Unstable regulatory T cells, enriched for naïve and Nr1^{neg} cells, are purged following fate challenge

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One Sentence Summary: Fate challenge purges regulatory T cells of incompletely committed cells, a complex subset enriched for naïve, Nr1-negative T_{reg}.

Abstract:

Regulatory T cells (T_{reg}) are indispensable for the control of immune homeostasis and have clinical potential as a cell therapy for treating autoimmunity. T_{reg} can lose expression of the defining Foxp3 transcription factor and acquire effector T cell (T_{eff}) characteristics, a process referred to as T_{reg} plasticity. The extent and reversibility of such plasticity during immune responses remain unknown. Here, using a murine genetic fate-mapping system, we show that T_{reg} stability is maintained even during exposure to a complex microbial/antigenic environment. Furthermore, we demonstrate that the observed plasticity of T_{reg} following adoptive transfer into a lymphopenic

environment is a property limited to only a subset of the T_{reg} population, with the non-converting majority of T_{reg} being resistant to plasticity upon secondary stability challenge. The unstable T_{reg} fraction is a complex mixture of phenotypically-distinct T_{reg} , enriched for naïve and $Nrp1^{neg}$ T_{reg} , and includes peripherally-induced T_{reg} and recent thymic emigrant T_{reg} . These results suggest that a “purging” process can be used to purify stable T_{reg} that are capable of robust fate-retention, with important implications for cell transfer therapy.

Introduction

Regulatory T cells (T_{reg}) are crucial players in creating immune tolerance and maintaining immune homeostasis. Malfunction or insufficient numbers of T_{reg} have been associated with diverse conditions, ranging from atopic disease such as allergic asthma to autoimmune diseases such as multiple sclerosis (1, 2). T_{reg} are characterized by expression of the transcription factor Foxp3, which is essential for establishing and maintaining their suppressive transcriptional program (3, 4). The importance of this transcription factor is demonstrated by the fact that loss of Foxp3 expression leads to the development of a lethal multi-organ autoimmune disease in both human and mice (4–6). Genetic deletion of Foxp3 in mature T_{reg} permitting the expression of interleukin 2 (IL-2) and effector cytokines such as T helper type 1 (Th1) cytokines demonstrated the function of Foxp3 in maintaining the T_{reg} program (7). T_{reg} are generally believed to be a terminally differentiated population with long-term stability in a healthy setting (8). However, several studies have suggested that signals in the microenvironment such as stress, danger or inflammation can trigger the loss of Foxp3 expression and acquisition of effector cytokines, resulting in generation of ex-Foxp3 cells (9–11).

Different models have been proposed to describe the origin and function of the ex-Foxp3 cell population. The generation of ex-Foxp3 cells may be a programmed response to acute inflammatory triggers, in order to aid in the initiation phase of the immune response (12). Several *in vitro* studies indicate that, upon microbial/antigenic challenge, T_{reg} can lose Foxp3 expression and convert into a T_{eff} phenotype, supporting this model (13, 14) as opposed to undergoing an IL-2-dependent population attrition and phenotypic plasticity (15). Likewise, Foxp3⁺ T_{reg} cells can downregulate Foxp3 expression and convert into ex-Foxp3 cells during the inflammatory context of experimental autoimmune encephalomyelitis (11). Alternatively, it has been argued that genuine T_{reg} are highly stable *in vivo*, and that “ex-Foxp3 cells” instead originate from an uncommitted population of Foxp3^{neg} T cells that transiently upregulate Foxp3 (8, 16). Whether ex-Foxp3 cells previously possessed suppressive function, and thus could be legitimately described as ex- T_{reg} , relies on a retrospective analysis of functional capacity, at the single cell level, that is currently not feasible.

The nature of T_{reg} instability thus remains highly controversial. The question is therapeutically relevant, with increasing utilization of T_{reg} in cell therapy (17). With latent autoimmune potential, retaining Foxp3 expression and suppressive status is key in any clinical use of T_{reg} as a cell therapy. It has been proposed that certain T_{reg} markers are associated with a higher potential for stability (16, 18, 19), however, it remains unclear as to whether these identify an unstable subset, or whether it represents an intermediate stage in fluctuating probabilistic deconversion. Here, we formally tested whether T_{reg} instability is a stochastic event or a subset property using an adoptive transfer model into a lymphopenic environment. By transferring T_{reg} and assessing their ability to become ex-Foxp3 cells over sequential challenge, we demonstrated that only a fraction of T_{reg} spontaneously deconverts. This unstable subset appeared to be enriched for, though not limited to, $Nrp1^{neg}$ T_{reg} and T_{reg} bearing a naïve phenotype. Finally, we

demonstrated that ex-Foxp3 cells are refractory towards re-conversion, both *in vitro* and *in vivo*, indicating that instability is a terminal, rather than transient, state.

Results

The ex-Foxp3 cell compartment arises independent of microbial challenge

Despite the stability of the T_{reg} lineage, spontaneous ex-Foxp3 cells arise even under homeostatic conditions. Using fate-mapping mice, with a *Foxp3*^{YFP-Cre} knock-in allele driving a *Rosa26*^{RFP} fate-mapping allele, ex-Foxp3 cells, with a previous history of Foxp3 expression, are readily detected as YFP^{neg} RFP⁺ (Fig. 1A, Fig. S1). In the unmanipulated baseline state, 80% of CD4⁺ T cells with a history of Foxp3 expression remain as T_{reg}, with the remaining 20% forming the ex-Foxp3 cell population (Fig. S2A, Fig. 1F). Loss of Foxp3 in this population, coupled with the self-recognition capacity of T_{reg} encoded for by an autoreactive T cell receptor (TCR) (20), drives an inflammatory cytokine profile in the ex-Foxp3 cell population (Fig. 1B). The inflammatory nature of the ex-Foxp3 cell population has been proposed to act as an amplification circuit during the acute phase of infections (10), trading more rapid induction of infection responses at the cost of transient autoimmunity.

The acute infection response model of ex-Foxp3 cell formation postulates a dynamic deconversion process, where T_{reg} to ex-Foxp3 cell plasticity is promoted by microbial challenge. This model is supported by *in vitro* stimulation experiments with PAMPs (13, 14), however *in vivo* data is limited, with infection by *Listeria monocytogenes* not leading to any notable ex-Foxp3 cell formation (8). Whether this discrepancy is due to *in vitro* culture conditions or the nature of the particular infection model used *in vivo* is contested. We therefore applied a more holistic approach to microbial challenge, using the model of “wild” microbiome seeding in transgenic mice (21). By co-housing *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOPflRFP} fate-mapper mice (*Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper) with wild-exposed pet store mice for eight weeks, we simulated the normal microbial challenge experienced by wild mice (Fig. 1C). Compared to SPF mice, co-housed wild-exposed mice experienced T cell challenge and activation, as evidenced by the activation profile of CD8⁺ T cells and, to a lesser extent, conventional CD4⁺ T cells (T_{conv}) as well as a by slight, albeit not significant, increased pro-inflammatory serum profile (Fig. S3A-H). Analysis of the T_{reg} population demonstrated an increase in absolute number (Fig. 1D), however, this was in line with the overall increase in the CD4⁺ T cell compartment (Fig. S3G), with no reduction in the frequency of T_{reg} within CD4⁺ T cells (Fig. 1E, S3I). An increase in the frequency of T_{reg} within RFP⁺ cells (Fig. 1F), with a corresponding decrease in frequency of ex-Foxp3 cells among RFP⁺ cells (Fig. 1I) was observed, which is likely a result of T_{reg} expansion in response to inflammation. Surprisingly, there was no increase in absolute number and frequency of ex-Foxp3 cells (Fig. 1G, H, Fig. S3J), despite the microbial/antigenic challenge, with an increase in microbial diversity, including several species commonly considered pathogens (Fig. S3K-N, Table S5, for pathogen screening see Materials and Methods). Ex-Foxp3 cells also maintained their phenotype upon co-housing, with loss of the T_{reg} markers CD25 and TGFβ, and acquisition of effector markers, including IL-2, IFNγ and TNFα, while Nr1 remained at an expression level intermediate between the T_{reg} and T_{eff} populations (Fig. 1J). T_{reg} and ex-Foxp3 cells from different immunological tissues (spleen, lymph nodes, Peyer’s patches) and lung were analyzed with similar conclusions on T_{reg} stability (Fig. S3I, J). Together, these data characterize ex-Foxp3 cell formation as a process refractory to microbial challenge. While not excluding ex-Foxp3 cell induction as an outcome of particular microbial stimuli, we find no support for the acute infection response model when considering the normal microbial challenges experienced by wild mice.

Ex-Foxp3 cell formation is a non-stochastic event

As an alternative to the acute infection response model of ex-Foxp3 cell formation, we sought to test whether T_{reg} plasticity presents a stochastic process. To address this question we performed a model of lymphopenic transfer, where a fraction of transferred T_{reg} lose stability when transplanted into a lymphopenic host (18). T_{reg} from Foxp3 reporter mice (*Foxp3^{YFP-Cre}Rosa^{RFP}* fate-mapper or *Foxp3^{Thy1.1}*) were transplanted in a 1:1 ratio with congenically-disparate naïve CD4⁺ T cells into *Rag1^{KO}* mice. This allows the tracking of T_{reg} and newly-generated ex-Foxp3 cells, with the naïve T cell population serving to control for any selective expansion of non-T_{reg} cells (Fig. 2A, Fig. S4A). Five weeks post-primary transfer, up to 27% of transferred T_{reg} descendants were newly-generated ex-Foxp3 cells (Fig. 2B, C), consistent with previous approaches (18, 22). These ex-Foxp3 cells showed a high expression of IL-2 and IFN γ (Fig. 2D), and were phenotypically similar to ex-Foxp3 cells from an unmanipulated mouse (Fig. 1B).

To determine whether T_{reg} instability is a stochastic event or a property of a subset of T_{reg}, we then performed a secondary lymphopenic transfer. T_{reg} from the transplanted mouse, sorted as Foxp3-expressing cells (and thus, excluding the population that had converted into ex-Foxp3 cells) were re-transplanted into new *Rag1^{KO}* recipients, together with new congenically-disparate naïve CD4⁺ T cells (Fig. 2A, Fig. S4A). This system recapitulates the original transfer, with the exception that the input T_{reg} population had been purged of those cells that became ex-Foxp3 cells in the initial transfer. Following this secondary transfer, the remaining T_{reg} population showed limited loss of Foxp3 expression with ex-Foxp3 cell formation on average reduced by >85% compared to the primary transfer, and no upregulation in inflammatory cytokine expression (Fig. 2B-D). As the lymphopenic transfer system provides a known time point for stability insult, we were able to document the kinetic of ex-Foxp3 cell formation and phenotype changes. Ex-Foxp3 cell formation occurred mainly during the 3-9 days post-transplantation period, coincident with a T_{reg} temporary proliferative burst and rapid loss of naïve phenotype (Fig. 2E, Fig. S4B, C). Similarly, a proliferative burst was observed in transferred T_{reg} upon secondary transfer (Fig. S4D), demonstrating that T_{reg} instability upon primary transfer was not proliferation-induced but reflects the presence of intrinsically unstable T_{reg} clones in the T_{reg} population. Phenotypically, ex-Foxp3 cells, from their first detection, demonstrated lower levels of the T_{reg} markers CD25, Nrp1, Eos, and Helios (Fig. 2F). These data demonstrate that there is an unstable Foxp3⁺ subset, revealed through the permissive environment of lymphopenia, prone to lose Foxp3 expression. This ex-Foxp3 cell formation is not entirely stochastic, as the remaining T_{reg} population, once purged of these unstable cells, becomes largely resistant to further ex-Foxp3 cell formation under identical conditions. Thus, rather than being a trait possessed by all T_{reg}, the majority of ex-Foxp3 cell potential under lymphopenic conditions is limited to a fixed subset of T_{reg}, without exclusion of additional stochastic effects making minor contributions. The full de-differentiation process of ex-Foxp3 cells undertaken by these unstable T_{reg} took several weeks, however even T_{reg} markers considered Foxp3-independent, such as Eos (23), were eventually lost.

