HHS Public Access

Author manuscript

Cell. Author manuscript; available in PMC 2017 August 11.

Published in final edited form as:

Cell. 2016 August 11; 166(4): 977–990. doi:10.1016/j.cell.2016.07.006.

Memory of inflammation in regulatory T cells

Joris van der Veeken[1], **Alvaro J. Gonzalez**[2], **Hyunwoo Cho**[2], **Aaron Arvey**[1], **Christina S. Leslie**[2], and **Alexander Y. Rudensky**[1,3]

[1]Howard Hughes Medical Institute and Immunology Program, New York, NY 10065, USA

[2]Computational Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

[3]Ludwig Center at Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Abstract

Eukaryotic cells can "remember" transient encounters with a wide range of stimuli, inducing lasting states of altered responsiveness. Regulatory T (Treg) cells are a specialized lineage of suppressive CD4 T cells that act as critical negative regulators of inflammation in various biological contexts. Treg cells exposed to inflammatory conditions acquire strongly enhanced suppressive function. Using inducible genetic tracing we analyzed the long-term stability of activation-induced transcriptional, epigenomic, and functional changes in Treg cells. We found that the inflammation-experienced Treg cell population reversed many activation-induced changes and lost its enhanced suppressive function over time. The "memory-less" potentiation of Treg suppressor function may help avoid a state of generalized immunosuppression that could otherwise result from repeated activation.

Graphical abstract

Address correspondence to: Alexander Rudensky (rudenska@mskcc.org), Christina Leslie (cleslie@cbio.mskcc.org).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Accession numbers

The GEO accession number of the sequencing data reported in this study is GSE83315.

Author Contributions

J.v.d.V. and A.Y.R conceived and designed the experiments, interpreted the results, and wrote the manuscript. J.v.d.V. performed the experiments. A.J.G. and H.C. performed transcriptome analysis. A.J.G analyzed stability of activation-induced chromatin modifications. H.C. analyzed association between Foxp3 and H3K27me3 deposition. A.A. performed preliminary analyses of ATAC and ChIP-seq datasets. C.S.L. designed and supervised computational analyses.

Author Manuscript

Author Manuscript

Author Manuscript

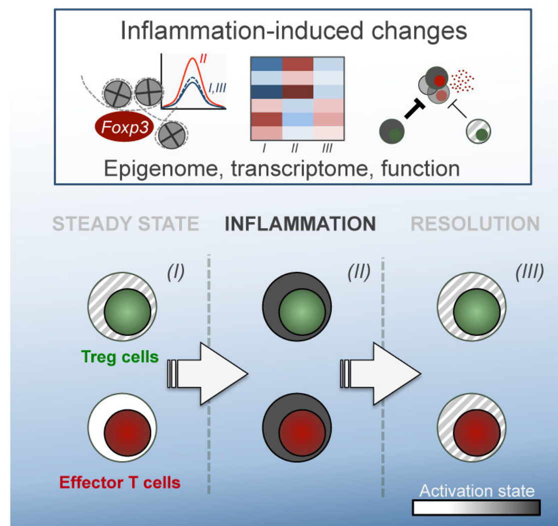
Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



Introduction

One of the hallmarks of the adaptive immune system in higher vertebrates is its ability to respond more rapidly and effectively to pathogens that have been previously encountered. The initial observation that clearance of an infection can lead to long-term protection against reinfection with the same or similar pathogens formed the basis for modern vaccination. Clonal selection theory subsequently provided an explanation for this immunological memory by postulating that clonally expanded activated lymphocytes could give rise to a population of long-lived antigen-specific cells capable of protecting the host from reinfection. It has since become clear that in addition to increasing the total number of pathogen-specific cells that can mount a recall response, lymphocyte activation also induces stable transcriptional, epigenetic, and metabolic changes that contribute in a cell-intrinsic manner to the survival and enhanced responsiveness of long-lived memory cells (Chang et al., 2014).

While the term “immunological memory” typically refers to the enhanced ability to protect against re-infection, immune cells may “remember” a host of stimuli and prior activations leading to states of altered responsiveness. For example, recognition of self-antigen by CD8 T cells can imprint a state of tolerance, characterized by the persistent inability to respond to cognate antigen even after it is encountered in a highly immunogenic context. This state can be “remembered” after cells are removed from the tolerizing environment (Schietinger et al., 2012). Another example is T cell exhaustion, a state of functional hyporesponsiveness resulting from persistent exposure to antigen and inflammatory signals, best characterized in the context of chronic viral infection (Wherry and Kurachi, 2015). This exhausted state becomes increasingly irreversible upon long-term exposure to the stimulatory environment and may eventually persist even in the absence of external cues (Angelosanto et al., 2012; Utzschneider et al., 2013). Importantly, this type of cellular memory of prior activation states is not restricted to the adaptive immune system, but can also influence innate immune cell function in both vertebrate and invertebrate animals Thus, mechanisms that mediate short-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and long-term cellular memory of prior challenges act broadly across cell types and may enable context-dependent fine-tuning of immune responsiveness (Monticelli and Natoli, 2013; Quintin et al., 2014). The extent to which different immune and non-immune cell types diverge in their ability to maintain a cell-intrinsic memory of physiological challenges, and the content of these "memories" are largely unclear.

Regulatory T (Treg) cells are a specialized subset of immunosuppressive CD4 T cells that express the X-chromosome-encoded, lineage-specific transcription factor Foxp3 (Josefowicz et al., 2012). Treg cells maintain peripheral tolerance by providing essential suppression of autoreactive CD4 T cells that have escaped negative selection in the thymus. In addition, Treg cells act as critical negative regulators of inflammation in various biological contexts, including infection, metabolic disease, tissue repair, and cancer (Belkaid and Tarbell, 2009; Burzyn et al., 2013). Importantly, Treg cells respond to inflammation by sharply increasing their suppressive function. Activated Treg cells upregulate immunosuppressive molecules and tissue homing receptors, and undergo polycomb-mediated repression of Foxp3-bound genes, which may prevent the acquisition of pro-inflammatory functions (Arvey et al., 2014). Whether these changes represent stable differentiation or a transient adaptation to the inflammatory environment is currently unclear.

Conventional antigen-specific CD4 and CD8 T cells maintain a large fraction of activation-induced transcriptional changes after pathogen clearance, resulting in increased cytolytic and proinflammatory potential and enhanced responsiveness to cytokine stimulation (Berg et al., 2003; Crawford et al., 2014; Hale et al., 2013; Kaech et al., 2002; Lertmemongkolchai et al., 2001; Sprent and Surh, 2002). This provides a clear biological benefit to the host by enhancing the ability to respond to previously encountered pathogens to afford protection from re-infection. In contrast, the potential functional importance of an equivalent memory mechanism in Treg cells is not intuitively obvious. Thymic differentiation of Treg cells requires relatively strong T cell receptor (TCR) stimulation, such that the Treg TCR repertoire is enriched for self-reactivity (Hsieh et al., 2004). A long-lasting increase in the suppressive function of these self-reactive cells, persisting after the resolution of inflammation, may not just negate the benefits of generating effector T cell memory, but could also lead to generalized immunosuppression. Thus, we hypothesized that many inflammation-induced changes in Treg cells, in particular the marked augmentation of their suppressor function, may be transient.

To test this hypothesis, we used inducible genetic lineage tracing to investigate features of Treg cells before, during, or after exposure to an acute inflammatory environment. We found that the inflammation-experienced Treg cell pool reversed many activation-induced transcriptional changes and lost its enhanced suppressive function over time. Transcriptional changes were largely similar during primary and secondary Treg cell activation, and not commonly associated with stable chromatin modifications that would facilitate reactivation. In contrast, Treg cell activation induced a limited number of stable changes, including a long lasting preference for non-lymphoid tissue localization. We found that resting Treg cells shared a transcriptional program with conventional memory T cells, which was not further reinforced upon secondary stimulation. These observations suggest that loss of a "naïve" state during Treg cell development may underlie the limited differentiation and memory

Author Manuscript

potential of these cells. Thus, the extent and contents of cellular memory generated in response to transient environmental challenges may markedly differ between closely related cell types, based on their developmental history.

