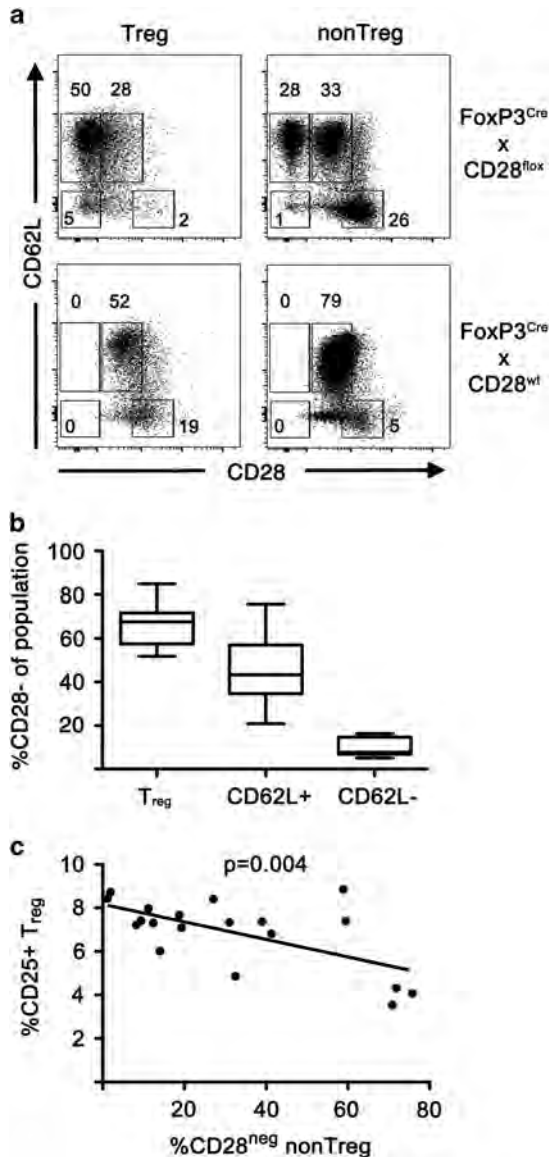


Figure 1 Foxp3^{Cre} activity is not restricted to Foxp3⁺ Treg. (a, b) Lymph node CD4⁺ lymphocytes were analysed for the presence of CD28⁻ cells among YFP⁺ Treg and YFP⁻ non-Treg in *Foxp3^{Cre}Cd28^{fl/fl}* males. (a) representative flow cytometric analysis for CD28⁻ CD4 T cells is shown. (b) The plot summarises three independent experiments with a total of $n=9$ (Treg) and $n=6$ (CD62L⁺ and CD62L⁻ non-Treg); mean \pm s.e.m. (c) The graph depicts the inverse relationship between the presence of CD28⁻ non-Treg and CD25⁺ Treg in *Foxp3^{Cre}/wtCd28^{fl/fl}* females. Graph represents six independent experiments with a total of $n=19$. $P=0.004$ (**), $r^2=0.394$.

T cells (Figures 1a and b). Deletion was observed in both naive and, to a lesser extent, antigen-experienced conventional T cells, with considerable variation in the level of deletion (Figures 1b and c), indicative of low-level stochastic Cre activation in non-Tregs or a lineage precursor. Notably, the level of CD28 excision is higher in naive T cells than in antigen-experienced CD62L^{low} T cells (Figure 1b), consistent with the function of CD28 in allowing transition between these activation states. Furthermore, we observed an indirect correlation between the level of non-specific CD28 excision and the frequency of CD4⁺CD25⁺ T cells (Figure 1c). As CD28-deficient T cells are poor IL-2 producers,^{11,19} this reduction in Treg numbers in mice with high levels of non-specific CD28 excision is likely due to impaired IL-2-mediated Treg homeostasis.¹⁴

Despite the promiscuous activity of Cre, which should be considered when using *Foxp3^{Cre}* mice, we assessed the function for CD28 in Treg after selection. Male *Foxp3^{Cre}Cd28^{fl/fl}* mice developed a lymphoproliferative disorder, including lymphadenopathy, mild splenomegaly, T-cell activation and lymphocytic organ infiltrations (Figures 2a and b and data not shown). In rare cases, mice presented with disease progression similar to scurfy mice (with fatal disease at 4 weeks of age). However, disease onset and severity were highly variable (Figures 2c and d), with other mice showing a milder phenotypic appearance, similar to the observations of Turka and colleagues.¹⁵ Correlation between the degree of T-cell activation and promiscuous *Cd28* excision (Figure 2e) indicates that lineage-specific Treg excision resulted in severe disease, whereas promiscuous *Cd28* excision resulted in the milder form of disease, probably due to the intrinsic defect in T-cell activation caused by the loss of CD28 (akin to the *Cd28* knockout mouse).¹⁹