A formal possibility of the transfer experiment is that T_{conv} transiently expressing Foxp3 are captured in the original sort, with ex-Foxp3 generation occurring as a byproduct of the normal reversion process. We sought to distinguish between the possibilities of active deconversion of T_{regs} upon transfer, versus passive continuation of reversion of transient Foxp3 expression, with mathematical modelling. Using the experimental data above, we developed a computational model of Foxp3-expressing cell behavior after transfer. Mathematical modelling of the kinetics of ex-Foxp3 T cell generation showed an optimal fit to kinetic models with active deconversion initiated at transfer, and taking a median length of 10 days to complete (adjusted mean squared error (MSE) = 0.001, Prob = 0.961). By comparison, models based on the assumption of uninterrupted reversion

from transient Foxp3 expression gave a relatively poor fit (adjusted MSE = 0.008, Prob = 0.852), and required a median length of 29 days for transient expression (Fig. S5). Models built on the assumption of transient Foxp3 expression in conventional T cells, with the transient expression lasting 48 hours or less, had very poor fits with the experimental data (adjusted MSE > 0.26, Prob <= 0.220). The mathematical modelling is therefore most consistent with the active deconversion of T_{reg} than reversion of transient Foxp3-expressing conventional T cells captured in the original T_{reg} sort. This suggests that low numbers of T_{conv} cells, transiently expressing Foxp3, theoretical contaminants in the T_{reg} sort, are insufficient to modify the outcome of these transfer assays.

Ex-Foxp3 cells are largely resistant to regaining lost identity

The identification of T_{reg} plasticity as a subset property is inherently problematic for usage of T_{reg} in cell therapy applications. As T_{reg} possess self-reactive TCRs (20), an ex-Foxp3 cell inherits both the inflammatory potential of a conventional T cell and the potential for recognition of self-antigens of a T_{reg}, with commensurate risk of autoimmunity. To reconcile this risk with the observation of ex-Foxp3 cell formation, we previously proposed a “transient flexibility” model, whereby ex-Foxp3 cells can aid in acute inflammation, but are biased towards returning to the T_{reg} fate once the destabilising condition is removed (12). To directly test whether ex-Foxp3 cells possess such a “fate memory” and return to become T_{reg}, we tested the capacity of ex-Foxp3 cells to re-enter the T_{reg} lineage, *in vitro* and *in vivo*. *In vitro*, we purified ex-Foxp3 cells 14 days after lymphopenic transfer and cultured them under T_{reg} polarizing conditions. Naïve and activated CD4⁺ T cells were subjected to the same conditions, the latter in their activated, inflammatory state being more similar to ex-Foxp3 cells. Surprisingly, no apparent affinity for the re-expression of Foxp3 within ex-Foxp3 cells was observed, with Foxp3 induction rates far lower than naïve T cells or even activated T cells (Fig. 3A, B, Fig. S6).

As a complementary *in vivo* approach, we modified the lymphopenic transfer system to allow for simultaneous relief of the T_{reg}-destabilizing condition and tracking of ex-Foxp3 cell potential for T_{reg} fate-rebound (Fig. 3C, Fig. S7A). We started with the basic system of lymphopenic transfer of T_{reg}, but using *Foxp3^{GFP-DTR}* mice as the source mice for T_{reg} donors. As with other T_{reg} sources, this results in the generation of divergent T_{reg} and ex-Foxp3 populations, however unlike the standard system, diphtheria toxin receptor (DTR) is only expressed on the cells remaining as T_{reg}, with ex-Foxp3 cells losing DTR expression along with Foxp3. This allowed us to deplete T_{reg} (DTR⁺) with diphtheria toxin (DT) 14 days post-transfer, while leaving ex-Foxp3 cells (DTR^{neg}) untouched (Fig. S7B). Simultaneous with depletion of the original T_{reg} population, we replenished the lymphoid compartment with splenocytes from *Foxp3^{YFP-Cre}Rosa^{RFP}* fate-mapper mice. This replenishment allows us to monitor the behavior of the remaining DTR^{neg} ex-Foxp3 cell population in a lymphocyte-containing system. Splenocyte transfer in this system reduced serum levels of inflammatory cytokines such as IFN γ , IL-2, IL-6 and CXCL1 (Fig. S7C). While lymphocyte profiles were not entirely normalized by splenocyte transfer (Fig. S7D-F), the T_{reg}-destabilizing environment of lymphopenia was negated, as measured by tracking ex-Foxp3 cell formation from *Foxp3^{YFP-Cre}Rosa^{RFP}* fate-mapper T_{reg} (YFP⁺RFP⁺ T_{reg} to YFP^{neg}RFP⁺ ex-Foxp3 cells). The lack of enrichment for YFP^{neg} RFP⁺ ex-Foxp3 cell numbers in the lymphorepleting splenocytes (Fig. 3D, E) demonstrates that the system is lymphoreplete to the degree that it no longer drives spontaneous ex-Foxp3 formation, although the lack of spontaneous T_{reg} induction in co-transferred congenically disparate naïve T cells indicates the environment was not T_{reg}-inducing either (Fig. 3G). This system therefore allowed us to ask the question whether ex-Foxp3 cells, de-differentiated under lymphopenic conditions, spontaneously revert back to

Foxp3 expression if the environment becomes neutral in terms of T_{reg} stability. Despite the partial resolution of the environment to a neutral milieu, we observed no evidence of DTR^{neg} ex-Foxp3 cell to T_{reg} re-conversion, with T_{reg} numbers from the DTR^{neg} ex-Foxp3 cell population equivalent to that from the control congenic naïve T cells (Fig. 3F, G). Even if we reverted lymphopenia at an earlier time-point, day 5 after *Foxp3*^{GFP-DTR} T_{reg} transfer (the earliest point where ex-Foxp3 cells are consistently identified, Fig. 2E), ex-Foxp3 cells did not demonstrate any propensity to re-convert to the Foxp3⁺ T_{reg} lineage (Fig. S8). Overall, this data demonstrates that, both *in vitro* and *in vivo*, the ex-Foxp3 cell population induced by lymphopenia did not maintain a fate memory for the T_{reg} lineage.

Single cell RNA sequencing identifies a potential intermediate state in ex-Foxp3 cell generation

The persistence of the inflammatory status in ex-Foxp3 cells (Fig. 1B), even following amelioration of the original insult, highlights the importance of characterizing the unstable subset present within the original heterogeneous T_{reg} population. It has previously been proposed that CD25^{low} cells among T_{reg} are distinctly unstable (18, 24). To test this, we sorted CD25^{high} and CD25^{low} T_{reg} from congenically-disparate mice, reconstituted the mixture in proportions reflecting the initial make up, and challenged stability through lymphopenic co-transfer with congenic naïve T cells into *Rag1*^{KO} mice. However, unlike previous reports, no difference was observed in the propensity of CD25^{high} or CD25^{low} T_{reg} to form ex-Foxp3 cells (Fig. S9). Therefore, we turned to a targeted single-cell multi-omics approach to identify cellular candidates for the precursor to ex-Foxp3 cell formation. To identify the molecular T_{reg} signature associated with instability, we performed sc-RNAseq after adoptive transfer using a custom gene panel enriched for T_{reg} signature genes (Table S1) combined with single cell proteomics (BDTM AbSeq). Based on the kinetic data obtained by flow cytometry (Fig. 2E, F), we assessed changes in gene expression in T_{reg} before they undergo loss of Foxp3 (CD4⁺ YFP⁺ RFP⁺ cells from an unmanipulated *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mouse) and CD4⁺ YFP^{neg} RFP⁺ ex-Foxp3 cells at 5, 10 and 15 days post-lymphopenic co-transfer of *Foxp3*^{YFP-Cre} *Rosa*^{RFP} T_{reg} and congenic naïve T cells into *Rag1*^{KO} mice (Fig. 4A, Fig. S10A). Unsupervised clustering analysis identified six major clusters visualized using Uniform Manifold Approximation and Projection (UMAP) (Fig. 4B). *Ex vivo* T_{reg} formed two clusters, distinct from ex-Foxp3 cells. Day 5 ex-Foxp3 cells formed a third cluster, disparate from both *ex vivo* T_{reg} and later ex-Foxp3 cell time points, while day 10 and day 15 ex-Foxp3 cells were intermingled in the remaining three clusters. These results indicate that the reprogramming of T_{reg} occurs rapidly, with the T_{reg} signature partially lost by day 5, and the reprogramming plateaued by day 10. *Foxp3* gene expression was absent among all ex-Foxp3 cell populations, corresponding with the reporter-based sorting (Fig. 4C). Differential expression analysis between the different time points identified 3 major classes of genes: rapidly lost genes, transiently-enriched genes and late-enriched genes (Fig. 4D, Fig. S10B, G, Table S2). Rapidly lost genes mainly included T_{reg} markers (e.g. CD25, *Foxp3*, *Ikzf4*, *Ctla4*, PD-1, *Ikzf2*), indicating an immediate shutdown of T_{reg} signature genes at onset of instability. At day 5, a transiently upregulated gene set included genes with major roles in stability loss (including *Satb1* and *Pde3b*), potentially contributing to the rapid reprogramming of the T_{reg} identity (3, 25–27). Interestingly, day 5 ex-Foxp3 cells were of a naïve phenotype, reflected by high expression of CD62L and *Ccr7*, while expression of CD44 and *Cxcr3* were absent (Fig. 4D, E, Fig. S10B, C). Finally, after 10 days, ex-Foxp3 cells had lost a substantial fraction of the T_{reg} signature, lost the transiently upregulated gene set and had reprogrammed into a highly activated phenotype that showed little evolution at the later time point analyzed (Fig. 4D, Fig. S10B, C, Table S2).

The custom gene and AbSeq panel enriched for T_{reg} signature genes allowed us to quantify the temporal loss of the T_{reg} signature in ex-Foxp3 de-differentiation. Considering previously identified T_{reg} (core) signatures (28, 29), our panel contains 219 T_{reg} signature markers (214 genes and 5 AbSeq, Table S3). The unmanipulated T_{reg} population expressed 99.5% of the T_{reg} signature genes evaluated here. This frequency gradually declined upon loss of Foxp3 expression with T_{reg} signature gene expression: day 5 ex-Foxp3 cells expressed 72%, day 10 ex-Foxp3 cells expressed 54% and day 15 ex-Foxp3 cells expressed 56% (significance threshold = 0.0001, FC threshold = 2, Fig. S10D). This data supports a model where ex-Foxp3 cells can arise from bona-fide T_{reg} : the de-differentiation does not begin until more than 3 days post-transfer (Fig. 2E) and the emergent cells have intermediate expression of signature genes, suggesting transcriptional reprogramming beyond merely loss of Foxp3. In addition, given the homogenously high CD62L level of day 5 ex-Foxp3 cells and further given that transient Foxp3 expression in non-Treg is related to activation, our data support the hypothesis that unstable T_{reg} are the main contributor to the observed ex-Foxp3 cell population. The early changes in T_{reg} -specific gene signature are therefore likely to readout downregulation rather than lack of expression in Foxp3-expressing precursors. In the specific case of CD25 expression, this conclusion was formally validated in transfer experiments (Fig. S9).