Results

Generation and fate mapping of inflammation-experienced Treg cells

T cell memory has traditionally been studied in the context of clonally expanded cells with defined specificities for pathogen-derived or model antigens. Unlike conventional naïve CD4 T cells, Treg cells may be viewed as “antigen-experienced”, as their generation in the thymus or peripheral lymphoid organs requires strong TCR stimulation. Consistently, Treg TCRs exhibit heightened self-reactivity compared to conventional CD4 T cells. While recognition of non-self antigens may contribute to Treg cell activation in certain settings (Liu et al., 2014; Shafiani et al., 2013), it is not clear if TCR-mediated activation is always the sole driver of Treg cell responses. Consistently, TCR-dependent genes account for only a quarter of the activated Treg cell-specific gene signature (Levine et al., 2014). To study memory of past inflammatory exposures *in vivo,* we used a model in which Treg cells become activated in response to the widespread inflammatory autoimmune responses that they normally control. Administration of diphtheria toxin (DT) to *Foxp3*$^{\text{GFP-DTR}}$ mice results in Treg cell depletion followed by rapid expansion and activation of cytokine-producing effector T cells, granulocytes, and myeloid cells (Kim et al., 2007). After clearance of DT, Treg cells rebounding in this inflammatory environment acquire an activated phenotype characterized by widespread transcriptional and epigenomic changes and enhanced suppressive function (Arvey et al., 2014).

To track Treg cells over time we made use of previously described *Foxp3*$^{\text{GFP-Cre-ERT2}}$ x *R26Y* mice. Treg cells in these mice express a GFP-Cre-ERT2 triple-fusion protein, which undergoes nuclear translocation upon tamoxifen treatment and mediates deletion of a loxp-flanked stop cassette preceding a YFP reporter allele knocked into the *Rosa26* locus (Rubtsov et al., 2010). We generated mixed bone marrow chimeric (BMC) mice by reconstituting irradiated *Foxp3*$^{\text{GFP-DTR}}$ mice with a 9:1 mixture of *Foxp3*$^{\text{GFP-DTR}}$ and *Foxp3*$^{\text{GFP-Cre-ERT2}}$ x *R26Y* bone marrow (Fig 1a). In these animals, 90% of the Treg cells can be depleted by DT treatment to induce transient inflammation and a cohort of the remaining Treg cells can be irreversibly labeled to allow isolation of inflammation-experienced YFP^{+} Treg cells after recovery (Fig 1a). Transient Treg cell ablation in these mice led to the expansion of activated effector CD4 T cells on day 11 post-treatment (Fig 1b, c, S1a, b). By day 60, the frequency and total number of effector CD4 T cells returned to baseline, indicating recovery from the challenge. YFP^{+} cells made up less than 1% of resting splenic Treg cells in mice receiving tamoxifen, but not DT (Fig 1d, e). Labeled Treg cells rapidly expanded upon DT treatment and only marginally contracted after resolution of inflammation (Fig 1d, e, S1c, d). Consistently, when we tracked inflammation-experienced Treg and Teff cells in DT treated *Foxp3*$^{\text{GFP-DTR}}$*CD4*$^{\text{CreERT2}}$*R26*$^{\text{tdTomato}}$ mice, we found that inflammation-experienced Tomato^{+} Treg cells were maintained to a much greater degree than Foxp3^{-} cells in the same mice (Fig S1e-g), suggesting that inflammation-experienced Treg cells are maintained long-term. Thus, our experimental model allowed for isolation of

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

resting Treg (rTr), activated Treg (aTr), and inflammation-experienced "memory" Treg (mTr) cells from mice before, during, or after exposure to an acute inflammatory challenge, respectively (Fig 1a).

Stability of inflammation-induced transcriptional changes in Treg cells

Treg cells undergo numerous functional, transcriptional and epigenomic changes in response to inflammation (Arvey et al., 2014). To assess the stability of these changes, we first characterized splenic YFP^{+} rTr, aTr, and mTr cell populations by flow cytometry. Compared to rTr cells, aTr cells expressed increased levels of the costimulatory molecules ICOS and GITR, as well as increased levels of Foxp3 (Fig 2a-c). These changes were completely or partially reversed after inflammation was resolved (Fig 2a-c). In contrast, activation-induced downregulation of the secondary lymphoid tissue homing receptor CD62L and the high-affinity IL-2 receptor α-chain CD25 persisted two months post-challenge (Fig 2d,e). These results suggested that Treg cells may maintain only a subset of activation-induced changes.

To account for the stability of activation-induced gene expression changes genome-wide, we performed RNA-sequencing (RNA-seq) on bulk rTr, and YFP^{+} aTr, and mTr cells isolated from mixed BMC mice. We found that 61% of upregulated genes, and 43% of downregulated genes had reversed activation-induced expression changes fully or to a significant degree in mTr cells, while 6% and 8% of up- and down-regulated genes, respectively, maintained activation-induced changes after resolution of inflammation (Fig 2f-h, Table S1, see Experimental Procedures for statistical definitions of stable and transient regulation). The remaining gene expression changes could not be confidently classified as either transient or stable. Genes transiently affected by inflammation included proven and putative regulators of Treg cell function such as Granzyme B, Furin, and Il10 (Fig 2h). Previously defined TCR-dependent genes, as well as genes repressed by Foxo1 underwent transient upregulation in response to activation (Fig 2i, j) (Levine et al., 2014; Luo et al., 2016).

Our observations suggested that the bulk Treg cell population had a rather limited capacity to maintain inflammation-induced transcriptional changes. Alternatively, it was possible that autoimmune inflammation in *Foxp3*$^{GFP\text{-}DTR}$ mice simply did not support formation of functional memory. However, we found that a stable activation-induced gene expression program defined in virus-specific GP66-77/I-A^{b} (GP66) tetramer^{+} CD4 T cells isolated 7 or 75 days after LCMV infection was also induced and maintained to a significant degree in inflammation-experienced effector T cells isolated from DT- and tamoxifen-treated *Foxp3*$^{GFP\text{-}DTR}$*CD4*^{CreERT2}*R26*^{tdTomato} mice (Fig S2a-d, Table S2). Moreover, these cells maintained a CD44^{hi}CD62L^{lo} cell surface phenotype and an enhanced capacity to produce IFNγ (Fig S2e), suggesting that inflammation in the Foxp3^{DTR} model could support the generation of functional memory cells. To show that the limited maintenance of activation-induced transcriptional changes in mTr cells was not restricted to autoimmune inflammation, we tracked LCMV-experienced Treg cells in tamoxifen-treated *Foxp3*$^{GFP\text{-}DTR}$*CD4*^{CreERT2}*R26*^{tdTomato} animals (Fig S2f). Similar to the Foxp3^{DTR} model, infection-experienced Treg cells were better maintained than their Foxp3-counterparts in the same mice. Infection induced a modest upregulation of CD44 and downregulation of CD62L

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

and CD25, which were stably maintained over time (Fig S2g,h). In contrast, many infection-induced transcriptional changes in Treg cells were reversible, including changes in immune response genes such as *Gzmb*, *Ccr5*, and *Ikzf3* (Fig S2i-k, Table S3). While the magnitude of transcriptional changes was generally much less dramatic, the transient activation program defined in the *Foxp3*^{DTR} model was also induced in Treg cells upon LCMV-infection (Fig S2l). In contrast, stable expression changes were model-specific (Fig 2m). Thus, while long term-maintenance and acquisition of a CD44^{hi}CD62L^{lo}CD25^{lo} cell surface phenotype may represent common characteristics of activation-experienced Treg cells regardless of the activating stimulus, a limited number of additional stable transcriptional changes may be induced in a response-specific manner. In contrast, regulation of transient activation-induced changes, accounting for a large fraction of transcriptional changes, may be similar in Treg cells responding to different inflammatory challenges.