Peripheral CD28 expression is required for Treg proliferation but is dispensable for stability and survival

Despite the variability in the disease caused by non-specific Cre activity in *Foxp3^{Cre} Cd28^{fl/fl}* mice, we were able to assess the impact of *Cd28* excision in Treg by limiting analysis to female *Foxp3^{Cre}/wtCd28^{fl/fl}* mice, where X chromosome inactivation restricts Cre activity to 50% of the hematopoietic compartment. In these mice, wild-type and *Cd28*-deleted Treg are generated in equal proportions and survive within the same microenvironment. In *Foxp3^{Cre}/wtCd28^{fl/fl}* mice, CD28-deficient Treg registered a profound competitive disadvantage, with a relative decrease in number of CD28-deficient Treg cells from the default 50 to ~20% of the total Treg (Figure 3). These results demonstrate that post-Treg selection CD28 expression is critical for homeostasis, indicating a function either in lineage stability, proliferation or apoptosis.

As a serendipitous observation, we found that CD28-deficient Treg in female *Foxp3^{Cre}/wtCd28^{fl/fl}* mice had reduced Foxp3 protein expression when compared with wild-type Treg in the same mice (Figure 4a). However, a similar reduction was observed also in *Foxp3^{Cre}Cd28^{+/+}* Treg when co-stained with congenically marked wild-type Treg (Figure 4b). Therefore, decreased Foxp3 expression was not caused by the loss of CD28 signalling and rather represents a mild hypomorphic feature of the *Foxp3^{Cre}* allele. Despite the false lead of depressed Foxp3 expression, the *Foxp3* locus contains a CD28 response element in the conserved non-coding DNA sequence 3,²⁰ which still allowed for the possibility that CD28 directly modulates the stability of Foxp3 expression, and hence the stability of the Treg lineage. We therefore investigated whether the loss of CD28 signalling would result in reduced Treg lineage stability and whether ex-Foxp3⁺ cells, such as described by Miyao *et al.*,²¹ contributed to the above-described population of CD28-deficient non-Treg. To this end, Treg