To gain insight into the origin of the unstable T_{reg} subset, we performed a network-based analysis to identify day 0 T_{reg} with the greatest similarity to day 5 ex-Foxp3 cells (Fig. 4F, Fig. S10E-G). Using this approach, the T_{reg} population divided into 3 clusters, which we annotated as ‘pure’ T_{reg} (not connected to ex-Foxp3 cells), interconnected or ‘inter’ T_{reg} (mostly connected to day 5 ex-Foxp3 cells) and ‘other’ T_{reg} (connected to day 10/15 ex-Foxp3 cells, but not to day 5 ex-Foxp3 cells). The ‘other’ T_{reg} population shared a CD62L expression phenotype with day 10/15 ex-Foxp3 cells, suggesting a convergent activation-induced basis for connectivity (Fig. S10E, F). The ‘inter’ T_{reg} , having the greatest transcriptional relatedness to day 5 ex-Foxp3 cells and a distinct gene expression profile, were identified as the candidate population enriched in unstable T_{reg} . The ‘inter’ T_{reg} population expressed 88% of the analysed T_{reg} signature genes and therefore ‘inter’ Treg still represent bona fide T_{reg} despite their apparent similarity to day 5 ex-Foxp3 cells. ‘Inter’ T_{reg} were enriched for naïve $Nrp1^{neg}$ T_{reg} (Fig. 4G), consistent with an enrichment of naïve cells in flow cytometric profiles of splenic $Nrp1^{neg}$ T_{reg} compared to $Nrp1^{+}$ T_{reg} (Fig. 4H). While we detected *Nrp1* in 22% total day 0 T_{reg} (which, based on coexpressed genes, extrapolates out to 89% of the population), only in 13% of ‘inter’ T_{reg} and 7% of day 5 ex-Foxp3 cells *Nrp1* was detected (cell numbers precluded prediction) (Fig. 4I). Considering that *Nrp1* does not present a lagging marker upon loss of Foxp3 (Fig. 2F), this suggests that $Nrp1^{neg}$ T_{reg} may preferentially give rise to ex-Foxp3 cells. However, within the ‘inter’ T_{reg} subset, both $Nrp1^{+}$ as well as $Nrp1^{neg}$ cells displayed high connectivity with day 5 ex-Foxp3 cells and little connectivity with day 10/15 ex-Foxp3 cells (Fig. 4 I). Further, we find that day 5 ex-Foxp3 cells express high levels of CD62L (Fig. 4E), an expression level that compares to the subset of ‘inter’ T_{reg} (Fig. S10E). While, compared to day 0 T_{reg} , the majority of genes that are differentially expressed in all ‘inter’ T_{reg} are also differentially expressed in the CD62L^{high} fraction of ‘inter’ T_{reg} , *Nrp1* is downregulated specifically in the latter (Table S4). Together, this data raises the hypothesis that naïve $Nrp1^{neg}$ T_{reg} are the subset present in the heterogenous T_{reg} population that are most prone to deconvert following lymphopenic challenge.

The unstable T_{reg} population is enriched for peripheral Treg

Lack of Nrp1 expression has been suggested to enrich for peripheral-induced T_{reg} (pT_{reg}) (30, 31). To formally test whether latent instability resides within the pT_{reg} compartment, we used the BAC-Foxp3^{Cre-GFP} fate-mapping strain, which, unlike the *Foxp3*^{YFP-Cre} strain, has been demonstrated to preferentially label thymus-derived T_{reg} (tT_{reg}) (32, 33) (Fig. S11A,B). Compared to *Foxp3*^{YFP-Cre} fate-mappers, BAC-Foxp3^{Cre-GFP} fate-mapping mice have lower spontaneous ex-Foxp3 cell numbers (Fig. 5A), suggestive of increased stability of the labelled population of tT_{reg}. By crossing the BAC-Foxp3^{Cre-GFP} transgene to the CD45.1⁺CD45.2⁺*Foxp3*^{Thy1.1} background and sorting CD4⁺Thy1.1⁺ T cells, we could sort the T_{reg} population as a whole, containing both the BAC(GFP)⁺ Foxp3(Thy1.1)⁺ population (enriched for tT_{reg}) and BAC(GFP)^{neg} Foxp3(Thy1.1)⁺ population (enriched for pT_{reg}), retaining the original ratio when reconstituting *Rag1*^{KO} mice (Fig. 5B). Following lymphopenic co-transfer with congenically-disparate naïve T cells, a substantial proportion of the pT_{reg}-enriched population had formed ex-Foxp3 cells, compared to only a minor fraction of tT_{reg}-enriched cells (Fig. 5C, D, Fig. S11C). This ex-Foxp3 cell formation was accordingly accompanied by a relative depletion of pT_{reg}-enriched cells from the remaining T_{reg} pool (Fig. 5E). Notably, while the pT_{reg}-enriched population was inherently more prone to ex-Foxp3 cell formation (Fig. 5D), the distinction was not absolute, and due to the greater numbers of tT_{reg}-enriched cells in the input fraction, in terms of absolute number both fractions contribute nearly equally to the ex-Foxp3 cell population (Fig. 5F). Upon transfer, ex-Foxp3 cells demonstrated a differential expression based on T_{reg} of origin, with a higher expression of Nrp1 and Eos in thymic-derived ex-Foxp3 cells, reflecting the different starting expression patterns, and enhanced production of IL-17 by peripheral-induced ex-Foxp3 cells (Fig. 5G, H). Using Nrp1 as an independent marker for tT_{reg} enrichment (30, 31), transfer of Nrp1⁺ thymic or Nrp1^{neg} gut T_{reg} into *Rag1*^{KO} mice reproduced these results (Fig. S12).

Our single cell analysis suggested that instability in the Nrp1^{neg} T_{reg} fraction resided particularly within the naïve compartment. We therefore tested the instability of Nrp1^{neg} T_{reg} and Nrp1⁺ T_{reg} populations based on their activation status (Fig. 6A, Fig. S13). Congenically-disparate naïve Nrp1⁺ T_{reg} (CD4⁺ CD44^{neg} CD62L⁺ Nrp1⁺ Foxp3-Thy1.1⁺) and activated Nrp1⁺ T_{reg} (CD4⁺ CD44⁺ CD62L^{neg} Nrp1⁺ Foxp3-Thy1.1⁺) were co-transferred with naïve, conventional T cells (CD4⁺ CD44^{neg} CD62L⁺ Foxp3-Thy1.1^{neg}) into *Rag1*^{KO} mice. Similarly, congenically-disparate naïve Nrp1^{neg} T_{reg} (CD4⁺ CD44^{neg} CD62L⁺ Nrp1^{neg} Foxp3-Thy1.1⁺) and activated Nrp1^{neg} T_{reg} (CD4⁺ CD44⁺ CD62L^{neg} Nrp1^{neg} Foxp3-Thy1.1⁺) were adoptively transferred with naïve, conventional T cells into *Rag1*^{KO} mice. Validating our results above, the Nrp1^{neg} T_{reg} fraction displayed heightened instability (Fig. 6B, C). Nrp1⁺ T_{reg}-derived ex-Foxp3 cells disproportionately retained expression of certain T_{reg} signature markers (such as Eos, GITR, CTLA-4, CD25, Helios) at 14 days post-adoptive transfer (Fig. 6D), suggestive of a more gradual loss of T_{reg} identity in the Nrp1⁺ T_{reg} fraction compared to the Nrp1^{neg} T_{reg} fraction. When further separating each subset based on activation status, we did not observe a heightened instability in the CD62L⁺ CD44^{neg} Nrp1^{neg} T_{reg} fraction (Fig. 6E). However, the naïve nature of the majority of Nrp1^{neg} T_{reg} in spleen and lymph nodes can explain why we find an enrichment of CD62L⁺ cells within the ‘inter’ T_{reg} population (Fig. 4G). Together, these results validate the molecular model generated through our single cell analysis, with Nrp1^{neg} T_{reg} demonstrating enhanced intrinsic instability. However, while naïve T_{reg} trend to give rise to more ex-Foxp3 cells than activated T_{reg}, a possible enrichment of the unstable subset within naïve T_{reg} may be diluted.

Recent thymic emigrant T_{reg} contribute to the unstable T_{reg} population

While pT_{reg} disproportionately contribute to the ex-Foxp3 population, tT_{reg} were a substantial contributor to the total ex-Foxp3 cell population (Fig. 5F). Therefore, we sought to determine which unstable tT_{reg} population could be contributing to the ex-Foxp3 cell population. In our single-cell analysis we observed that early ex-Foxp3 cells showed a heightened expression of *Pdlim4*, an adaptor protein of S1P1 which is essential for egress from secondary lymphoid organs and the thymus (Fig. S10B) (34, 35). Considering the highly naïve phenotype of early ex-Foxp3 cells with high CD62L and *Ccr7* (Fig. 4E, Fig. S10B, C), we hypothesized that recent thymic emigrants may contain unstable T_{reg} cells. Notably, recent thymic emigrant (RTE) T_{reg} were highly naïve and enriched for Nrp1^{neg} cells and Nrp1⁺ RTE T_{reg} expressed low levels of Nrp1 compared to non-RTE T_{reg} (Fig. 7A, B, Fig. S14 A-C). To test whether RTE T_{reg} are enriched for unstable T_{reg} clones compared to non-RTE T_{reg}, we utilized *Rag2*^{GFP} *Foxp3*^{Thy1.1} mice in which T_{reg} can be identified via Thy1.1 expression and delayed decay of the GFP protein after termination of *Rag2* expression allows for identification of recent thymic emigrant T cells (36). Sort-purified RTE T_{reg} (CD4⁺ Rag2-GFP⁺ Foxp3-Thy1.1⁺) and non-RTE T_{reg} (CD4⁺ Rag2-GFP^{neg} Foxp3-Thy1.1⁺) were each subjected to a lymphopenic challenge of 14 days by co-transfer with congenic naïve T cells into *Rag1*^{KO} mice (Fig. 7C, Fig. S14D). RTE T_{reg} indeed possessed a heightened instability compared to their non-RTE T_{reg} counterpart (Fig. 7D, E, Fig. S14 E, F). This indicates that instability in the tT_{reg} fraction is likely to result mainly from early and incompletely committed RTE T_{reg} as compared to established tT_{reg}.

Discussion

The question of origin for ex-Foxp3 cells has key therapeutic consequences. Several groups have suggested pT_{reg} to possess a higher intrinsic instability than tT_{reg} based on Nrpl expression, and to dominantly contribute to ex-Foxp3 cells through loss of Foxp3 in uncommitted clones (1, 16, 18) with lineage commitment being intimately linked to the demethylation of T_{reg}-specific demethylated regions (11, 16, 37, 38). Overall, ex-Foxp3 cells have been suggested to arise from early uncommitted pT_{reg} (1), from committed T_{reg} or tT_{reg} (11, 16), activated T_{conv} with low and promiscuous Foxp3 expression (16), CD25^{lo/-} T_{reg} as a whole (34) or CD25^{lo/-} Foxp3^{lo} pT_{reg} (16), respectively. Our data builds upon these initial findings, with the addition that instability is especially predominant in a naïve population of Nrpl^{neg} T_{reg} with contribution of both pT_{reg} and tT_{reg}. These findings are in line with preferential loss of Foxp3 expression in early, uncommitted T_{reg} from both the pT_{reg} subset and from recent thymic emigrant tT_{reg} which present as Nrpl^{neg/low}, without excluding the possibility of contributions from other T_{reg} subsets.

Ex-Foxp3 cell development is thought to be driven by TCR stimulation and inflammatory or lymphopenic environments. The extent to which ex-Foxp3 cells develop during acute infection or microbial challenge is unclear and *in vivo* data is limited (8, 13, 14). Our study contradicts previous *in vitro* results using PAMPs and calls for caution when extrapolating strong *in vitro* stimuli to *in vivo* conditions. Here, co-housing with wild-exposed mice imposed a complex microbial challenge combined with T cell activation and T_{reg} expansion. This challenge did not increase ex-Foxp3 cell formation to a detectable level. The origin of ex-Foxp3 cells has been highly debated, with proposals that deconversion is a stochastic event (10, 11), or a feature of an uncommitted T_{reg} population (8, 16). We addressed the stability of the T_{reg} population as a whole, applying a sequential stability challenge. This experimental design is different from, but complementary to previous approaches (1, 16), addressing the key question of potential stochastic instability. Our data demonstrates that the heterogeneous T_{reg} population is purged of unstable clones during the first stability insult, and T_{reg} maintained during this first transfer have a remarkable stability when subjected to a subsequent challenge. These data undoubtedly support the heterogeneity model of T_{reg} stability (39) in which uncommitted clones can give rise to ex-Foxp3 cells, while fully committed T_{reg} remain stable even under harsh lymphopenic conditions.

Due to the self-reactive nature of the TCR of T_{reg} (20), it is important to understand the reversion potential of the ex-Foxp3 cell population. The potential of ex-Foxp3 cells to regain Foxp3 expression has been addressed previously with varying outcomes depending on the source of ex-Foxp3 cells (16, 18). Here, we used both an *in vitro* as well as an *in vivo* approach. Our data are in concordance with previous results with only a minor fraction of lymphopenia-induced ex-Foxp3 cells possessing T_{reg} lineage memory (18). In contrast, ex-Foxp3 cells generated similarly under lymphopenic conditions failed to reconvert upon lymphorepletion and stabilization of the environment. This argues that T_{reg} fate memory is lost in the majority of ex-Foxp3 cells.