Maintenance of inflammation-induced chromatin modifications

While relatively few inflammation-induced transcriptional changes were maintained, it was possible that transiently regulated genes underwent chromatin remodeling that poised them for more rapid or enhanced responses to subsequent challenges. We therefore analyzed the stability of inflammation-induced changes in genome-wide chromatin accessibility, and histone modifications indicative of a repressed (H3K27me3), active (H3K27Ac), or poised (H3K4me1) state. Using an assay for transposase accessible chromatin (ATAC-seq) (Buenrostro et al., 2013), we characterized chromatin accessibility in bulk rTr, and YFP^{+} aTr, and mTr cells and identified a total of 1159 ATAC-seq peaks differentially accessible (FDR 5%) in at least one cell type (Fig 3a, b). ATAC-seq libraries showed the expected pattern of fragment-length distribution (Fig S3a), and accessibility changes measured by ATAC-seq were associated with expression changes in nearby genes (Fig S3b). Treg cell activation induced a large number of both transient and stable chromatin accessibility changes (Fig S3c), and deposition of permissive and repressive histone modifications in regions flanking accessible sites (Fig S3d-g). Stable and transient accessibility changes were similarly distributed over promoters, introns, and intergenic regions (Fig S3h).

To determine if transient expression changes were associated with stable chromatin-level memory, we performed aggregated analysis of chromatin states based on ATAC-seq, and ChIP-seq of aforementioned histone modifications surrounding ATAC-seq peaks (Fig 3c-e, S4a-c, Table S4). Chromatin regions associated with transiently upregulated genes became more accessible, gained H3K4me1 and H3K27Ac, and lost H3K27me3 upon Treg cell activation (Fig 3d). However, these modifications were lost in inflammation-experienced mTr cells. We observed qualitatively similar changes at stably upregulated genes; however, in accordance with their expression pattern, chromatin changes were stably maintained (Fig S4b). Importantly, stably upregulated genes underwent quantitatively greater changes in chromatin accessibility than transiently upregulated genes, suggesting that overall, stable chromatin changes may have been easier to detect. Regulatory elements of transcriptionally repressed genes showed decreased accessibility and H3K27Ac, and accumulated H3K27me3 upon Treg cell activation (Fig 3e, S4c). At transiently downregulated genes these changes were completely lost two months after challenge (Fig 3e). In contrast, stable transcriptional repression was associated with a persistent loss of H3K27Ac and gain in H3K27me3, and a

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

continuing decline in accessibility (Fig S4c). Thus, stable and transient changes in gene expression were associated with quantitatively or kinetically distinct modifications of chromatin. Critically, our results suggest that genes undergoing transient expression changes upon Treg cell activation do not commonly maintain memory at a chromatin level.

We next analyzed the stability of activation-induced H3K27me3 at Foxp3-bound sites. Foxp3 targets are largely unchanged in activated compared to resting Treg cells; however, a subset of these sites acquires activation-induced H3K27me3. This process is Foxp3-dependent and accounts for a large fraction of aTr cell-specific H3K27me3 (Arvey et al., 2014). As previously reported, Treg cell activation led to increased H3K27me3 deposition at peaks and domains near Foxp3-bound sites (Fig 4a) and this effect was greatest for regions associated with highly bound Foxp3 targets (Fig 4b). Moreover, regions in closer proximity to highly bound Foxp3 targets showed the greatest gain in H3K27me3 upon Treg cell activation (Fig 4c). Importantly, activation-induced changes in H3K27me3 were completely reversed in the inflammation-experienced mTr cell population (Fig 4a-c).

Reactivation of inflammation-experienced Treg cells

While our results suggested that Treg cells stably maintained only few activation-induced transcriptional and epigenomic changes, it remained possible that the transiently responding gene set maintained certain epigenetic marks not accounted for in our study and was poised to mount a “recall” or “memory” response upon secondary stimulation. Moreover, stable chromatin modifications could be maintained at distal regulatory elements that were functionally important, but could not be readily linked to the genes undergoing transient expression changes. Finally, it was possible that the bulk population of inflammation-experienced Treg cells contained only a small subpopulation of “memory” cells. This subpopulation would not be detectable by a population-based biochemical assay such as RNA-seq, but would preferentially expand and respond differentially to secondary activation.

To account for these possibilities, we compared Treg cell responses to primary and secondary inflammatory challenges. We adoptively transferred rTr cells together with inflammation-experienced mTr cells and congenically marked pre-activated CD4 effector T cells into T cell-deficient mice, so that the primary and secondary responses of Treg cells to inflammation could be directly compared in the same environment (Fig 5a). Importantly, recipient mice receiving activated effector T cells alone showed severe weight loss and uncontrolled effector T cell proliferation, indicating that Treg cells in this setting were actively preventing autoimmune inflammation. We isolated cells two weeks post-transfer based on expression of fluorescent protein reporters and congenic markers (Fig 5b) and analyzed their transcriptional responses by RNA-seq.

We first analyzed the responses of sets of genes that we identified as transiently and stably changed in response to inflammation (Fig 2g). Many transcriptional changes resulting from transient Treg cell depletion in the mixed BMC setting were also induced in the adoptive cell transfer model (Fig 5c-f, Table S5). Strikingly, genes undergoing transient activation-induced expression changes, including TCR-dependent genes, were almost identically affected during primary or secondary activation (Fig 5d-f). In contrast, the few stable

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

transcriptional changes induced by Treg cell activation in the mixed BMC model (Fig 2g) were maintained or reinforced during a secondary challenge (Fig S5a). Moreover, statistically significant gene expression changes identified in Treg cells undergoing secondary versus primary activation were already present to an extent in mTr cells prior to transfer (Fig S5b,c). To determine if recall responses to viral infection were similarly regulated, we transferred CellTrace^{TM} labeled Tomato^{+} mTr cells isolated 60 days after LCMV infection of tamoxifen treated *Foxp3*$^{GFP\text{-}DTR}$*CD4*^{CreERT2}*R26*^{tdTomato} mice together with Tomato^{-} Foxp3^{+} rTr cells isolated from naïve animals into congenically marked recipients and infected them with LCMV (Fig 5g). A second group of mice received polyclonal Tomato^{+} LCMV-experienced effector cells ("mTe") together with naïve CD4 T cells prior to infection. When we analyzed the recall expansion of LCMV-experienced cells on day 11 post-infection (p.i), we found that LCMV-experienced effector cells had proliferated dramatically more than cotransferred naïve CD4 T cells (Fig 5h). In contrast, LCMV-experienced Treg cells and their inexperienced counterparts proliferated to a similar extent. Compared to primary responders, both Treg and Teff cells undergoing secondary activation showed increased expression of CD44, and CXCR3, and reduced expression of CD62L (Fig 5i,j), although differences were generally smaller in Treg cells. Additionally, Treg cells downregulated CD25. Importantly, these phenotypic changes were already initiated to some degree in LCMV-experienced Treg cells prior to transfer (Fig S2g,h). Together, our observations suggest that the Treg cell pool responds in a largely similar way to primary and secondary activation by either autoimmune inflammation or viral infection. Nevertheless, Treg cell activation can induce a limited degree of stable differentiation, which may be reinforced upon secondary stimulation. Importantly, these results do not argue against the notion that small subsets of Treg cells with stably altered phenotype or function may differentiate in response to inflammation or infection. However, our observations would suggest that such cells might not be able to undergo pronounced recall expansion to the same extent as conventional memory CD4 T cells.