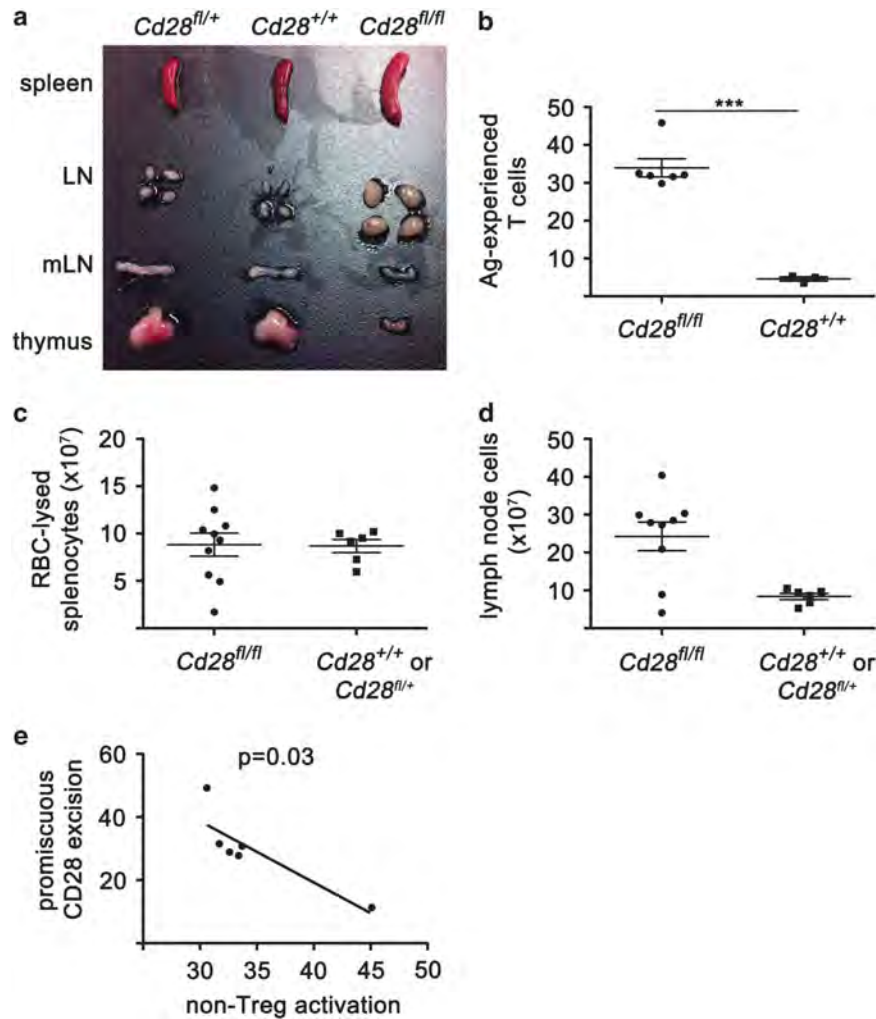


Figure 2 *Foxp3^{Cre}Cd28^{fl/fl}* mice develop autoimmune lymphadenopathy. (a) *Foxp3^{Cre}Cd28^{fl/fl}* mice displayed mild splenomegaly, pronounced lymphadenopathy and (b) strong T-cell activation. Antigen (Ag)-experienced CD4 T cells were defined as YFP⁻CD4⁺ CD44⁺CD62L⁻, $P < 0.0001$; mean \pm s.e.m. Number of red blood cell (RBC)-lysed splenocytes (c) and number of lymph node cells (d) from *Foxp3^{Cre}Cd28^{fl/fl}* males (*Cd28^{fl/fl}*) and *Foxp3^{Cre}Cd28^{fl/fl}* or *Cd28^{+/+}* males are given. (e) The relationship between the presence of CD28⁻ CD4 non-Treg and CD4 non-Treg activation measured as CD62L⁻, $P = 0.03$, $r^2 = 0.7298$. Data in a represent $n = 3$ for each genotype pooled from two independent experiments. Data in b and e represent two independent experiments with a total of $n = 6$ for *Foxp3^{Cre}Cd28^{fl/fl}* mice and $n = 3$ for *Foxp3^{Cre}Cd28^{+/+}* mice. Data in c and d represent four independent experiments with a total of $n = 10$ *Foxp3^{Cre}Cd28^{fl/fl}* mice and $n = 6$ *Foxp3^{Cre}Cd28^{fl/fl}* or *Cd28^{+/+}* mice.

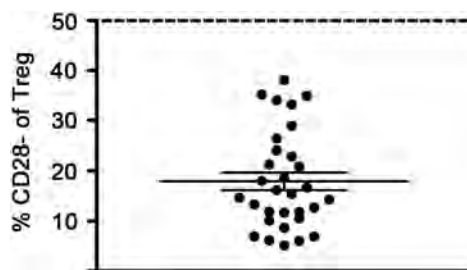


Figure 3 CD28-deficient Treg have a competitive disadvantage in the presence of CD28-sufficient Treg. Frequency of CD28-deficient Treg among total Treg in *Foxp3^{Cre/wt}Cd28^{fl/fl}* females. Treg was defined as CD25⁺CD4⁺ or Foxp3⁺CD4⁺. Data represent seven independent experiments with a total of $n = 31$ mice; mean \pm s.e.m.

from *Foxp3^{Cre}Cd28^{fl/fl}* and *Foxp3^{Cre}Cd28^{+/+}* mice were purified and the methylation status of the Treg-specific demethylated region (TSDR) was analysed (Figure 4c). The data showed complete demethylation of the Treg-specific demethylated region in the presence and absence of CD28 indicative of stable Foxp3 expression. We performed additional lineage tracing experiments by breeding *Foxp3^{Cre/wt}Cd28^{fl/fl}* mice to R26-RFP reporter mice.²² All RFP⁺ cells actively transcribed Foxp3, as determined by co-expression of YFP and RFP (Figure 4d). Taken together, these data demonstrate that the loss of CD28 in Foxp3⁺ Treg does not confer lineage instability.

