Correction

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Correction for "Imbalanced signal transduction in regulatory T cells expressing the transcription factor FoxP3," by Dapeng Yan, Julia Farache, Diane Mathis, and Christophe Benoist, which appeared in issue 48, December 1, 2015, of *Proc Natl Acad Sci USA* (112:14942–14947; first published November 16, 2015; 10.1073/pnas.1520393112).

The authors note that Michael Mingueneau should be added to the author list between Julia Farache and Diane Mathis. Michael Mingueneau should be credited with designing research, performing research, and analyzing data. The corrected author line, affiliation line, and author contributions appear below. The online version has been corrected.

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Imbalanced signal transduction in regulatory T cells expressing the transcription factor FoxP3

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FoxP3$^+$ T regulatory (Treg) cells have a fundamental role in immunological tolerance, with transcriptional and functional phenotypes that demarcate them from conventional CD4$^+$ T cells (Tconv). Differences between these two lineages in the signaling downstream of T-cell receptor-triggered activation have been reported, and there are different requirements for some signaling factors. Seeking a comprehensive view, we found that Treg cells have a broadly dampened activation of several pathways and signaling nodes upon TCR-mediated activation, with low phosphorylation of CD3ζ, SLP76, Erk1/2, AKT, or S6 and lower calcium flux. In contrast, STAT phosphorylation triggered by interferons, IL2 or IL6, showed variations between Treg and Tconv in magnitude or choice of preferential STAT activation but no general Treg signaling defect. Much, but not all, of the Treg/Tconv difference in TCR-triggered responses could be attributed to lower responsiveness of antigen-experienced cells with CD44^{hi} or CD62L^{lo} phenotypes, which form a greater proportion of the Treg pool. Candidate regulators were tested, but the Treg/Tconv differential could not be explained by overexpression in Treg cells of the signaling modulator CD5, the coinhibitors PD-1 and CTLA4, or the regulatory phosphatase DUSP4. However, transcriptome profiling in *Dusp4*-deficient mice showed that DUSP4 enhances the expression of a segment of the canonical Treg transcriptional signature, which partially overlaps with the TCR-dependent Treg gene set. Thus, Treg cells, likely because of their intrinsically higher reactivity to self, tune down TCR signals but seem comparatively more attuned to cytokines or other intercellular signals.

signal transduction | immunoregulation

FoxP3$^+$ T regulatory (Treg) cells help maintain lymphoid homeostasis in many immunological contexts: tolerance to self versus autoimmune deviation, foeto-maternal tolerance, allergy, responses to pathogens, and interactions with commensal microbes (1–4). Their importance is highlighted by the devastating multiorgan inflammation that occurs in FoxP3-deficient *scurfy* mice or immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) patients (5), or upon experimental lineage ablation. In addition, FoxP3$^+$ Treg cells partake in extraimmune regulatory activities, for instance by dampening inflammation in visceral adipose tissue or channeling tissue repair after muscle injury (6). Treg cells influence not only other T cells but also cells of the innate immune system such as natural killer or dendritic cells and macrophages. Treg cells differ transcriptionally from their "conventional" CD4$^+$ counterparts (Tconv) with respect to their transcriptomes, and a canonical "Treg signature" of differentially expressed transcripts has been defined, which collectively define and ensure the stability of the Treg phenotype (refs. 1, 3, and references therein).

The somatically rearranged T-cell receptor (TCR) expressed by Treg cells plays an important part in their differentiation and physiology. The TCR repertoire expressed by Treg cells is quite broad and largely, albeit not completely, distinct from that of Tconv cells (7–9). Treg differentiation requires TCR interactions with MHC-II molecules (ref. 10 and references therein), as engagement of the TCR by agonist ligands during thymic differentiation strongly favors clonal deviation into the FoxP3+ lineage, either by inducing differentiation along the lineage (11) or because FoxP3+ cells are inherently more resistant to clonal deletion (12). Accordingly, the analysis of mice expressing a Nurr77-GFP reporter, whose expression correlates with the strength of cell activation via the TCR, showed that the reporter was generally expressed at a higher level in Treg than in Tconv cells (13). For mature Treg cells, experiments involving genetically interrupted TCR expression or signaling showed that TCR engagement is not required for Treg survival (at least in the short term) and FoxP3 expression but is necessary for suppressor activity and expression of a component of the typical Treg transcriptional signature (14–16). Indeed, Treg-mediated suppression requires engagement by a cognate antigen, even if different from the antigen recognized by the T cell being suppressed (17).