The deconversion is likely an active process caused by environmental disruption, as supported by mathematical modelling, and is completed by day 10 after lymphopenic challenge. Our molecular data further supports the notion of loss of fate memory, and indicates an almost immediate shutdown of core T_{reg} signature genes upon lymphopenia-induced T_{reg} instability, of both Foxp3-dependent (such as *Il2ra*) and Foxp3-independent (such as *Ikzf2* and *Ikzf4*) genes. It is therefore plausible that the unstable T_{reg} population is unstable by virtue of loss of the Foxp3-independent feedback circuits, a property exacerbated by lymphopenia. Of note, ex-Foxp3 cells, soon after deconversion, possessed a high expression of CD62L and *Ccr7*, indicative of a naïve

phenotype. This argues that ex-Foxp3 formation is not necessarily a result of T_{reg} activation, but rather originates from the naïve T_{reg} population that has only recently converted and/or not yet received antigenic stimulation. Interestingly, ex-Foxp3 cells, while having lost expression of key T_{reg} signature genes, overall show differential expression of such genes in less than a third of the T_{reg} signature, suggesting that these cells indeed descent from bona fide T_{reg} and not, as previously suggested, from non-suppressive conventional T cells with promiscuous Foxp3 expression (16). The delayed kinetics of ex-Foxp3 appearance after transfer is also more consistent with a legitimate de-differentiation process, a conclusion further supported by mathematical modelling comparing different deconversion process options. Using a network-based approach we identified a T_{reg} subset closely related to the earliest ex-Foxp3 cells. Interestingly, these ‘inter’ T_{reg} are enriched for *Nrp1*^{neg}, CD62L^{high} and CD25^{low} cells. While this data thereby supports previous studies suggesting a stability dichotomy in pT_{reg} with CD25^{low} pT_{reg} possessing a higher degree of instability, we did not find evidence for increased instability in the CD25^{neg} T_{reg} subset. Further, while we show that Nrp1^{neg} T_{reg} from peripheral lymphoid tissues as well as Nrp1^{neg} gut T_{reg} are enriched in unstable cells, a picture that is complicated by the fact that also RTE T_{reg} are enriched in Nrp1^{neg/low} cells. Our data thus reflects the heterogeneity of the unstable T_{reg} population as well as the differential kinetic of T_{reg} identity loss in different T_{reg} subsets and calls for a cautious use of Nrp1 as a marker for pT_{reg} particularly in non-GALT organs. Overall, the unstable T_{reg} clones present a complex mixture but are enriched for naïve Nrp1^{neg} T_{reg} which are likely to represent recently converted but incompletely committed pT_{reg} as well as RTE tT_{reg} cells.

With increasing usage of T_{reg} cell-based clinical trials (40), and the identified risk of autoreactive T_{reg} becoming inflammatory ex-Foxp3 cells (10, 20), the need to avoid T_{reg} plasticity is paramount. The stochastic model would posit that T_{reg} for cell therapy should be treated in a manner to stabilize Foxp3 expression, limiting plasticity after injection, and indeed much effort has been made in this direction (17). Our results, demonstrating that T_{reg} plasticity is a property restricted to an unstable subset, would suggest a radically different approach: rather than aid T_{reg} stability, the ‘purging’ of the T_{reg} population of unstable clones by exposure to a destabilizing environment may leave behind a residual highly stable T_{reg} remnant population. A “destabilize and repurify” strategy may thus result in a population more capable of fate-retention and safer for clinical use than the previous “stabilize and treat” approach. These models would, however, require validation in human T_{reg} systems before incorporation into cell therapy trials.

Materials and Methods

Study design:

The main objective of this study was to investigate the extent to which stochasticity and/or population heterogeneity contribute to the phenomenon of T_{reg} fate loss. To this end, T_{reg} fate mapper mice were co-housed with wild-exposed mice to expose the T_{reg} fate mapper mice to pathogens and microbial challenge. We further performed sequential fate challenges by adoptively co-transferring T_{reg} from fate mapper mice with naïve T cells into lymphopenic hosts, re-isolating the stable, non-converting T_{reg} population and repeating the fate challenge. The T_{reg} population was then dissected into multiple subsets based on markers for origin or activation among others, to compare the intrinsic stability of various subsets upon adoptive transfer into lymphopenic mice. The extent to which ex-Foxp3 cells possess the ability to re-enter the T_{reg} lineage has been addressed *in vitro*, subjecting ex-Foxp3 cells to T_{reg} converting conditions, as well as *in vivo*,

ameliorating the T_{reg}-destabilizing milieu. Finally, we aimed to identify a molecular identity of putative unstable T_{reg}. To this end, ex-Foxp3 cells were subjected to single cell multi-omics at different time points after fate loss and their transcriptome and proteome were compared that of unmanipulated T_{reg}.

Animals:

Rag1^{KO} mice (41), *Foxp3*^{Thy1.1} mice (42), *Foxp3*^{YFP-Cre} mice (43), BAC-Foxp3^{Cre-GFP} mice (33), *Foxp3*^{GFP-DTR} mice (44), *Rosa26*^{flSTOP^{fl}RFP} mice (45) and *Rag2*^{GFP} mice (36) have been described previously and were used on the C57BL/6 and CD45.1⁺ backgrounds. All mice were maintained in specific pathogen-free facilities of the University of Leuven unless otherwise stated. All mice were used at 6 to 20 weeks of age. Donor mice for T_{reg}, naïve T cells and splenocytes for transfer into *Rag1*^{KO} mice were male. *Rag1*^{KO} recipient mice were male or female.

For co-housing experiments, wild-exposed pet store female mice were co-housed for a period of 8 weeks with *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} female mice in an L2 animal facility. Bedding was exchanged regularly between different cages to stabilize the microbiome between different cages. Co-housed (n=3) and SPF (n=1) *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} fate-mapper mice were subjected to health screening and tested for 47 pathogens. All mice tested positive for *Trichomonas* sp., but only co-housed mice tested additionally positive for *Rodentibacter* spp. (formerly *P. pneumotropica*) (2 out of 3), *Adenovirus* K87 (1 out of 3), *Murine norovirus* (3 out of 3), *Theiler's encephalomyelitis virus* (GD VII) (3 out of 3), *Aspiculuris tetraptera* (1 out of 3), *Myobia musculi* / *Radfordia* sp. (3 out of 3), *Myocoptes musculus* (3 out of 3), *Spironucleus muris* (2 out of 3), *Syphacia obvelata* (1 out of 3), *Helicobacter bilis* (2 out of 3), *Helicobacter* spp. (3 out of 3) and *Helicobacter typhlonicus* (1 out of 3). Ectopic excision of the *Rosa26*^{flSTOP^{fl}RFP} allele was analyzed using PCR and flow cytometry, and *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} mice showing unspecific recombination were excluded from *ex vivo* analysis (Fig. S2B).

In T_{reg} transfer experiments utilizing T_{reg} from *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} mice, unspecific, erratic recombination by the *Foxp3*^{YFP-Cre} allele is bypassed as T_{reg} were sort-purified based on the expression of YFP. Mice adoptively transferred with *Foxp3*^{GFP-DTR} T_{reg} were i.p. injected at day 14 and 15 post-transfer with a dose of 50 µg/kg of diphtheria toxin (DT, Native Antigen Company). For adoptive transfer experiments, T_{reg} were sorted from either *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} mice (CD4⁺ YFP⁺ RFP⁺), CD45.1 *Foxp3*^{Thy1.1} mice (CD4⁺ Thy1.1⁺), BAC *Foxp3*^{GFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} *Foxp3*^{Thy1.1} mice (CD4⁺ Thy1.1⁺) or CD45.1 *Foxp3*^{GFP-DTR} mice (CD4⁺ GFP⁺) (3-4 x 10⁵) and i.v. co-injected with naïve T cells from *Foxp3*^{Thy1.1} mice (CD4⁺ CD62L⁺ CD44^{neg} Foxp3-Thy1.1^{neg}; 3-4 x 10⁵) in a 1:1 ratio into *Rag1*^{KO} mice. Post-sort purity was an average of 96%. For the RTE adoptive transfer experiment, CD45.2 *Foxp3*^{Thy1.1} T_{reg} (CD4⁺ Thy1.1⁺) were sorted from lethally irradiated mice (9.5Gy) reconstituted with bone marrow from *Foxp3*^{Thy1.1} *Rag2*^{GFP} mice at 8 weeks after bone marrow transplantation. To assure that the transferred Foxp3-reporter-expressing cells from *Foxp3*^{YFP-Cre} *Rosa26*^{flSTOP^{fl}RFP} mice are bona-fide T_{reg}, we sorted YFP⁺ RFP^{high} cells (which have been shown to be committed to the T_{reg} lineage and to possess a fully demethylated TSDR (16)) (Fig. S4A). Mice were euthanized at the indicated time points and analyzed for ex-Foxp3 cell formation using high-parameter flow cytometry (gating strategy in Fig. S1C). All experiments were approved by the University of Leuven Animal Ethics Committee (P088/2018, P105/2018, P008/2019, P150/2019).

Flow cytometry and cell sorting:

Cells for adoptive transfer were CD4-enriched from spleen and mesenteric/peripheral lymph nodes using the MojoSort™ Mouse CD4 T Cell Isolation Kit (BioLegend) according to the manufacturer's instructions, followed by antibody staining and flow cytometric sorting for Foxp3 reporter expression. Thymi were CD8-depleted prior to sorting CD4⁺ CD8^{neg} Foxp3-Thy1.1⁺ Nrp1⁺ T_{reg}. In brief, single cell suspensions of thymi were stained for CD8-biotin (53-6.7, eBioscience) and subsequently CD8-expressing cells were captured using streptavidin-coated magnetic beads obtained from the Magnisort CD4 T cell enrichment kit (eBioscience). For sorting of T_{reg} from gut tissue, single cell suspensions were prepared from small intestine. Small intestines were first incubated in a pre-digestion step in HBSS containing 2.5% FCS and 10mM EDTA to remove the epithelial layer. Next, the single cell suspension was prepared by digestion for 30 minutes at 37°C with 1 mg/ml collagenase IV (ThermoFisher) and 40 mg/ml DNase I (Sigma-Aldrich) in IMDM (Thermo) supplemented with 2mM MgCl₂, 2mM CaCl₂, 20% FBS and 2 mM HEPES. Digested tissue was mechanically disrupted, filtered through 70 µm mesh and enriched for leukocytes by centrifugation (400 g, 10 minutes, no brakes) through 40% Percoll (Sigma-Aldrich). Afterwards cells were further enriched for CD4⁺ T cells using the MojoSort™ Mouse CD4 T Cell Isolation Kit (BioLegend) according to the manufacturer's instructions, followed by antibody staining and flow cytometric sorting for CD4⁺ Nrp^{neg} Foxp3-Thy1.1⁺ T_{reg}. For kinetic analyses, T_{reg} were labelled with CellTrace Violet (Thermo Fisher Scientific) according to the manufacturer's instructions before adoptive transfer into *Rag1*^{KO} mice.