With the exclusion of reduced lineage stability to explain the loss of CD28-deficient Treg in *Foxp3^{Cre/wt}Cd28^{fl/fl}* mice, we further investigated the effect of CD28 on Treg proliferation and apoptosis. Analysing *Foxp3^{Cre/wt}Cd28^{fl/fl}* female mice, we found that proliferation of CD28-deficient Treg was reduced by 68%, when compared with

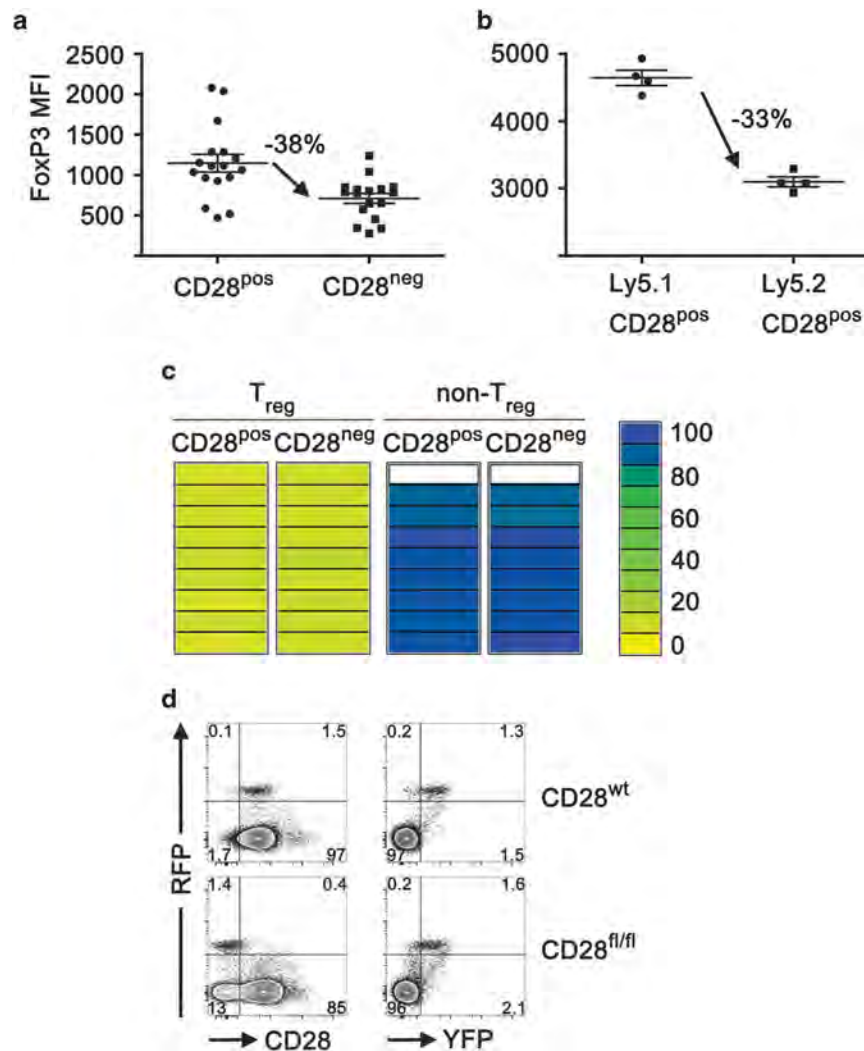


Figure 4 CD28-deficient Treg show intact lineage integrity. **(a)** Foxp3 MFI (median fluorescence intensity of intracellular Foxp3 staining) was measured on splenic CD4⁺Foxp3⁺CD28⁺ or CD28⁻ T cells, respectively, from *Foxp3^{Cre/wt}Cd28^{fl/fl}* females. Data represent a total of $n=17$ in four independent experiments; $**P=0.0015$; mean \pm s.e.m. **(b)** Splenocytes from Ly5.1 *Foxp3^{Cre}Cd28^{+/+}* and Ly5.2 *Foxp3^{Cre}Cd28^{+/+}* males were mixed before staining and analysis. Data represent one experiment with $n=4$ mice; $***P<0.0001$; mean \pm s.e.m. **(c)** The methylation status of the Treg-specific demethylated region (TSDR) was determined in CD28⁺ or CD28⁻ Treg (CD4⁺CD25⁺), and CD28⁺ or CD28⁻ conventional T cells (CD4⁺CD25⁻), purified by cell sorting from the spleen and peripheral lymph nodes. Data represent three to six biological replicates from three independent experiments. Amplicons are vertically arranged, each representing a single CpG motif. The degree of methylation at each CpG motif is represented according to the colour code. **(d)** Treg lineage tracing was performed using *Foxp3^{YFP/Cre}Cd28^{+/+}R26^{RFP}* (upper plots) and *Foxp3^{YFP/Cre}Cd28^{fl/fl}R26^{RFP}* (lower plots) mice. Splenocytes were analysed for RFP⁺ cells within and outside the YFP⁺ Treg population. Data represent three independent experiments each containing $n=1$ mouse.