Treg cell activation transiently potentiates suppressive function, but confers lasting preference for non-lymphoid tissue localization

We next sought to characterize the stability of inflammation-induced changes in Treg cell function. First, we assessed the suppressive capacity of rTr, aTr, and mTr cells on a per-cell basis by measuring their ability to suppress effector T cell proliferation *in vitro.* As previously reported, aTr cells showed enhanced suppressive function compared to their resting counterparts (Fig 6a) (Arvey et al., 2014). Consistent with the predominantly transient changes in expression of genes encoding proven and putative mediators of suppression and related molecules, such as Granzyme B, Galectin-1, Furin, and Fgl2, the suppressor function of mTr cells was equivalent to that of rTr cells. To compare long-term suppressive function *in vivo,* we transferred pre-activated CD4 effector T cells, alone, or in combination with either rTr or mTr cells into T cell-deficient recipients and monitored weight loss over time as a measurement of autoimmune wasting disease. Since rTr and mTr cells undergo rapid activation under lymphopenic conditions and effectively become activated or reactivated Treg cells, respectively (Fig 5c,d), we excluded the aTr cells from this experiment. Consistent with their comparable *in vitro* suppressor function, rTr and mTr populations were equally capable of preventing effector T cell expansion and wasting

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

disease in this model (Fig 6b, c). Together, our data suggest that Treg cells respond to an inflammatory environment by sharply, but transiently increasing their capacity to suppress until inflammation is resolved. This "memory-less" potentiation of Treg cell suppressor function may help avoid immunosuppressed states that could otherwise result from repeated inflammatory insults, including infections.

Because the small subset of genes undergoing stable activation-induced expression changes was enriched for genes involved in cell migration, adhesion, and extravasation (Fig 6d), we reasoned that inflammation-experienced Treg cells might have an altered ability to localize to primary and secondary lymphoid organs compared to their inexperienced counterparts. To test this idea, we analyzed the tissue distribution of resting and inflammation-experienced Treg cells two weeks after co-transfer into lymphopenic mice in the presence of pre-activated effector CD4 T cells (Fig 5a). In mice that received a mix of rTr and aTr cells, aTr cells preferentially accumulated in the intestinal lamina propria, liver, and lung, relative to the spleen and lymph nodes (Fig 6e). In mice receiving a mix of mTr and rTr cells we observed a similar trend (Fig 6e). Thus, exposure to an inflammatory environment imprints a stable non-lymphoid tissue localization preference. This observation is consistent with the downregulation of CD62L and CD25 on mTr compared to rTr cells, which may be indicative of a reduced non-lymphoid tissue homing potential and reduced dependency on IL-2, respectively (Smigiel et al., 2014). Together, our data suggest that activation under inflammatory conditions transiently enhances Treg cell suppressive function and promotes Treg cell localization to non-lymphoid tissues, where they may carry out specialized functions in response to local cues and engage in tissue protection and repair (Panduro et al., 2016).

Treg and conventional memory T cells share a gene expression signature not stably affected by secondary activation

Based on our observations, the Treg cell pool appeared to have a relatively limited ability to maintain activation-induced transcriptional, epigenomic and functional changes. Our recent finding that Foxp3 represses the expression of several transcriptional regulators implicated in memory T cell development, including TCF1, and OcaB, and can displace another important regulator of memory T cell differentiation, Foxo1, on chromatin raised the possibility that in Treg cells Foxp3 expression may prevent memory cell differentiation (Arvey et al., 2014; Samstein et al., 2012). To test this notion we transferred either aTr cells isolated 11 days after DT treatment of Foxp3$^{GFP-DTR}$ mice, or activated cells expressing a *Foxp3-GFP* reporter null allele (*Foxp3^{GFPKO}*) into lymphoreplete mice and analyzed maintenance of their activation program one month later. Compared to "fate-mapped" Treg cells in the BMC setting, aTr cells parked in lymphoreplete hosts maintained a greater fraction of activation-induced changes (Fig S6a,b). This difference may be due to the stronger activation stimulus induced by complete Treg ablation in Foxp3$^{GFP-DTR}$ mice compared to 90% Treg ablation in the mixed BMC setting, or to the shorter recovery time following transfer. Nevertheless, expression changes in many immune response genes, including Ccr2, Granzyme B, Galectin-3, and Tim-3 were already completely or partially reversed (Fig S6b). Importantly, Foxp3-independent activation-induced gene expression changes were maintained or reverted to an equal extent in both aTr cells and Foxp3^{GFPKO} cells one month post-transfer (Fig

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

S6c,d, Table S6), arguing against a non-redundant role for Foxp3 in preventing or erasing memory.

Because Treg cells are selected for self-reactivity, even Treg cells in naïve animals may have been exposed to their cognate antigens. Consistently, a substantial fraction of Treg cells from adult mice has an antigen-experienced $CD44^{+}CD62L^{-}$ cell surface phenotype (Fig S2g) and is long-lived (Luo et al., 2016). This raises the interesting possibility that a large fraction of the resting Treg cell pool in unmanipulated mice may be composed of cells that are more akin to conventional memory than to naïve T cells, and this feature may underlie their limited ability to "remember" inflammatory stimuli. A corollary of this hypothesis is that T cells should have a limited ability to accumulate stable changes in response to repeated stimulation. While intuitively sound, this notion seemed unlikely at first, given that memory CD8 T cells exposed to multiple rounds of *in vivo* antigen stimulation were shown to accumulate more and more stable transcriptional changes with each successive activation episode in an almost linear manner (Wirth et al., 2010). However, the side-by-side comparison of LCMV-specific memory CD4 T cells exposed to either one or two rounds of infection showed that secondary memory CD4 T cells had only limited capacity to further upregulate activation markers (Fig 7a-e), or increase cytokine production in response to *in vitro* stimulation (Fig 7f). Finally, the transcriptomes of primary and secondary memory CD4 T cells were largely similar with only a small fraction of activation-induced changes stably reinforced upon secondary activation (Fig 7g-I, Table S7). Critically, we found that a large portion of the differentially expressed (FDR<5%, FC>2) gene signature identified in Treg cells compared to naïve CD4 T cells was also present in conventional memory CD4 T cells (Fig 7j) and these genes were not stably up- or down-regulated upon secondary memory T cell stimulation (Fig 7k). Together, these observations suggest that in contrast to CD8 T cells, antigen-experienced CD4 T cells have a very limited ability to undergo long-lasting transcriptional changes in response to secondary stimulation and this feature may underlie the limited memory "potential" of the resting Treg cell pool.

Discussion

Cellular differentiation is associated with changes in gene expression induced in precursor cells by cell-intrinsic or –extrinsic cues and maintained in mature cells in the absence of the inducing signals. In addition to developmental processes, the ability to "remember" exposure to environmental cues can profoundly affect the function of differentiated cells. Such memory of previous challenges features prominently in the immune system. Through genetic fate mapping combined with analysis of transcriptional, epigenomic and functional changes we found that the bulk population of inflammation-experienced Tregs maintained only a limited subset of inflammation-induced changes, enabling their preferential accumulation in non-lymphoid organs after adoptive transfer. Non-lymphoid tissue Treg cells may acquire specific functions in response to local cues, for example, a tissue protective function mediated by Treg cell-derived amphiregulin (Arpaia et al., 2015). Interestingly, the transcriptome of Treg cells isolated from non-lymphoid tissues at steady state resembles that of aTr cells isolated from secondary lymphoid organs during an inflammatory challenge (X. Fan, and A.Y.R., unpublished observations). This observation suggests that Treg cells within non-lymphoid tissues may continuously receive signals, such

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

as antigens, cytokines, chemokines, alarmins and other factors that maintain their activated state, in addition to organ-specific signals that may confer other specialized functions.

While our experiments revealed that inflammation-experienced Treg cells largely lacked functional "memory", it remains possible that chronic exposure to activation signals could eventually imprint a more stable hyper-suppressive state. Although the effect of overall strength of inflammation and activation signals on the stability of gene expression changes remains unknown, it seems likely that stronger TCR stimulation in response to completely novel antigens or to previously encountered antigens, whose expression is markedly increased upon infectious challenge or injury, may increase the extent of stable differentiation or "memory" in Treg cells.