Samples were analyzed using the following antibodies: anti-BB220 (RA3-6B2), anti-CD11b (M1/70), anti-CD19 (1D3, B4), anti-CD25 (PC61), anti-CD3 (17A2), anti-CD4 (GK1.5, RM4-5, SK3), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD8α (53-6.7), anti-CD90.1 (anti-Thy1.1; OX-7, HIS51), anti-CD103 (M290), anti-CD304 (anti-Nrp1; 3DS304M, 3E12), anti-CTLA-4 (UC10-4F10-11), anti-Eomes (Dan11mag), anti-Eos (ESB7C2), anti-Foxp3 (REA788, FJK-16s), anti-GATA-3 (L50-823), anti-GFP (FM264G), anti-GITR (DTA-1), anti-Helios (REA829), anti-ICOS (C398.4A), anti-IFNγ (XMG1.2), anti-IL-2 (JES6-5H4), anti-IL4 (11B11), anti-IL-6 (MP5-20F3), anti-IL-10 (JES5-16A3), anti-IL-17A (TC11-18H10), anti-KI67 (16A8), anti-KLRG-1 (2F1), anti-NK1.1 (PK136), anti-PD-1 (29F.1A12), anti-T-Bet (4B10), anti-TCRβ (H57-597), anti-TCRγδ (GL3), anti-TGF-β (TW7-16B4), anti-TNF-α (MP6-XT22) and fixable Viability Dye eFluor™ 780 from Abcam, BioLegend, BD Bioscience, eBioscience and Miltenyi Biotec. For intracellular cytokine staining, lymphocytes were plated in 96-well tissue-culture plates in complete RPMI (Thermo Fisher Scientific) containing Phorbol 12,13-dibutyrate (500 ng/ml, Bio-Techne), ionomycin (750 ng/mL, Bio-Techne), and brefeldin A (2 µg/ml, Bio-Techne) for 4 h at 37°C. Cells were blocked with 2.4G2 hydridoma supernatant in combination with mouse IgG (ThermoFisher Scientific) for 20 min at 4°C. Cells were fixed with either 2% formalin prior to intracellular cytokine staining or the Transcription Factor Staining Buffer Set (eBioscience). Flow cytometry was performed on a BD FACSCanto II or BD FACSymphony and sorting was performed on a BD FACSAria III (BD Biosciences) and a Sony MA900 (Sony). Results were analyzed with FlowJo version 10.6.1 (Becton Dickinson, Ashland). Compensation for high parameter flow panels was performed using Autospill (46).

In vitro assays:

Ex-Foxp3 cells (CD4⁺ YFP^{neg} RFP⁺), naïve T cells (CD4⁺ CD62L⁺ CD44^{neg} RFP^{neg} GFP^{neg}) and activated T cells (CD4⁺ CD62L^{neg} CD44⁺ RFP^{neg} GFP^{neg}) from spleen, peripheral lymph node (pLN) and mesenteric lymph node (MLN) of adoptively transferred *Rag1*^{KO} (ex-Foxp3

cells) or *Foxp3^{YFP-Cre} Rosa26^{flSTOP^{fl}RFP}* mice (naïve/activated T cells) were purified using MagniSort-CD4⁺ T-cell enrichment kit (eBioscience) according to the manufacturer's instructions followed by FACS sorting. Next, cells were seeded in 96-well plate (3 x 10⁴ per well) with Dynabeads mouse T Activator CD3/CD28 (ThermoFisher) in a 1:4 beads to cell ratio in complete RPMI in presence of human TGFβ (0 or 10 ng/ml, R&D systems), and mouse IL-2 (20 ng/ml, eBioscience) for stimulation. Cells were cultured for 5 days after which flow cytometry analysis was performed for Foxp3 expression.

Serum cytokine and CRP analysis:

Serum cytokine levels were determined using the MSD pro-inflammatory mouse cytokine panel (Meso Scale Diagnostics, Rockville, Md), according to the manufacturer's instructions. CRP levels were determined using the Mouse CRP ELISA Kit (ThermoFisher) according to the manufacturer's instructions.

Microbiome sequencing:

Microbiome sequencing was performed as previously described (47). Fecal samples were frozen at the experimental end for subsequent microbiome analysis. The V4 region of the 16S rRNA gene was amplified with the primer pair 515F and 806R (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT, respectively), modified to contain a barcode sequence between each primer and the Illumina adaptor sequences to produce dual-barcoded libraries (48).

Size selection, before Illumina sequencing, was performed using Agencourt AMPure to remove fragments below 200 bases. Sequencing was performed on the Illumina MiSeq platform (MiSeq Reagent Kit v2, 500 cycles, 15.38 % PhiX, 2*250 PE) at the VIB Nucleomics core laboratory (Leuven, Belgium). After de-multiplexing with sdm without allowing for mismatches, as part of the LotuS pipeline (49), fastq sequences were further analyzed per sample using the DADA2 pipeline (v. 1.6) (50). In brief, after inspecting quality, sequences were trimmed to remove the primers and the first 10 bases after the primer, keeping only 200 bases and 130 for the R1 and R2 files, respectively. After merging paired sequences and removing chimeras, taxonomy was assigned using formatted RDP training set 'rdp_train_set_16'.

Sequences annotated to the class Chloroplast, family mitochondria or unknown bacteria were removed prior to the analyses. The sequencing data was rarefied to 10,000 observations to calculate inter-individual and intra-individual microbial diversity. All further analyses were performed at the genus level, such as richness, Shannon diversity index and Bray-Curtis dissimilarity index. Statistical analyses were performed using R, and packages phyloseq (51) and vegan (52). For microbiota and metadata association analysis, we excluded taxa present in <20% of samples. Correlations between bacteria and archaea genera and mice group status were performed using Wilcoxon test. Correction for multiple testing using Benjamini-Hochberg's procedure (false discovery rate (FDR))(53) was performed when applicable; significance was defined at FDR<10%.

Single cell RNA sequencing and AbSeq:

Splenic and peripheral/mesenteric LN T_{reg} (CD4⁺ YFP⁺ RFP⁺) from *Foxp3^{YFP-Cre} Rosa26^{flSTOP^{fl}RFP}* fate-mapper mice were co-transferred with congenic splenic naïve T cells (CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}) from CD45.1 *Foxp3^{Thy1.1}* mice into *Rag1^{KO}* mice. For single cell RNA sequencing (scRNA-seq) and AbSeq, ex-Foxp3 cell samples (CD4⁺ YFP^{neg} RFP⁺, spleen/LNs pooled) at the indicated time points after adoptive transfer and T_{reg} (CD4⁺ YFP⁺ RFP⁺,

spleen/LNs pooled) from an unmanipulated *Foxp3^{YFP-Cre} Rosa26^{flSTOP^{fl}RFP}* mouse were obtained by FACS sorting and stained with sample tag antibodies (BD Single-Cell Multiplexing Kit, BD biosciences). Next, samples were pooled and stained using the following oligonucleotide-conjugated antibodies according to manufacturing instructions: anti CD25 (PC61), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-GITR (DTA-1), anti-CD4 (RM4-5), anti-TCR β (H57-597), anti-CD69 (H1.2F3), anti-TIGIT (1G9), anti-CD95 (Jo2), anti-CD122 (TM-1 β), anti-Tim-3 (5D12), anti-CCR7 (4B12), anti-CD103 (M290), anti-CD279 (J43), anti-CD274 (MIH5), anti-CD233 (C9B7W), anti-CD71 (C2), anti-CD278 (7E7G9), anti-ITGB7 (M293), anti-CD137 (1AH2), anti-CD40 (3/23), anti-CD3 ϵ (145-2C) from BD Biosciences. Multiplexing, subsequent pooling and simultaneous processing avoided sample to sample batch effects.

Single-cell capture and cDNA library preparation was performed using the BD Rhapsody Single-Cell Analysis System (BD Biosciences), according to the manufacturer's instructions, using a custom gene panel (591 genes, Table S1). In short, bead-captured mRNA and oligo-conjugated antibodies were reverse transcribed and treated with exonuclease Exo I. A first round of 12 PCR cycles was performed with BDTM Abseq primer, sample tag PCR1 primers and a custom PCR1 primer panel. Next, the PCR product was purified by double-sided size selection with AMPure XP beads (Agencourt) to separate the oligo-conjugated antibodies PCR1 product (~170 bp) from mRNA-targeted PCR1 products (350–800 bp). The mRNA-targeted PCR1 products were subjected to a second round of PCR using a custom PCR2 primer panel, upon which the PCR product was purified with AMPure XP beads. A fraction of the oligo-conjugated antibodies PCR1 product was also subjected to a second round of PCR using the Sample Tag PCR2 Primer and the PCR product was purified with AMPure XP beads. The mRNA targeted PCR2 products, Sample Tag PCR2 products and BDTM Abseq PCR1 products were then amplified by PCR with library primers containing the Illumina Index 1 (i7). Finally, the three libraries were purified with AMPure XP beads. Quality check was performed on Agilent Bioanalyzer with the High Sensitivity Kit for oligo-conjugated antibodies PCR1 product and for the three final libraries. The libraries were then pooled to aim for 8000 reads per cell for the targeted mRNA library, 300 reads per tagging antibody per cell for the sample tag library and 300 reads per BDTM Abseq antibody per cell for the BDTM Abseq library. Sequencing was performed on an Illumina NextSeq500 instrument using a Mid Output kit v2.5 (150 cycles, paired-end). Sample demultiplexing, barcode processing, alignment, filtering, UMI counting were done using the standard BD Biosciences Rhapsody analysis pipeline on Seven Bridges (www.sevenbridges.com).

Further analysis was done using the Seurat 3.0 R toolkit (54) and Heatmapper (55) for clustering analysis. For network-based analysis, BioLayout Express3D (56) was used. In brief, the expression matrix file was preprocessed and imported into BioLayout Express3D, as well as the file containing the sample tag calls, which was used for the creation of a ".classsets" file. Based on a user-defined Pearson correlation coefficient threshold ($r = 0.95$), an undirected network graph of the data was generated whereby the nodes represent cells and the edges between them represent Pearson correlation relationships ($r \geq 0.95$). After multiple filtering iterations by node degree ($\delta(G) = 3$), fringe nodes were removed and cells with related expression profile clustered together in distinct regions of the graph. First, the day 0 cells that are connected only with day 0 cells were distinguished ("pure" T_{reg}) and afterwards, the day 0 cells that are connected with at least one "pure" T_{reg} and one day 5 ex-Foxp3 cell were identified, which resulted in 506 intermediate T_{reg} cells ("inter" T_{reg}), while the remaining day 0 cells were classified as "other" T_{reg}. Volcano plots (significance versus fold-change) were created with ggplot2 (57), by plotting the $-\log_{10}$ of the p-value on the y-axis, and the $\log_2$ of the fold-change in gene expression between two cell subsets

on the x-axis. Significantly differentially expressed genes (FDR < 0.0001 and fold-change > 2) are indicated in red for up-regulated, and in blue for down-regulated genes.

Mathematical modelling of ex-Foxp3 kinetics:

A probabilistic model was generated to model the kinetics of ex-Foxp3 T cell expansion. We generated two sets of models, in order to compare the consistency of the experimental data with a model of active Foxp3 deconversion (i.e., one where ex-Foxp3 cells arise from stable T_{reg}) to a model of Foxp3 expression homeostasis (i.e., one where ex-Foxp3 cells arise from transient expression of Foxp3 in T_{conv}). First, we fitted a model assuming that Foxp3 loss starts from time $t = 0$ days, i.e. modelling active deconversion at day 0. The length of Foxp3 expression in each cell was modelled as $N(\mu, \sigma^2)$, where we searched for optimal μ, σ parameters over a grid of possible values. As an alternative, we considered a homeostatic model allowing continual flipping between Foxp3 expression, where $t = 0$ did not change the biological context, but only allowed the discrimination of ex-Foxp3 cells from never-Foxp3 cells. Under this scenario, the start time of Foxp3 expression was modelled as uniformly distributed random variable $\sim U(-\mu-4\sigma, 0)$. The length of Foxp3 expression was modelled as $N(\mu, \sigma^2)$, where we searched for optimal μ, σ parameters. To model Foxp3 selection, we filtered for random variables spanning $t = 0$ only, leading to a population with a median Foxp3 expression time $t = 0$. The goodness of fit for each tested parametric set and model was evaluated by mean squared error divided by the standard error ex-Foxp3 cells at each time point (adjusted MSE): $(1/4) \times \sum_i (\text{mean}(y_{t(i)}) - \text{fitted_mean}(y_{t(i)}))^2 / \text{se}(\text{mean_}y_{t(i)})$ to reflect the ex-Foxp3 data heteroskedasticity. Probability (Prob) of observing the ex-Foxp3 cell data under the tested parameters and model was calculated assuming normal distribution at each time point. Data and simulation runs are available as Supplementary Dataset 1, and the code used to generate the simulations is available as Supplementary Code 1.