CD28-sufficient Treg (Figures 5a and b). To exclude the possible influence of the hypomorphic *Foxp3^{Cre}* allele on Treg homeostasis, we analysed the proliferation status of *Foxp3^{Cre}Cd28^{+/+}* Treg and found that reduced Foxp3 expression does not affect the proliferation of these Treg (data not shown). In contrast to the proliferation, the survival of Treg was not affected by the lack of CD28 (Figure 5c). Treg with reduced proliferation and increased apoptosis strongly accumulate in a Foxp3^{low} population and we speculate that this population represents dying Treg (Figure 5a). The Foxp3^{hi} population among Foxp3⁺CD28^{pos} and Foxp3⁺CD28^{neg} Treg is comparable in relative frequency (Figure 5d). However, unlike CD28^{pos} Treg, where proliferation is higher among the Foxp3^{hi} cells, the proliferation of CD28^{neg} Tregs is equally impeded in both the Foxp3^{low} and Foxp3^{hi} populations (Figure 5e). Interestingly, the non-specific excision of

CD28 in a stochastic subset of conventional T cells allowed a comparison to be made as to the relative function of CD28 in regulatory and conventional lineages. Both conventional CD4⁺ T cells and Foxp3⁺ Treg showed reduced proliferation following the loss of CD28, however, the degree of the effect was more pronounced in Treg than in non-Treg (Figure 5f). Together these results identify enhanced proliferation, and not lineage stability or survival, as the key post-selection function of CD28 in Treg.

DISCUSSION

Thymic Treg development depends on co-stimulation by CD28.¹¹ Although these experiments have proven a requirement for CD28 for Treg apart from being an IL-2 inducer in conventional T cells, they do not allow the discrimination of pre- or post-selection functions of