We propose that a largely "memory-less" potentiation of Treg cell suppressor function may help avoid not only enhanced suppression of effector memory T cells during a secondary challenge, but also generalized immunosuppression, which may otherwise result from repeated Treg cell activation. While selectively enforcing maintenance of an activated state in inflammation-experienced Treg cells is not technically feasible, published and unpublished observations support the notion that maintaining Treg cells in a perpetually activated or "super-suppressive" state long-term may be undesirable. For example, while Foxo1 inactivation is required for Treg cell activation, its genetic deletion results in Treg cell dysfunction (Ouyang et al., 2012). Additionally, we have observed that a stable gain in suppressor function due to continuous Treg cell-restricted activation of STAT5 results in diminished immune responses to bacterial and viral infections ((Chinen et al., 2016)). However, a different form of immune regulatory memory driven by clonal expansion of small populations of antigen-specific Treg cells is likely of importance in certain contexts and may ensure tolerance to tissue restricted self-antigens, pregnancy associated alloantigens, or antigens from food and commensal microbiota (Lathrop et al., 2011; Legoux et al., 2015; Rosenblum et al., 2011; Rowe et al., 2012).

Our findings that Treg cells and T effector cells differ in both the extent and the content of "memory" of inflammation likely extend to other cell types. We propose that a distinct capacity to maintain a cellular "memory" of environmental challenges may be found in other closely related immune cells, such as monocyte-derived macrophages vs. tissue resident macrophages, based on their divergent developmental history. We further speculate that cell types characterized by continuous generation from undifferentiated precursors are likely to display pronounced "memory" of gene expression and functional changes induced by transient environmental challenges, while those largely maintained via self-renewal may have only a limited ability to "remember".

Experimental Procedures

See the Extended experimental procedures for further information

Mice

Mice were bred and housed under SPF conditions in the animal facility at Memorial Sloan-Kettering Cancer Center. Experiments were performed in accordance with institutional

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

guidelines. *Foxp3*$^{\text{GFP-DTR}}$, *Foxp3*$^{\text{GFP-Cre-ERT2}}$ x *R26Y*, and *CD4*$^{\text{CreERT2}}$ mice have been described (Kim et al., 2007; Rubtsov et al., 2010; Sledzinska et al., 2013). *TCRβδ*$^{-/-}$ mice and C57Bl/6 Ly5.1 mice were purchased from Jackson Laboratories and bred in-house.

In vivo activation and fate mapping of regulatory and effector T cells

At 8 weeks post-transfer, BMC mice were intraperitoneally injected with 1μg diphtheria toxin (DT) in PBS. Remaining *Foxp3*$^{\text{GFP-Cre-ERT2}}$ x *R26Y* Treg cells were labeled by administrating tamoxifen (40mg/ml, olive oil solution) by oral gavage on days 3 and 4 post-DT treatment. Activated and "memory" Treg cells were isolated on day 11 or day 60 post-DT, respectively, using fluorescence-activated cell sorting (FACS). Resting Treg cells were isolated from mice that received only tamoxifen, but no DT. For isolation of inflammation-experienced Treg and effector T cells from *Foxp3*$^{\text{GFP-DTR}}$*CD4*$^{\text{CreERT2}}$R26$^{\text{tdTomato}}$ mice we injected animals with DT, followed by tamoxifen treatment on day 7 and 8 post-challenge. Activated and inflammation-experienced effector T cells were FACS-sorted based on expression of tdTomato and lack of GFP expression on day 11 and day 60 post-DT, respectively. Resting effector cells were isolated based on the same criteria from mice receiving tamoxifen only.

Adoptive transfer

For adoptive transfer experiments, 1×10^5 FACS-sorted activated or "memory" Treg cell populations were transferred together with 1×10^5 resting Treg cells and 8×10^5 congenically marked effector CD4 T cells into *TCRβδ*$^{-/-}$ recipients.

Activated and "memory" Treg cell populations were isolated as GFP$^+$YFP$^+$ CD4 T cells from mixed BMC mice 11 days or 2 months post-DT treatment, respectively. Resting Treg cells were isolated as GFP$^+$YFP$^-$ CD4 T cells from BMC mice without DT treatment. Effector CD4 T cells were isolated as GFP-Ly5.1$^+$ CD4 T cells from *Foxp3*$^{\text{GFP-DTR}}$ Ly5.1 mice, 11 days post-DT treatment. Recipient mice were analyzed 2 weeks after adoptive transfer.

Comparison of primary and secondary memory of LCMV-infection

GP66 tetramer$^+$ CD4 T cells were isolated from LCMV infected animals on day 60 p.i. and 1×10^4 cells were transferred into congenically marked recipients infected with LCMV one day post transfer. Primary and secondary memory cells were isolated 60 days p.i. and were compared by flow cytometry and RNA-seq. For *in vitro* restimulation, cells were incubated with GP61-80 peptide for 6 hours at 37 degrees in the presence of brefeldin A.

In vitro suppression assay

Treg cell populations and naïve CD4 T responder cells were cultured at various ratios in the presence of irradiated splenocytes and soluble anti-CD3 antibody (1μg/ml) for 72h. Responder cell proliferation was measured by incorporation of ^{3}H-Thymidine during the final 8 hours of culture.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In vivo suppression assay

4×10^5 pre-activated effector CD4 T cells isolated from *Foxp3*^GFP-DTR^Ly5.1 on day 11 post-DT treatment were transferred together with 1×10^5 tdTomato$^+$ Treg cells isolated from *Foxp3*$^{\text{GFP-DTR}}$*CD4*$^{\text{CreERT2}}$*R26*$^{\text{tdTomato}}$ mice into *TCRβδ*$^{-/-}$ recipients. Effector T cell numbers in lymphoid and non-lymphoid tissues were analyzed 50 days post-transfer and weight was monitored throughout the experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the MSKCC integrated genomics operation for sequencing RNA-, ChIP-, and ATAC-seq libraries and preprocessing data. *CD4*$^{\text{CreERT2}}$ mice were generated in the laboratory of Thorsten Buch. We thank C. Campbell, S. Dikiy, A. Levine, S. Lee, K. Wu, and W. Hu for help with tissue lymphocyte isolations and all members of the Rudensky lab for helpful discussions and technical assistance. This study was supported by NIH grants R37 AI034206, P30 CA008748, U01 HG007893. AYR is an investigator with the Howard Hughes Medical Institute.

References

Angelosanto JM, Blackburn SD, Crawford A, Wherry EJ. Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. J Virol. 2012; 86:8161–8170. [PubMed: 22623779]

Arpaia N, Green JA, Moltedo B, Arvey A, Hemmers S, Yuan S, Treuting PM, Rudensky AY. A Distinct Function of Regulatory T Cells in Tissue Protection. Cell. 2015; 162:1078–1089. [PubMed: 26317471]

Arvey A, van der Veeken J, Samstein RM, Feng Y, Stamatoyannopoulos JA, Rudensky AY. Inflammation-induced repression of chromatin bound by the transcription factor Foxp3 in regulatory T cells. Nat Immunol. 2014; 15:580–587. [PubMed: 24728351]

Belkaid Y, Tarbell K. Regulatory T cells in the control of host-microorganism interactions (*). Annual review of immunology. 2009; 27:551–589.

Berg RE, Crossley E, Murray S, Forman J. Memory CD8+ T cells provide innate immune protection against Listeria monocytogenes in the absence of cognate antigen. The Journal of experimental medicine. 2003; 198:1583–1593. [PubMed: 14623912]

Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods. 2013; 10:1213–1218. [PubMed: 24097267]

Burzyn D, Benoist C, Mathis D. Regulatory T cells in nonlymphoid tissues. Nat Immunol. 2013; 14:1007–1013. [PubMed: 24048122]

Chang JT, Wherry EJ, Goldrath AW. Molecular regulation of effector and memory T cell differentiation. Nat Immunol. 2014; 15:1104–1115. [PubMed: 25396352]

Chinen T, Kannan A, Levine A, Fan X, Klein U, Zheng Y, Gasteiger G, Feng Y, Fontenot J, Rudensky A. An essential role for IL-2 receptor in regulatory T cell function. Nat Immunol. 2016 In press.

Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, Wherry EJ. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. Immunity. 2014; 40:289–302. [PubMed: 24530057]

Hale JS, Youngblood B, Latner DR, Mohammed AU, Ye L, Akondy RS, Wu T, Iyer SS, Ahmed R. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. Immunity. 2013; 38:805–817. [PubMed: 23583644]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity. 2004; 21:267–277. [PubMed: 15308106]

Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annual review of immunology. 2012; 30:531–564.

Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell. 2002; 111:837–851. [PubMed: 12526810]

Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol. 2007; 8:191–197. [PubMed: 17136045]

Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, Peterson DA, Stappenbeck TS, Hsieh CS. Peripheral education of the immune system by colonic commensal microbiota. Nature. 2011; 478:250–254. [PubMed: 21937990]

Legoux FP, Lim JB, Cauley AW, Dikiy S, Ertelt J, Mariani TJ, Sparwasser T, Way SS, Moon JJ. CD4+ T Cell Tolerance to Tissue-Restricted Self Antigens Is Mediated by Antigen-Specific Regulatory T Cells Rather Than Deletion. Immunity. 2015; 43:896–908. [PubMed: 26572061]

Lertmemongkolchai G, Cai G, Hunter CA, Bancroft GJ. Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens. J Immunol. 2001; 166:1097–1105. [PubMed: 11145690]

Levine AG, Arvey A, Jin W, Rudensky AY. Continuous requirement for the TCR in regulatory T cell function. Nat Immunol. 2014; 15:1070–1078. [PubMed: 25263123]

Liu J, Cao S, Peppers G, Kim SH, Graham BS. Clonotype-specific avidity influences the dynamics and hierarchy of virus-specific regulatory and effector CD4(+) T-cell responses. Eur J Immunol. 2014; 44:1058–1068. [PubMed: 24510524]

Luo CT, Liao W, Dadi S, Toure A, Li MO. Graded Foxo1 activity in Treg cells differentiates tumour immunity from spontaneous autoimmunity. Nature. 2016; 529:532–536. [PubMed: 26789248]

Monticelli S, Natoli G. Short-term memory of danger signals and environmental stimuli in immune cells. Nat Immunol. 2013; 14:777–784. [PubMed: 23867934]

Ouyang W, Liao W, Luo CT, Yin N, Huse M, Kim MV, Peng M, Chan P, Ma Q, Mo Y, et al. Novel Foxo1-dependent transcriptional programs control T(reg) cell function. Nature. 2012; 491:554–559. [PubMed: 23135404]

Panduro M, Benoist C, Mathis D. Tissue Tregs. Annual review of immunology. 2016; 34:609–633.

Quintin J, Cheng SC, van der Meer JW, Netea MG. Innate immune memory: towards a better understanding of host defense mechanisms. Curr Opin Immunol. 2014; 29:1–7. [PubMed: 24637148]

Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. Response to self antigen imprints regulatory memory in tissues. Nature. 2011; 480:538–542. [PubMed: 22121024]

Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature. 2012; 490:102–106. [PubMed: 23023128]

Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, Rudensky AY. Stability of the regulatory T cell lineage in vivo. Science. 2010; 329:1667–1671. [PubMed: 20929851]

Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. Cell. 2012; 150:29–38. [PubMed: 22770213]

Schietinger A, Delrow JJ, Basom RS, Blattman JN, Greenberg PD. Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. Science. 2012; 335:723–727. [PubMed: 22267581]

Shafiani S, Dinh C, Ertelt JM, Moguche AO, Siddiqui I, Smigiel KS, Sharma P, Campbell DJ, Way SS, Urdahl KB. Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. Immunity. 2013; 38:1261–1270. [PubMed: 23791647]

Sledzinska A, Hemmers S, Mair F, Gorka O, Ruland J, Fairbairn L, Nissler A, Muller W, Waisman A, Becher B, et al. TGF-beta signalling is required for CD4(+) T cell homeostasis but dispensable for regulatory T cell function. PLoS Biol. 2013; 11:e1001674. [PubMed: 24115907]

Smigiel KS, Richards E, Srivastava S, Thomas KR, Dudda JC, Klonowski KD, Campbell DJ. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. The Journal of experimental medicine. 2014; 211:121–136. [PubMed: 24378538]

Sprent J, Surh CD. T cell memory. Annual review of immunology. 2002; 20:551–579.

Utzschneider DT, Legat A, Fuertes Marraco SA, Carrie L, Luescher I, Speiser DE, Zehn D. T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. Nat Immunol. 2013; 14:603–610. [PubMed: 23644506]

Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015; 15:486–499. [PubMed: 26205583]

Wirth TC, Xue HH, Rai D, Sabel JT, Bair T, Harty JT, Badovinac VP. Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. Immunity. 2010; 33:128–140. [PubMed: 20619696]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

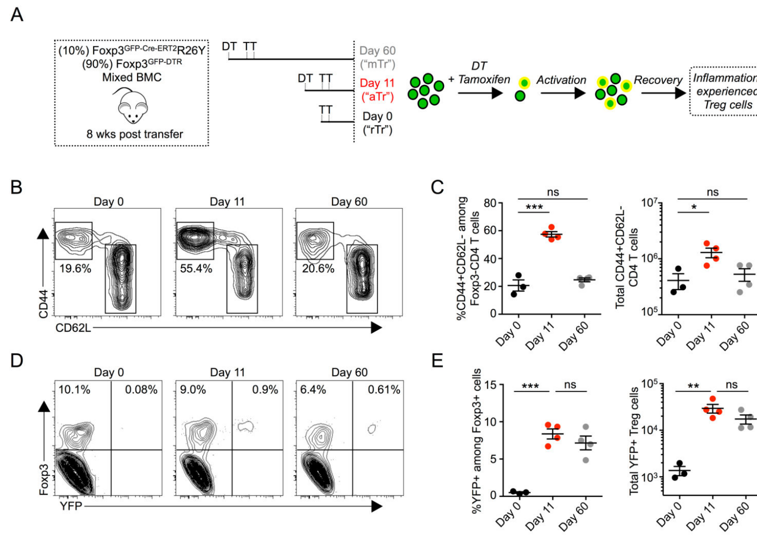


Figure 1. Generation and fate mapping of inflammation-experienced Treg cells

a) Experimental design schematic. Bone marrow chimeric (BMC) mice were treated with diphtheria toxin (DT) followed by tamoxifen (T) on days 3 and 4 post-DT treatment.

b, c) Frequency and total numbers of CD44$^+$CD62L$^-$Foxp3$^-$ CD4 T cells in spleen on day 0, day 11, and day 60 post-DT treatment.

d, e) Frequency and total numbers of YFP$^+$ Treg cells in spleen on day 0, day 11, and day 60 post-DT. Data are representative of 2 independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. P-values were calculated using one-way ANOVA with Tukey's multiple comparisons test. Error bars show mean with S.E.M.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

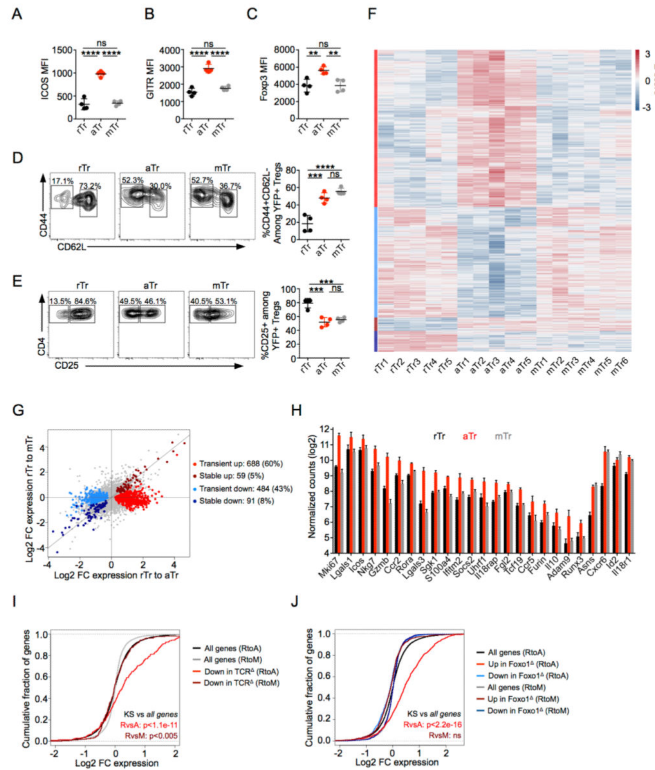


Figure 2. Stability of inflammation-induced transcriptional changes in Treg cells

a-e) FACS analysis of ICOS (a), GITR (b), Foxp3 (c), CD44 and CD62L (d), and CD25 (e) protein levels in YFP^{+} Treg cells from the spleen of BMC mice on day 0 (rTr), day 11 (aTr), and day 60 (mTr) post DT treatment. *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001. P-values from one-way ANOVA with Tukey's multiple comparisons test.