Statistics:

Statistical analyses were performed with Prism software (GraphPad Software) using the Mann-Whitney U test. For flow cytometric analysis of cytokine markers of the co-housing experiment min-max normalization was used, where each feature was normalized as the range from 0 to 1, to account for variability between experimental repeats.

Supplementary Materials

Table S1. Custom panel single cell RNA sequencing for the BD Rhapsody.

Table S2. Differentially expressed genes during Treg de-differentiation.

Table S3. List of analysed T_{reg} signature genes/AbSeq

Table S4. Differential expression analysis.

Table S5. Wilcoxon tests results for phylum and genera differentially associated to SPF and CoH mice.

Table S6. Raw data file (Excel spreadsheet).

Fig. S1. Gating schemes with representative example plots from flow cytometric analyses.

Fig. S2. Characterization of *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice.

Fig. S3. Changes in microbiome and T cell activation upon co-housing with wild-exposed pet store mice.

Fig. S4. Early T_{reg} proliferation following adoptive transfer into a lymphopenic environment.

Fig. S5. Mathematical modelling of ex-Foxp3 kinetics after transfer is consistent with a stable T_{reg} source.

Fig. S6. Gating scheme and post-culture analysis of *in vitro* T_{reg} conversion assay.

Fig. S7. Ex-Foxp3 cells do not display fate memory *in vivo* upon lymphorepletion.

Fig. S8. Ex-Foxp3 cells do not display fate memory *in vivo* immediately after Foxp3 loss.

Fig. S9. CD25 expression is insufficient to describe the unstable T_{reg} subset.

Fig. S10. Network-based analysis identifies a subset of Treg with high molecular similarity to day 5 ex-Foxp3 cells.

Fig. S11. Phenotypic profiling of CD45.1^{+/.}2⁺ BAC-Foxp3^{GFP-Cre} *Rosa^{RFP} Foxp3^{Thy1.1}* mice.

Fig. S12. Nrp1^{neg} gut T_{reg} possess increased instability compared to Nrp1⁺ thymic T_{reg}.

Fig. S13. Sorting of naïve and activated cells within Nrp1⁺ and Nrp1^{neg} T_{reg} populations.

Fig. S14. Phenotyping of RTE- and non-RTE Treg and ex-Foxp3 cells derived thereof.

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Figure legends:

Figure 1

Fig. 1. No induction of ex-Foxp3 cells following physiological *in vivo* microbial challenge. (A) Schematic overview of the *Foxp3*^{YFP-Cre} and *Rosa26*^{flSTOP^{fl}RFP} loci and representative flow cytometry plots of the *Foxp3*^{YFP-Cre} live YFP reporter vs the RFP fate-mapper cassette expression. (B) Cytokine expression comparing splenic T_{reg} (CD4⁺ YFP⁺ RFP⁺), ex-Foxp3 cells (CD4⁺ YFP^{neg} RFP⁺) and conventional T cells (T_{conv}, CD4⁺ YFP^{neg} RFP^{neg}) from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice (n=10). (C) *Foxp3*^{YFP-Cre} *Rosa*^{RFP} mice were either SPF-housed (n=13) or co-housed with wild-exposed pet store mice for 8 weeks (n=9) and analyzed using high-parameter flow cytometry. Absolute number (D), frequency of CD4⁺ (E) and frequency of RFP⁺ (F) of the splenic T_{reg} population, and absolute number (G), frequency of CD4⁺ (H) and frequency of RFP⁺ (I) of the splenic ex-Foxp3 cell population. (J) T_{reg}, ex-Foxp3 cells and T_{conv} were analyzed for expression of CD25, TGFβ, IL-2, IFNγ, TNFα and Nr1p. Cytokine expression was normalized using min-max normalization. Data are plotted as means ± SEM, and are representative of at least two independent experiments. p-values by unpaired Mann-Whitney U test. Refer to Fig. S1A, B for gating strategy.

Figure 2

Fig. 2. Elevated stability of T_{reg} following *in vivo* purging of the unstable subset. (A) In a primary transfer, CD4⁺ YFP⁺ RFP⁺ T_{reg} from *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice or CD4⁺ Thy1.1⁺ T_{reg} from *Foxp3^{Thy1.1}* mice were sorted and transferred in a 1:1 ratio with naïve T cells from congenic *Foxp3^{Thy1.1}* mice (CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}) into *Rag1^{KO}* mice. After 5 weeks, spleen, peripheral and mesenteric lymph nodes were pooled and sorted for T_{reg} (CD4⁺ YFP⁺ RFP⁺ or congenically-identified CD4⁺ Thy1.1⁺, respectively) as progeny of the transferred T_{reg}. The retrieved T_{reg} population was subsequently transferred in a 1:1 ratio with naïve T cells from congenic *Foxp3^{Thy1.1}* mice (CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}) into *Rag1^{KO}* mice in a secondary transfer and analyzed after 5 weeks for the development of ex-Foxp3 cells. Representative plots (B) and frequencies (C) of the ex-Foxp3 cells (congenically-identified CD4⁺ Foxp3^{neg}) post primary (1°) and secondary (2°) transfer (p < 0.0001, unpaired Mann-Whitney U test on

merged experiments A-F). **(D)** Cytokine profile of T_{reg} and ex-Foxp3 cells post primary and secondary transfer. Percentage of ex-Foxp3 cells **(E)** and change in expression of CD25, Nrpl, Eos and Helios in ex-Foxp3 cells, remaining T_{reg} and congenic T_{conv} **(F)** on days 3, 5, 9, 14, 20, 28 and 40 after adoptive transfer of T_{reg} from *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice and conventional CD4⁺ T cells from CD45.1 *Foxp3^{Thy1.1}* mice (T_{conv}, CD4⁺ Thy1.1^{neg}) into *Rag1^{KO}* mice. For the sequential adoptive transfer experiments **(A–D)**, data plotted for experiment A represent 12 mice pooled into a single sample for 1° transfer or 4 mice pooled into a single sample for 2° transfer, for experiments B-F data represent 2-9 individual mice for 1° transfer or 1-2 individual mice for 2° transfer. For kinetic experiments **(E, F)**, all data are plotted as means ± SEM and represent cells analyzed from 3 to 8 mice per time point (note: day 5 is a concatenated time point of data from 5 individual mice), and are representative of at least two independent experiments, except for samples collected at day 5. p-values by unpaired Mann-Whitney U test. Refer to Fig. S1C for gating strategy and to Fig. S4 for sorting gates and post-sort purity.

Figure 3

Fig. 3. Ex-Foxp3 cells show limited fate memory both *in vitro* and *in vivo*. (A-B) After lymphopenic transfer of T_{reg} (CD4⁺ YFP⁺ RFP⁺) from Foxp3^{YFP-Cre} Rosa^{RFP} fate-mapper mice, ex-Foxp3 cells (CD4⁺ YFP^{neg} RFP⁺) were sorted, cultured with α CD3/ α CD28 activation beads, IL-2 and TGF β for 5 days, followed by assessment of Foxp3 induction (n = 2). As controls, splenic naïve T cells (naïve CD4⁺, CD4⁺ YFP^{neg} RFP^{neg} CD62L⁺ CD44^{neg})

and activated T cells (act. CD4⁺, CD4⁺ YFP^{neg} RFP^{neg} CD62L^{neg} CD44⁺) were sorted from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice (technical repeats, n = 5,8) and cultured under identical conditions. Refer to Fig. S6A for sorting gates and Fig. S6B for analysis gating strategy. Shown are representative plots **(A)** and frequencies of CD4⁺ Foxp3⁺ cells **(B)**. **(C-G)** CD45.1 *Foxp3*^{GFP-DTR} T_{reg} (CD4⁺ GFP⁺) were transferred in a 1:1 ratio with naïve T cells from CD45.2 *Foxp3*^{Thy1.1} mice (CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}) into *Rag1*^{KO} mice. After 14 days, mice were injected with 3x10⁷ CD45.2⁺ splenocytes from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice and 1x10⁵ CD45.1⁺/CD45.2⁺ naïve T cells from *Foxp3*^{Thy1.1} mice (CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}). DT was injected on days 14 and 15 to deplete the *Foxp3*^{GFP-DTR} T_{reg} population, without impacting the DTR^{neg} ex-Foxp3 population. Mice were analyzed on day 28 post-transfer. **(C)** Schematic overview. **(D)** Representative plots for ex-Foxp3 cell formation in the splenocytes from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice used for lymphorepletion, comparing the pre-transfer (left) to post-transfer (right). **(E)** Frequency of CD4⁺ YFP^{neg} RFP⁺ ex-Foxp3 cells from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper T_{reg} among: transferred CD4⁺ YFP⁺ RFP⁺ T_{reg} progeny after 14 days of lymphopenia (n=7), donor splenocytes used for lymphorepletion at d14 prior transfer (n=4), and donor splenocytes at 14 days post-transfer (n=9). **(F)** Representative plots of Foxp3 expression in T_{reg} progeny pre and post T_{reg} depletion and lymphorepletion. **(G)** Foxp3⁺ cells among: transferred T_{reg} after 28 days of lymphopenia without other treatment (n=4, CD4⁺ YFP⁺ RFP⁺ T_{reg} from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice into *Rag1*^{KO} recipients), ex-Foxp3 cells generated from *Foxp3*^{GFP-DTR} mice after 14 days of lymphopenia followed by 14 days of lymphorepletion with *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper splenocytes (n=9), control naïve T cells from *CD45.1*^{+/2} *Foxp3*^{Thy1.1} mice introduced during lymphorepletion (n=5). Frequencies of Mean ± SEM; p-values by unpaired Mann-Whitney U test.

Figure 4

Fig. 4. Targeted multi-omics identifies naïve, Nrp1^{neg} T_{reg} as a potential unstable T_{reg} subset.

(A) T_{reg} from an unmanipulated *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mouse (day 0) and ex-Foxp3 cells (CD4⁺ YFP^{neg} RFP⁺) (day 5, 10 and 15 post co-transfer of T_{reg} from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} mice with naïve T cells [CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}] from congenic *Foxp3*^{Thy1.1} mice into *Rag1*^{KO} recipients) were assessed using a custom BD Rhapsody single cell panel. (B) UMAP plot (5260 cells), clustered according to sample (color) or Seurat clustering (dashed lines) (left). Day 0 T_{reg} and day 5/10/15 ex-Foxp3 cells projected on UMAP (right). (C) Expression of Foxp3 per time point (with % positive). (D) Heatmap of the differentially expressed genes between the different time points. Each column represents gene expression for an individual cell. (E) Expression level of CD62L (based on antibody binding) per time point. (F) Network-based analysis based on Pearson correlation relationship ($r \geq 0.95$). T_{reg} clusters were annotated as ‘pure’ (no connections to ex-Foxp3 cells), ‘inter’ T_{reg} (connected mostly to the day 5 and less day 10 or d15 ex-Foxp3 cells) or ‘other’ T_{reg}. (G) Status as activated or naïve Nrp1^{neg} T_{reg} or Nrp1⁺ T_{reg}, based on expression of CD62L (Ab) and *Nrp1* (left panel). (H) Corresponding data based on flow cytometry analysis of wildtype mice, based on Nrp1, CD44 and CD62L (n=9). (I) Left: *Nrp1* expression projected on network analysis matrix. Right: Weighted correlation network analysis based on Pearson correlation coefficient ($r \geq 0.95$). ‘inter’ Treg were dissected into Nrp1⁺ / predicted Nrp1⁺ as well as Nrp1^{neg} ‘inter’ Treg to measure the connectivity to day 5 ex-Foxp3 cells as well as day 10/15 ex-Foxp3 cells. p-values by unpaired Mann-Whitney U test. The sc-RNAseq data represent T_{reg} obtained from one mouse (day 0), ex-Foxp3 cells pooled from 8 mice (day 5), ex-Foxp3 cells pooled from 6 mice (day 10) or ex-Foxp3 cells pooled from 5 mice (day 15).