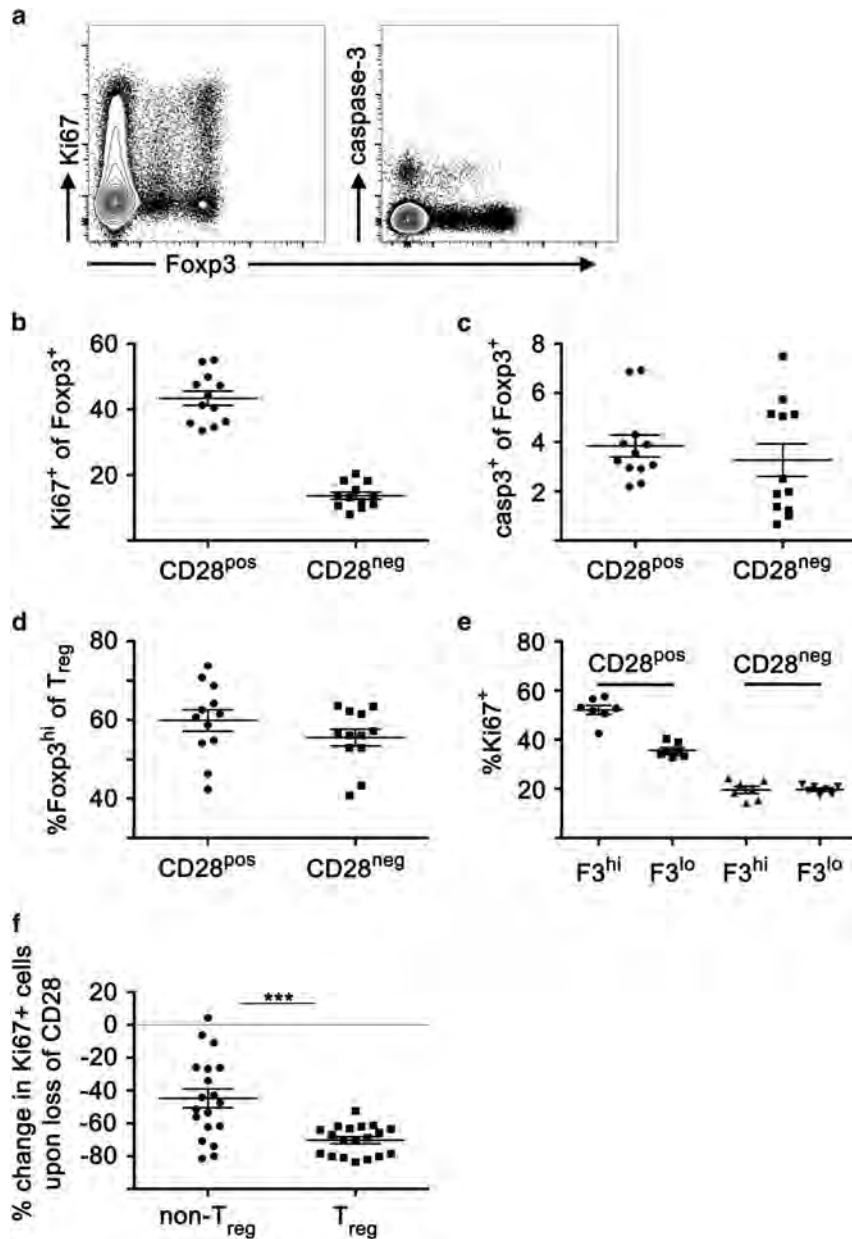


Figure 5 Treg proliferation, but not survival, is critically dependent on CD28. (a) CD4⁺ splenocytes were gated for Fxp3 versus Ki67 or active caspase-3, respectively, to illustrate the Fxp3 protein level in proliferating or dying Treg. (b, c) Splenic CD28⁺ and CD28⁻ Treg (CD4⁺Fxp3⁺) were analysed for the frequency of proliferating Ki67⁺ ($***P < 0.0001$) or active caspase-3⁺ ($P = 0.476$) cells. (d) Frequency of Fxp3^{hi} cells of CD28⁺ or CD28⁻ Treg (CD4⁺Fxp3⁺ splenic lymphocytes). Data in **a–d** represent two independent experiments containing a total of $n = 12$ mice. (e) CD4⁺ T cells were analysed for the frequency of proliferating Ki67⁺ cells of CD28-sufficient and -deficient Fxp3^{hi} (F3^{hi}) and Fxp3^{lo} (F3^{lo}) Treg; one of two experiments is shown ($n = 7$). (f) Ki67⁺ expression in CD28⁺ and CD28⁻ cells in *Fxp3^{Cre/WT}Cd28^{fl/fl}* females. Non-Treg were defined as CD4⁺Fxp3⁻ and Treg as CD4⁺Fxp3⁺. Graph indicates the relative reduction of Ki67⁺ cells amongst CD28⁻ cells compared with Ki67⁺ of CD28⁺ cells. Zero line indicates the baseline. Data represent five independent experiments containing a total of $n = 19$ mice. $***P = 0.0002$; mean \pm s.e.m. in **b–f**.

CD28. Here we addressed the peripheral requirement of CD28 signalling in Treg after this inductive stage through the generation and use of mice with Treg-specific CD28 deletion. We demonstrate that post-selection CD28 signalling is critical for the competitive fitness of Treg, consistent with the conclusion of previous studies.^{15,23} A competitive disadvantage can be the consequence of reduced proliferation or survival, or reduced lineage integrity. We report here a profound reduction in proliferation of Treg in the absence of CD28. Interestingly, Treg and non-Treg show differential dependence for CD28 signalling in homeostatic proliferation, with the non-Treg

proliferation being less affected by the loss of CD28. However, although CD28 can enhance T-cell survival,^{8,24} our data do not imply a dominant role for CD28 signalling in Treg survival. Overall, we show here that peripheral CD28 signalling, additionally to its critical role during thymic Treg induction, is critical for the maintenance of normal Treg numbers. This post-selection function of CD28 is of such importance that it can manifest in a scurfy-like fatal lymphadenopathy (in mice where non-specific Cre activity is low). Notably, this severe phenotype was not observed in previous studies,¹⁵ which may reflect variation in Cre activity.