f) Heatmap of stable and transient gene expression changes in bulk GFP+ rTr, and YFP+ aTr, and mTr populations from peripheral lymphoid organs analyzed by RNA-seq.

g) Gene expression changes in rTr vs aTr cells (x-axis) and rTr vs mTr cells (y-axis). All present genes are shown in grey. Gene group classifications require statistically significant (FDR<5%) changes in at least two cell state comparisons to avoid bias for detection of either stable or transient changes (see Experimental Procedures).

h) Log2 normalized RNA-seq counts for selected genes.

i-j) activation-induced changes in TCR- (l), and Foxo1- (m) dependent gene signatures. P-values calculated using one-sided Kolmogorov–Smirnov (KS) test comparing signature genes to all genes. Plots are generated using pooled RNA-seq data from two independent experiments (Experiment 1: rTr1-3, aTr1-3, mTr1-4; Experiment2: rTr4-5, aTr4-5, mTr5,6)

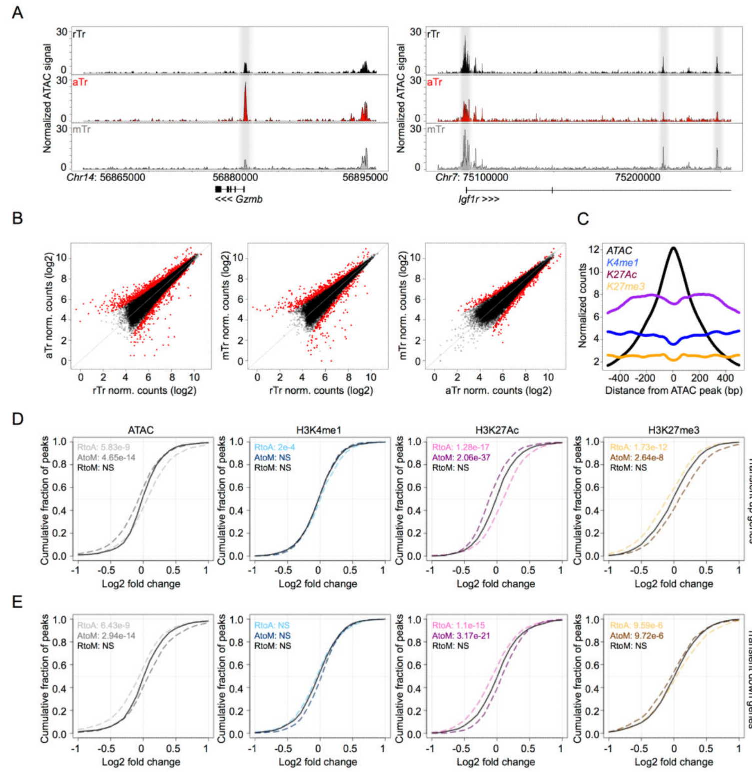


Figure 3. Transient gene expression changes are not commonly associated with chromatin-level memory

a) ATAC-seq tracks for genes undergoing transient activation-induced up-(*Gzmb*) or downregulation (*Igf1r*).

b) Scatter plots of ATAC-seq changes. Significant changes (FDR 5%) are highlighted in red.

c) Metapeak plot of histone modifications in 1kb window surrounding ATAC-seq peaks in rTr cells.

d,e) Distribution of chromatin changes in a 1kb window surrounding ATAC-seq peaks associated with transiently up- (d) or downregulated (e) genes. P-values calculated using one-sided KS test comparing peaks associated with transiently regulated genes to all peaks. Directionality of the KS tests logically corresponds with the directionality of gene expression changes. RtoA (rTr to aTr), AtoM (aTr to mTr), RtoM (rTr to mTr). NS (p>0.001).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

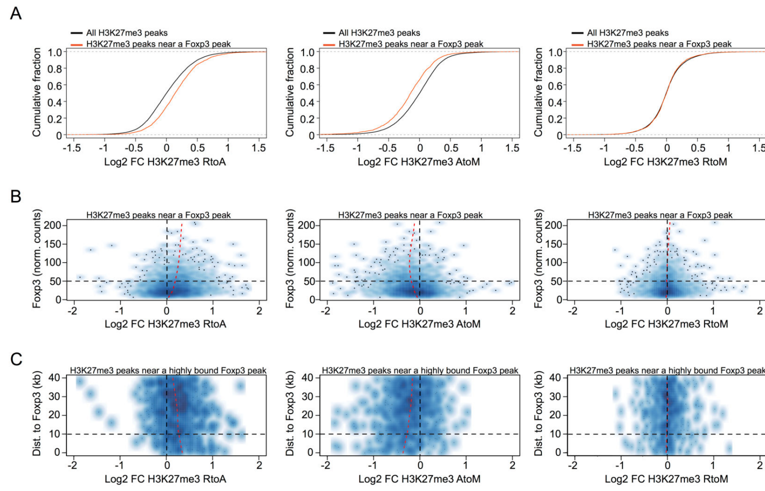


Figure 4. Transient activation-induced increase in H3K27me3 at Foxp3 bound sites

a) Association between Foxp3 binding and activation-induced H3K27me3. Comparing all (24999) H3K27me3 peaks and domains to those near (<40kb) a Foxp3 target (6656).

b) Relationship between Foxp3 ChIP-seq read count and activation-induced H3K27me3 changes for regions near a Foxp3 target site.

c) Relationship between distance to nearest Foxp3 peak and activation-induced H3K27me3 changes for regions near a highly occupied (normalized counts >50) Foxp3 target (561 peaks and domains total).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

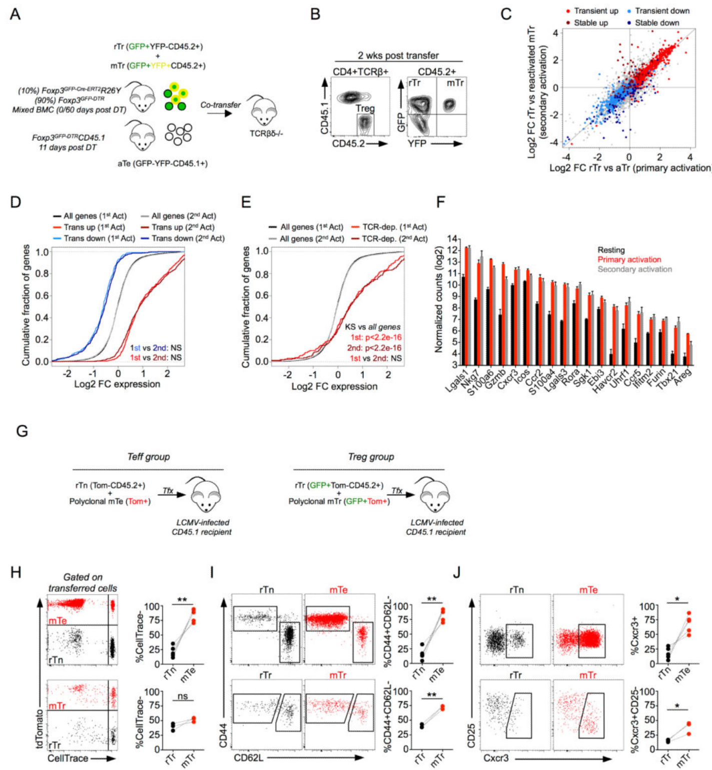


Figure 5. Transient Treg cell activation signature is regulated similarly upon secondary challenge

a, b) Experimental design schematic. $GFP^{+}YFP^{+}$ aTr or mTr cells were transferred together with resting $GFP^{+}YFP^{-}$ Treg cells and pre-activated $Foxp3^{-}$ $Ly5.1^{+}$ CD4 effector T cells into *TCRβδ*$^{-/-}$ recipients (a). Treg cells were isolated from secondary lymphoid organs two weeks post-transfer and analyzed by RNA-seq.