Figure 5

Fig. 5. Enhanced intrinsic instability of pT_{reg}-enriched cells following lymphopenic challenge. (A) Frequency of *Foxp3^{neg} RFP⁺* ex *Foxp3* cells in spleen, pLN and MLN of 8-week-old *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice and *BAC-Foxp3^{Cre-GFP} Rosa^{RFP} Foxp3^{Thy1.1}* fate-mapper (n=3, 7). (B) Total T_{reg} (CD4⁺ Thy1.1⁺) from *BAC-Foxp3^{Cre-GFP}*

Rosa^{RFP} Foxp3^{Thy1.1} mice were co-transferred with naïve T cells (nTc, CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}) from CD45.1 *Foxp3^{Thy1.1}* mice into *Rag1^{KO}* mice (n=9). Two weeks post-transfer, mice were sacrificed and pooled spleen, LNs and MLNs analyzed for T_{reg} (CD4⁺ Foxp3⁺) and ex-Foxp3 cells (CD4⁺ Foxp3^{neg}) originating from the RFP^{neg} pT_{reg}-enriched fraction and RFP⁺ tT_{reg}-enriched fraction. Representative plots (C) and frequencies (D) of ex-Foxp3 cells of tT_{reg}-enriched origin (RFP⁺ Foxp3^{neg}) or pT_{reg}-enriched origin (RFP^{neg} Foxp3^{neg}). (E) Representative plot and (F) average ratio between pT_{reg}- and tT_{reg}-enriched cells at D0 (n = 6), among remaining T_{reg} on day 14 post-transfer and ex-Foxp3 cells on day 14. (G) Ex-Foxp3 cells and T_{reg} originating from both fractions were analyzed for expression of Nrp1, Eos, and KLRG1 as well as (H) of IFN γ , IL-2, IL-17 and TGF β . Data are plotted as mean $\pm$ SEM and are representative of at least two independent experiments. p-value by unpaired Mann-Whitney U test.

A

CD45.1⁺ naïve Nrp1⁺ T_{reg}
 CD45.1/.2⁺ activated Nrp1⁺ T_{reg}
 CD45.2⁺ nTc

2 weeks

Rag1^{KO}

analyze stability of different populations

CD45.1⁺ naïve Nrp1^{neg} T_{reg}
 CD45.1/.2⁺ activated Nrp1^{neg} T_{reg}
 CD45.2⁺ nTc

2 weeks

Rag1^{KO}

B

% ex-Foxp3 cells

$p = 0.0006$

Nrp1⁺ T_{reg} Nrp1^{neg} T_{reg}

C

Nrp1⁺ T_{reg} origin

89.2%
5.93%

Nrp1^{neg} T_{reg} origin

58.0%
37.4%

Foxp3

CD4

D

% ex-Foxp3 cells

$p = 0.0181$ $p = 0.0041$ $p = 0.0012$ $p = 0.0023$ $p = 0.0012$ $p = 0.0006$

Eos GITR Nrp1 CD103 CD62L CTLA-4 CD25 CD44 ICOS KLRG1 CD69 Helios

ns ns ns ns ns

● Nrp1⁺ T_{reg}
 ■ Nrp1^{neg} T_{reg}

E

Nrp1⁺ T_{reg}

ns

% ex-Foxp3 cells

naïve activated

Nrp1^{neg} T_{reg}

ns

% ex-Foxp3 cells

naïve activated

Fig. 6. Dissection of naïve T_{reg} does not substantially enrich for instability within the Nrp1^{neg} T_{reg} population. (A) T_{reg} from spleen, LNs and MLNs (CD4⁺ Thy1.1⁺) were sort-purified from either CD45.1⁺ or CD45.1/2⁺ *Foxp3*^{Thy1.1} reporter mice as Nrp1⁺ or Nrp1^{neg} CD62L⁺ CD44^{neg} (naïve) or Nrp1⁺ or Nrp1^{neg} CD62L^{neg} CD44⁺ (activated). Naïve T cells from

spleen were sort-purified as $CD4^+ Thy1.1^{neg} CD62L^+ CD44^{neg}$ from $CD45.2 Foxp3^{Thy1.1}$ reporter mice. Cells were mixed as shown in a T_{reg} -to-naïve T cell 1:1 ratio and transferred into $Rag1^{KO}$ mice (recipients of $Nrp1^+$ T_{reg} $n = 7$, recipients of $Nrp1^{neg}$ T_{reg} $n = 7$). Two weeks post-transfer $CD4^+$ cells from spleen, LNs and MLNs were pooled for analysis. Frequencies (B) and representative plots (C) of ex-Foxp3 cells among $Nrp1^+$ and $Nrp1^{neg}$ T_{reg} . (D) Differential expression of T_{reg} and activation markers among ex-Foxp3 cells originating from $Nrp1^+$ or $Nrp1^{neg}$ T_{reg} , respectively. (E) Frequencies of ex-Foxp3 cells among naïve and activated $Nrp1^+$ and $Nrp1^{neg}$ T_{reg} . Please refer to Fig. S13 for representative sorting gates and post-sort purity examples. Data are plotted as mean $\pm$ SEM and are representative of two independent experiments; p-value by Mann-Whitney U test.

Figure 7

Fig. 7. Recent thymic emigrant T_{reg} display increased fate instability. (A) Frequency of naïve (CD62L⁺ CD44^{neg}), effector-memory T cells (TEM, CD62L^{neg} CD44⁺) and central-memory T cells (TCM, CD62L⁺ CD44⁺) of CD4⁺ Foxp3-Thy1.1⁺ splenic T_{reg} subdivided into RTE (GFP⁺) and non-RTE (GFP^{neg}) of *Rag2*^{GFP} *Foxp3*^{Thy1.1} origin (n = 6). (B) Frequency of Nrp1⁺ (left) and median fluorescent intensity (MFI) of Nrp1 in Nrp1⁺ cells (right) shown for RTE and non-RTE T_{reg}. (C) Scheme of experimental design. RTE T_{reg} and non-RTE T_{reg} were sort-purified from pooled spleen, LNs and MLN of *Rag2*^{GFP} *Foxp3*^{Thy1.1} origin and co-transferred with splenic naïve T cells sort-purified as CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg} from CD45.1⁺ *Foxp3*^{Thy1.1} reporter mice in a 1:1 ratio into *Rag1*^{KO} mice. (non-RTE T_{reg} n = 7, RTE T_{reg} n = 6). (D) Representative plots 14 days after lymphopenic transfer for Foxp3 expression as well as (E) frequency of ex-Foxp3 cells among RTE and non-RTE T_{reg}. Please refer to Fig. S14D for representative GFP⁺ and GFP^{neg} sorting gates and post-sort purity examples. Data are plotted as mean ± SEM and represent two independent experiments; p-value by Mann-Whitney U test.

Supplementary figure 1

A Gating strategy for analysis of co-housing experiment on transcription factors and surface markers

B Gating strategy for analysis of co-housing experiment on cytokine expression

C Gating strategy for analysis of adoptive transfer experiments

Fig. S1: Gating schemes with representative example plots from flow cytometric analyses. Gating strategy and representative plots for the definition of T_{reg} and ex-Foxp3 cells prior to further downstream analysis of **(A)** transcription factors and surface markers or **(B)** cytokines after co-housing of SPF *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice with wild-exposed pet store mice, and **(C)** of adoptively transferred mice after co-transfer of *Foxp3^{Thy1.1}* T_{reg} or *Foxp3^{YFP-Cre} Rosa^{RFP}* T_{reg} with naïve T cells into *Rag1^{KO}* mice (dump channel contains CD11b and B220).

Supplementary figure 2

Fig. S2. Characterization of *Foxp3^{YFP-Cre} Rosa^{RFP}* fate-mapper mice. **(A)** Histogram of YFP expression gated on RFP⁺ cells. **(B)** Histogram of RFP expression on CD4⁺ Foxp3⁺ cells, CD4⁺ Foxp3^{neg} cells, CD8⁺ cells and CD19⁺ cells.

Supplementary figure 3

Fig. S3. Changes in microbiome and T cell activation upon co-housing with wild-exposed pet store mice. (A) Serum samples of SPF mice and co-housed mice was analyzed for IFN γ , IL-1 β , IL-4, IL-6, IL-2, TNF α , IL-10 and C-reactive protein (CRP). (B-G) Splenic T cells of SPF mice were compared to mice co-housed for 8 weeks with wild-exposed pet store mice (n=13,9) by high-parameter flow cytometry. (B) The frequency of naïve (CD62L⁺ CD44^{neg}), effector-memory (TEM, CD62L^{neg} CD44⁺) and central memory (TCM, CD62L⁺ CD44⁺) CD8 T cells. (C) The ratio of TEM to naïve T cells among CD8 T cells. (D) The total number of CD8 T cells in SPF and co-housed mice are shown. Similarly, for CD4 T cells frequencies of naïve, TEM and TCM (E), TEM to naïve T cell ratio (F) and total CD4 T cell number are shown (G). (H) Expression levels of activation markers on CD8 and CD4 T cells in SPF and co-housed mice. Frequency of T_{reg} (I), and ex-Foxp3 cells (J) in peripheral lymph nodes (pLN), mesenteric lymph nodes (MLN), lung and Peyer's Patches (PP). (K-N) The microbial diversity of caecal contents in specific pathogen-free (SPF) and wild-cohoused (CoH) mice was compared through 16S rRNA analysis. (K) Upon comparing the microbial communities from SPF and CoH mice groups, we detected differences in richness but not in Shannon diversity index ($p < 0.00027$ and $p > 0.36$, respectively, Wilcoxon test). (L) Principal coordinate analysis (PCoA) of the mice microbiota community variation based on Bacteria and Archaea genus-level Bray-Curtis dissimilarity distances revealed two different clusters. 19 % of the variation on the microbial composition can be explained by this grouping (adonis test, $R^2 = 0.18791$, $p = 0.001$). Differential abundance analysis at the phylum level identified Proteobacteria enriched and Verrucomicrobia depleted in CoH mice (q-values < 0.1 , Wilcoxon test). Twelve genera were identified with differences in relative abundance, seven enriched and five depleted in CoH mice (q-values < 0.1 , Wilcoxon test). Phylum (M) and genus (N) distribution barplots. Only the top 9 phyla and top 14 genera are displayed. Data are plotted as means $\pm$ SEM and represent two independent experiments. p-values by unpaired Mann-Whitney U test. (P-values and q-values of (K-N) are shown in Table S5).

Supplementary figure 4

Fig. S4. Early T_{reg} proliferation following adoptive transfer into a lymphopenic environment. (A) Representative flow plots of pre-sort gating and post-sort purity of T_{reg} (CD4⁺ YFP⁺ RFP⁺ T_{reg}) from *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice and T_{conv} cells (CD45.1⁺ CD4⁺ Foxp3-Thy1.1^{neg} T_{conv}) from CD45.1 *Foxp3*^{Thy1.1} mice at primary (1°) and secondary (2°) transfers. Note: T_{reg} donor *Foxp3*^{YFP-Cre} *Rosa*^{RFP} fate-mapper mice show unspecific recombination outside of the T_{reg} lineage. However, this leakiness can be considered irrelevant as T_{reg} are sort-purified based on the expression of YFP. (B) For kinetic transfer experiments, the 1:1 T_{reg}:nTc mix was labelled with CellTrace Violet prior to transfer into *Rag1*^{KO} mice. Representative plots of T_{reg} and ex-Foxp3

cells on indicated days. **(C)** T_{reg} , ex-Foxp3 cells and T_{conv} were assessed for expression of Ki67 and $CD62L^+ CD44^{neg}$ cells post-transfer at the indicated time points (day 3 $n = 3$, day 5 $n =$ concatenated timepoint of 5 mice, day 9 $n = 8$, day 14 $n = 7$, day 20 $n = 3$, day 28 $n = 28$, day 40 $n = 3$) (note: day 5 is a concatenated time point of data from 5 individual mice). **(D)** After 1° transfer, T_{reg} and T_{conv} were mixed at a 1:1 ratio and labelled with CellTrace Violet to analyze proliferation rate post 2° transfer in experiment C and D presented in Fig. 2C. Shown is a representative plot of CellTrace Violet labelling vs. Foxp3 expression gated on either transferred T_{reg} (left) or transferred T_{conv} (right). All data are plotted as means $\pm$ SEM and are representative of at least two independent experiments.