On a technical note, we report here for the first time that the *Foxp3^{CreYFP}* allele is both hypomorphic and 'leaky', with some activity outside the Treg lineage. Reduced *Foxp3* expression can lead to an autoimmune syndrome similar to the phenotype of scurfy mice.²⁵ This is of particular interest in light of continuous *Foxp3* expression being a requirement for Treg lineage stability.²⁶ In lineage tracing experiments supported by Treg-specific demethylated region demethylation data we could demonstrate that there are no synergistic effects of the *Foxp3* hypomorphic allele with a lack of CD28 signalling with regard to lineage stability, however, the potential contribution of *Foxp3* hypomorphism to other Cre-LoxP experiments (both published and future) should be considered when interpreting the results. The other major limitation of the *Foxp3^{Cre}* allele discovered in this study, that is, major deletion outside the Treg population by stochastic Cre recombinase activity, potentially has more serious consequences on interpretation of published results. Notably, this is not found when crossing to *R26^{RFP}* or *R26^{YFP}* Cre reporter strains, however, we have also observed leaky Cre activity in the *Bcl6* allele²⁷ (own unpublished data), demonstrating that the stochastic expression is sufficient to drive the recombination of some but not other conditional alleles. As the degree of unwanted deletion in extra-Treg lineages is likely dependent on the sensitivity to Cre recombination of the conditional allele used, care should be taken when analysing conditional knock-outs generated using this particular *Foxp3* knockin allele (and potentially others²⁸) to exclude the off-target effects.

METHODS

Mice

Cd28^{fl/fl} embryonic stem cells were obtained from the EUCOMM Consortium and used to generate chimeric animals from which the *Cd28^{fl/fl}* strain was derived.¹⁷ *Cd28^{fl/fl}* mice were bred to *Foxp3^{YFP-Cre}* transgenic mice (kindly provided by A Rudensky, Memorial Sloan-Kettering Cancer Center, NY, USA) to generate mice with CD28-deficient Tregs. Mice were intercrossed with *R26^{RFP}* Cre recombinase reporter mice. Mice were housed in specific-pathogen free conditions. All experiments were performed in accordance with the University of Leuven Animal Ethics Committee guidelines.

Flow cytometry

Surface staining for flow cytometry was performed after blocking with mouse IgG (Jackson ImmunoResearch, Suffolk, UK) using the following reagents (all eBioscience, Hatfield, UK): anti-CD4-APC and -APC-H7 (RM4-5), anti-CD25-PerCP-Cy5.5 and -PE-Cy7 (PC61.5), anti-CD44-APC (1M7), anti-CD62L-PE-Cy7 (MEL-14) and anti-*Foxp3*-APC (FJK-16s). Following surface staining, cells were treated with the *Foxp3* staining kit (eBioscience) before staining with anti-*Foxp3*-APC (FJK-16s) and anti-Ki-67-PE (SolA15). For staining activated caspase-3, cells were plated at 10⁶ cells per 50 µl and well in 96-well U-bottom tissue culture plates in RPMI/10% fetal calf serum and incubated for 1 h at 37 °C with FITC-DEVD-FMK (Abcam, Cambridge, UK) before continuing with surface staining. Data were collected on a FACSantoII flow cytometer (Becton Dickinson, Erembodegem, Belgium) and analysed using Flowjo (Treestar, Ashland, OR, USA) software.

Treg-specific-demethylated region methylation analysis

CD4⁺CD25⁺CD28⁺YFP⁺, CD4⁺CD25⁺CD28⁻YFP⁺ and CD4⁺CD25⁻CD28⁻YFP⁻ cells were sorted from male *Foxp3^{Cre}Cd28^{fl/fl}* and *Foxp3^{Cre}Cd28^{+/-}* mice. Cells were first enriched for CD4⁺ cells using the mouse CD4⁺ Negative Selection Enrichment Kit (Stemcell Technologies, Grenoble, France) before cell sorting on a BD FACSAriaIII cell sorter (Becton Dickinson). Post-sort purity was greater than 97% and confirmed by post-sort intracellular *Foxp3* staining using the *Foxp3* staining kit (eBioscience). DNA was extracted using the DNA/RNA AilPrep Kit (Qiagen, Venlo, Netherlands) and bisulfite sequencing of the Treg-specific-demethylated region was performed as previously described.²⁹

Statistics

A significance threshold of 5% in an unpaired Student's *t*-test was maintained throughout the study and the statistical analysis was performed using Prism software (Graphpad, La Jolla, CA, USA). Error bars represent mean ± s.e.m.

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