c) Gene expression changes in rTr cells before and after transfer ("primary activation") on x-axis and changes in gene expression in rTr compared to reactivated mTr cells ("secondary activation") on y-axis. Gene expression groups defined in Fig 2g are highlighted.

d,e) Distribution of expression changes induced by primary and secondary activation. Transiently regulated genes (d) and TCR-dependent genes (e) are shown. P-values calculated using a one-sided KS test.

f) Log2 normalized RNA-seq counts for selected genes.

g) Experimental design schematic to compare recall responses of LCMV-experienced Treg and Teff cells. Polyclonal mTe and mTr populations were isolated 60 days after LCMV infection and tamoxifen treatment of *Foxp3*$^{\text{GFP-DTR}}$*CD4*$^{\text{CreERT2}}$*R26*$^{\text{tdTomato}}$ mice (see Fig S2f) and mixed at equal ratios with Tomato^{-} naïve CD4 (rTn) or rTr cells, respectively. Cells were labeled with CellTrace™ and transferred into congenically marked, LCMV-infected recipients. h-j) FACS analysis of (h) CellTrace™, (i) CD44 and CD62L, (j) CD25 and CXCR3 staining on day 11 p.i. *, $P<0.05$; **, $P< 0.01$. P-values calculated using T-test. Error bars show mean with S.E.M. Representative of two (i,j) or three (h) independent experiments.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

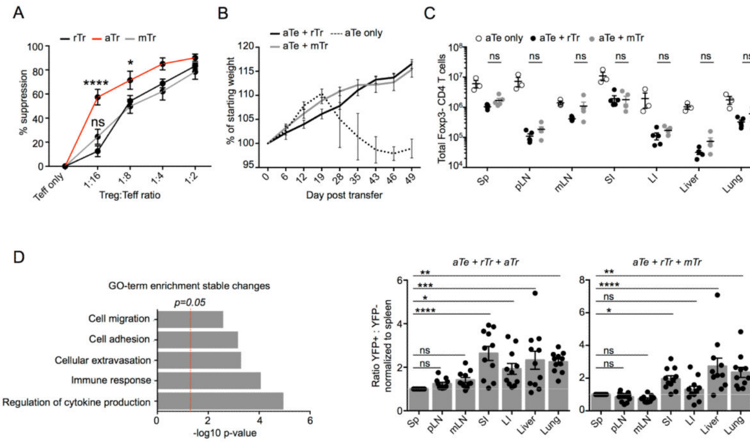


Figure 6. Treg cell activation transiently enhances suppressive function and imprints stable non-lymphoid tissue preference

a) *In vitro* suppression assay using rTr, aTr, and mTr cells from mixed BMCs. Pooled data from two independent experiments with a total of 6 replicates per condition are shown. Statistical tests compare mTr vs rTr, or mTr vs aTr.

b, c) *In vivo* suppression assay using rTr and mTr populations from $Foxp3^{GFP\text{-}DTR}CD4^{CreERT2}R26^{tdTomato}$ mice. Prevention of weight loss (wasting disease) (b) and suppression of T cell expansion (c) are shown. The data represent one of two independent experiments with at least 3 mice per group.

d) Selection of GO-terms enriched among genes undergoing stable transcriptional changes (from Fig 2g).

e) GFP$^+$YFP$^+$ aTr or mTr cells were transferred together with GFP$^+$YFP$^-$ rTr cells and activated Foxp3$^-$ Ly5.1$^+$ CD4 effector T cells into $TCR\beta\delta^{-/-}$ recipients, as shown in Fig 5a. Mice were analyzed two weeks post-transfer. Ratios of YFP$^+$ to YFP$^-$ Treg cells were normalized to the ratio in the spleen. The pooled data from two independent experiments are shown with a total of 11 mice per group. *, $P<0.05$; **, $P< 0.01$; ***, $P<0.001$; ****, $P<0.0001$. P-values calculated using two-way ANOVA (a, c) or one-way ANOVA (e) with Tukey's multiple comparisons test. Error bars show mean with S.E.M.

Author Manuscript

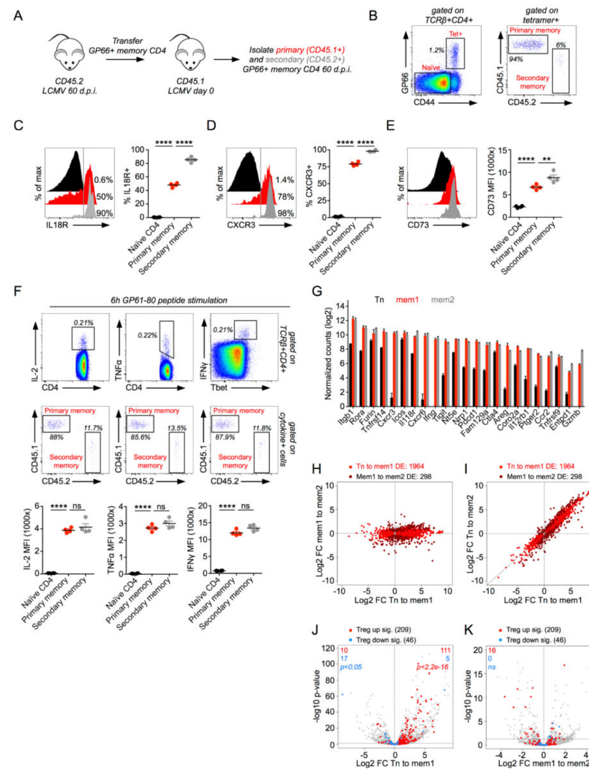


Figure 7. Treg cells and conventional memory T cells share a gene signature not stably affected by secondary activation

a,b) Experimental design schematic. LCMV-specific GP66-tetramer^{+} memory CD4 T cells were isolated 60 days p.i and transferred to congenically marked recipients. Recipients were infected with LCMV and 60 days later primary and secondary memory T cells were isolated.

c-d) Flow cytometric analysis of IL18R (c), CXCR3 (d), CD73 (e) staining.

f) Cytokine production after *in vitro* stimulation with GP61-80 peptide for 6h at 37 degrees in the presence of brefeldin A. Representative of two independent experiments. Error bars show mean with S.E.M.

g-k) RNA-seq of primary and secondary memory T cells. (g) Expression of a set of immune response genes, (h) FC-FC plot showing log2 fold change in naïve CD4 (Tn) vs primary memory (mem1) and in mem1 vs secondary memory (mem2). Differentially expressed genes (FDR<5%) are highlighted. (i) FC-FC plot showing Tn vs mem1 and Tn vs mem2.

j,k) Volcano plots showing differential gene expression in Tn vs mem1 (j) and mem1 vs mem2 (k). Genes differentially expressed in Treg compared to Tn (FDR<5% and FC>2) are highlighted. Number of significantly (FDR<5%) up- or downregulated Treg-signature genes is shown.

**, P< 0.01; ****, P<0.0001. P-values were calculated using one-way ANOVA (c-f) with Tukey's multiple comparisons test or Chi-square test (j,k).