Supplementary figure 5

Fig. S5. Mathematical modelling of ex-Foxp3 kinetics after transfer is consistent with a stable T_{reg} source. Results of mathematical modelling of active Foxp3 deconversion from previously stable T_{reg} at the point of transfer ($t = 0$) vs uninterrupted transient Foxp3 expression in non- T_{reg} .

(A) Dependence of adjusted MSE on median Foxp3 expression length for active Foxp3 deconversion, or (B) uninterrupted transient Foxp3 expression. (C) Dependence of the probability of observing the experimental data under the fitted model vs median Foxp3 expression length, for active Foxp3 deconversion, or (D) uninterrupted transient Foxp3 expression. (E) Best fit model (blue) plotted versus experimental results (black) for active Foxp3 deconversion, (F) uninterrupted transient Foxp3 expression, or (G) short-term (1.2 days) transient Foxp3 expression.

Supplementary figure 6

A ex-Foxp3 cell sort – gating strategy:

Naïve T cell and activated T cell sort – gating strategy:

B 5 days of culture with TGF- β and IL-2 – gating strategy:

Fig. S6. Gating scheme and post-culture analysis of *in vitro* T_{reg} conversion assay. (A) Gating strategies for sorting ex-Foxp3 cells (CD4⁺ YFP^{neg} RFP⁺) after lymphopenic transfer of *Foxp3*^{YFP-Cre}*Rosa*^{RFP} fate-mapper T_{reg} into *Rag1*^{KO} mice as well as naïve (CD4⁺ YFP^{neg} RFP^{neg} CD44^{neg} CD62L⁺, nTc) and activated (CD4⁺ YFP^{neg} RFP^{neg} CD44⁺ CD62L^{neg}, aTc) non-T_{reg} from unmanipulated *Foxp3*^{YFP-Cre}*Rosa*^{RFP} fate-mapper mice. (B) Gating strategy for post-culture analysis of Foxp3 expression.

Supplementary figure 7

Fig. S7. Ex-Foxp3 cells do not display fate memory *in vivo* upon lymphorepletion. (A)

Representative flow plots of sort gating and post-sort analysis of GFP⁺ T_{reg} sorted from CD45.1 *Foxp3^{DTR-GFP}* mice (top), naïve T cells (CD4⁺ CD44^{neg} CD62L⁺ Foxp3-Thy1.1^{neg}, nTc) sorted from CD45.2⁺ *Foxp3^{Thy1.1}* mice (middle) at day 0 and naïve T cells (CD4⁺ CD44^{neg} CD62L⁺ Foxp3-Thy1.1^{neg}, nTc) sorted from CD45.1/.2⁺ *Foxp3^{Thy1.1}* mice at day 14. **(B)** Representative plot of DT-mediated depletion efficiency of DTR⁺ T_{reg} at day 16 (2 days after DT treatment). **(C)** Serum samples of unmanipulated T_{reg}-reporter mice (WT, n = 10), *Rag1^{KO}* recipient mice after 14 days of adoptive transfer of T_{reg} (n = 5), *Rag1^{KO}* recipient mice after 28 days of adoptive transfer of T_{reg} (n = 4), *Rag1^{KO}* recipient mice after adoptive transfer of *Foxp3^{GFP-DTR}* T_{reg}, followed by 14 days of lymphorepletion with *Foxp3^{YFP-Cre Rosa^{RFP}}* fate-mapper splenocytes (n = 8). **(D-F)** Splenocytes from CD45.1⁺ *Foxp3^{Thy1.1}* mice (3x10⁷ cells) were injected into *Rag1^{KO}* mice (n = 6, referred to as *Rag1^{KO}* lymphorepleted) and compared to CD45.1⁺ *Foxp3^{Thy1.1}* mice (WT, n = 4) for immune activation and cytokine production. **(D)** Expression of CD69, CD103, Eomes, GATA-3, Nrp1, T-Bet, presence of naïve T cells, effector memory T cells (TEM), central memory T cells (TCM), expression of KI67, IFN γ , IL-2, IL-4, IL-6, IL-10, IL-17, TGF β , TNF α in spleen (blue, red) and LN (light grey, dark grey) of WT and *Rag1^{KO}* lymphorepleted mice were assessed. Frequency of CD4⁺ T_{reg}, CD4⁺ T_{conv}, and CD8⁺ T_{conv} of CD3⁺ cells in spleen in WT and *Rag1^{KO}* lymphorepleted in the spleen **(E)** and LN **(F)** were assessed. Mean $\pm$ SEM; p-values by unpaired Mann-Whitney U test.

Supplementary figure 8

Fig. S8. Ex-Foxp3 cells do not display fate memory *in vivo* immediately after Foxp3 loss. (A) *Foxp3*^{GFP-DTR} T_{reg} (4x10⁵) were transferred in a 1:1 ratio with naïve T cells from CD45.2⁺ *Foxp3*^{Thy1.1} mice (4x10⁵) into a *Rag1*^{KO} mice. After 5 days, mice were injected with 3x10⁷ CD45.2⁺ *Foxp3*^{YFP-Cre} *Rosa*^{RFP} splenocytes and 1x10⁵ CD45.1⁺/CD45.2⁺ naïve T cells from CD45.1/.2⁺ *Foxp3*^{Thy1.1} mice. DT was injected on days 5 and 6 to deplete the *Foxp3*^{GFP-DTR} DTR⁺ T_{reg} population, without impacting the DTR^{neg} ex-Foxp3 cell population. Mice were analyzed on day 19 after T_{reg} transfer. **(B)** Representative flow plots of sort gating and post-sort analysis of GFP⁺ T_{reg} sorted from CD45.1 *Foxp3*^{DTR-GFP} mice (top), naïve T cells (CD4⁺ CD44^{neg} CD62L⁺ Foxp3-Thy1.1^{neg}, nTc) sorted from CD45.2⁺ *Foxp3*^{Thy1.1} mice (middle) at day 0 and naïve T cells (CD4⁺ CD44^{neg} CD62L⁺ Foxp3-Thy1.1^{neg}, nTc) sorted from CD45.1/.2⁺ *Foxp3*^{Thy1.1} mice at day 5. **(C)** Representative plots for T_{reg} (Foxp3 expression) after a 2-day DT treatment of a CD45.1 *Foxp3*^{GFP-DTR} mouse treated alongside with the T_{reg}-transferred *Rag1*^{KO} recipient mice to control for DT batch activity. **(D)** Frequencies of ex-Foxp3 cells among: progeny of *Foxp3*^{YFP-Cre} *Rosa*^{RFP} transferred T_{reg} after 14 days of lymphopenia without further treatment (n = 7, as shown in Fig. 3E), donor *Foxp3*^{YFP-Cre} *Rosa*^{RFP} splenocytes at the point of collection for lymphorepletion (n = 1), and donor *Foxp3*^{YFP-Cre} *Rosa*^{RFP} splenocytes at 14 days post-transfer (n = 4). **(E)** Frequency of Foxp3⁺ expression among: progeny of transferred CD45.1 *Foxp3*^{Thy1.1} T_{reg} after 19 days of lymphopenia without further treatment (n = 3), ex-Foxp3 cells after 5 days of lymphopenia and 14 days of lymphorepletion (n = 4), control naïve T cells introduced during lymphorepletion (n = 4). Mean ± SEM; p-values by unpaired Mann-Whitney U test.

Supplementary figure 9

Fig. S9. CD25 expression is insufficient to describe the unstable T_{reg} subset. (A) Congenically disparate CD25⁺ and CD25^{neg} T_{reg} from CD45.1 Foxp3^{Thy1.1} mice (CD4⁺ Thy1.1⁺, 4 x 10⁵) were co-transferred with naïve T cells from CD45.2 Foxp3^{Thy1.1} mice (nTc, CD4⁺ Thy1.1^{neg} CD62L⁺ CD44^{neg}, 4 x 10⁵) into *Rag1*^{KO} mice and T_{reg} progeny was analysed 14 days after transfer (n = 7,

two independent experiments). **(B)** Representative flow plots of sort gates and post-sort analysis of CD25⁺ and CD25^{neg} T_{reg} and pre-injection mix analysis. **(C)** Two weeks post-transfer, mice were assessed for T_{reg} and **(D)** ex-Foxp3 cells originating from CD25⁺ T_{reg} (n = 7) and CD25^{neg} T_{reg} (n = 8). **(E)** Post-transfer T_{reg} originating from CD25⁺ and CD25^{neg} T_{reg} were analyzed for CD25 expression. **(F)** Ex-Foxp3 cells originating from transferred CD25⁺ and CD25^{neg} T_{reg}, were assessed for expression of CD25, Nr1, GITR, Eos, Helios and CD69. Mean ± SEM. p-values by unpaired Mann-Whitney U test.

Supplemental figure 10

Supplementary figure 12

Fig. S12. Nrp1^{neg} gut T_{reg} possess increased instability compared to Nrp1⁺ thymic T_{reg}. (A) T_{reg} were sort-purified from thymus (CD4⁺ CD8^{neg}) or gut (CD4⁺) as Fcγp3-Thy1.1⁺ Nrp1⁺ (thymus) or Fcγp3-Thy1.1⁺ Nrp1^{neg} (gut) and co-transferred with congenically-disparate naïve T cells (CD4⁺ CD62L⁺ CD44^{neg}) into *Rag1*^{KO} mice. Two weeks post-transfer, T_{reg} progeny was analysed for the loss of Fcγp3 expression. (B) Representative flow plots of sort gates and post-sort analyses. (C) Representative plots and (D) fold increase of ex-Fcγp3 cells from gut T_{reg} normalized on frequency of ex-Fcγp3 cells from thymic T_{reg}. (adoptively transferred mice: exp 1 n = 3 thymic T_{reg}, n = 1 gut T_{reg}; exp 2 n = 3 thymic T_{reg}, n = 1 gut T_{reg}).

Supplementary figure 13

Fig. S13. Sorting of naïve and activated cells within Nrp1⁺ and Nrp1^{neg} T_{reg} populations. Representative flow plots of sort gates and post-sort analysis of naïve Nrp1⁺ T_{reg} (CD4⁺ CD44^{neg} CD62L⁺ Nrp1⁺ Thy1.1⁺), activated Nrp1⁺ T_{reg} (CD4⁺ CD44⁺ CD62L^{neg} Nrp1⁺ Thy1.1⁺), naïve Nrp1^{neg} T_{reg} (CD4⁺ CD44^{neg} CD62L⁺ Nrp1^{neg} Thy1.1⁺), activated Nrp1^{neg} T_{reg} (CD4⁺ CD44⁺ CD62L^{neg} Nrp1^{neg} Thy1.1⁺).

Supplementary figure 14

Fig. S14. Phenotyping of RTE- and non-RTE T_{reg} and ex-Foxp3 cells derived thereof. *Rag2-GFP^{neg}* non-RTE T_{reg} and *Rag2-GFP⁺* RTE T_{reg} were assessed for expression of CD25, CD69, CD103, CTLA-4, Eos, Helios, ICOS and KLRG1 (**A**) and expression of IFN γ , IL-2, IL-4, IL-10, IL-17 and TGF β (n = 6) (**B**). (**C**) Median fluorescent intensity of Foxp3 in *Rag2-GFP^{neg}* non-RTE T_{reg} and *Rag2-GFP⁺* RTE T_{reg}. (**D**) Representative flow plots of sort gates (pre-gated on CD4⁺ Thy1.1⁺) and post-sort analysis of *Rag2-GFP^{neg}* non-RTE T_{reg} (CD4⁺ Thy1.1⁺ GFP^{neg}) and *Rag2-GFP⁺* RTE T_{reg} (CD4⁺ Thy1.1⁺ GFP⁺). (**E**) Ex-Foxp3 cells derived from either *Rag2-GFP^{neg}* non-RTE T_{reg} (n = 7) or *Rag2-GFP⁺* RTE T_{reg} (n = 6) were assessed for expression of CD25, CD69, CD103, CTLA-4, Eos, Helios, ICOS and KLRG1 and (**F**) for expression of IFN γ , IL-2, IL-4, IL-10, IL-17 and TGF β . Data representative of two independent experiment. Mean $\pm$ SEM; p-values by unpaired Mann-Whitney U test.