Accordingly, there are indications that, although Treg cells express largely the same canonical signaling armamentarium as Tconv cells, they differ in the quantitative balance of these pathways. Integration of results from many gain- or loss-of-function experiments suggests that, overall, signaling along the NF-κB pathways favors Treg differentiation and activity, whereas AKT/mTORC2 signals are inhibitory (reviewed in ref. 3). Several reports also pointed to differences in Treg signaling architecture, such as a different involvement of PKC-Θ (18), DGKζ (19), SHIP (20), or Dlgh1 (21). Finally, there have been reports that signaling intensity downstream of TCR engagement is reduced in Treg relative to Tconv cells (22–24). Here, we set out to assess the spread of signaling particularities in Treg cells and track their possible molecular origins.

Results

Dampened Signals Elicited by the TCR in Treg Cells. To analyze Treg responsiveness in a tightly controlled manner, whole splenocytes were stimulated in vitro by binding of biotinylated anti-CD3 and -CD28 antibodies, followed by cross-linking with streptavidin,

Significance

The homeostasis of FoxP3$^+$ Treg cells, essential controllers of immune and autoimmune responses, integrates inputs from the antigen-specific T-cell receptor (TCR) and from trophic cytokines or chemotactic cues. Expanding upon previous indications that Treg cells might be hyporesponsive, we find that TCR triggering induces dampened responses along all tested pathways in Treg compared with conventional CD4$^+$ T cells, whereas their responses to IFN-I, IL6, or IL2 were equal or stronger. This imbalance indicates that Treg cells are less sensitive to direct recognition of novel antigens, for instance of an infectious nature, and more to cytokine-based cues or intercellular communication.

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The authors declare no conflict of interest.

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while sharply raising the temperature to 37 °C to initiate signal transduction (24). At various times following the addition of streptavidin, cells were fixed, permeabilized, and stained with antibodies to distinguish Treg and Tconv cells coactivated in the same reactions or to probe phosphorylation levels at different TCR-triggered signaling nodes. FoxP3$^+$ Treg cells were distinguished from Foxp3-negative Tconv by staining with anti-FoxP3 antibody (for experiments in inbred C57BL/6 or mutant mice) or with anti-Thy1.1 for experiments in B6.*Foxp3.Cd90.1* reporter mice (25). As illustrated in Fig. 1*A*, and as expected from much prior literature, this cross-linking led to rapid and very synchronous Erk1/2 phosphorylation in Tconv cells, which then waned within the next 10 min. In contrast, Erk activation was much less extensive in Treg cells, as quantitated in Fig. 1*B*, with a lower proportion of responding cells and a lower mean fluorescence intensity [the minority of Treg cells that did respond also showed lower levels of phospho-Erk (pErk) than Tconv]. The same results were observed in C57BL/6 mice (Fig. S1*B*). Higher responses in Tconv were reflected quantitatively, over a number of experiments, by a higher induction ratio (Fig. 1*C*). On the other hand, the pErk induced in Treg cells persisted longer than in Tconv, as visible in Fig. 1*B* and manifest in low rates of decay in an exponential fit of the pErk postpeak data, probably reflecting reduced operation of negative feedback loops at these lower induction levels (Fig. 1*C* and Fig. S1*A*).

This lower response in Treg cells was not due to a requirement for a stronger activation signal, as it was observed across a range of doses of anti-CD3/28 or of cross-linking streptavidin (Fig. 1*D*). To extend these observations, we also assessed Erk1/2 phosphorylation in T cells from BDC2.5 TCR transgenic mice, stimulated with whole splenocytes loaded with mimotope agonist peptide (26). The response was more persistent than that elicited by antibody-mediated cross-linking, yet the same Treg/Tconv difference was present with this more physiological mode of activation (Fig. 1*E* and Fig. S1*C*).

We then compared the response in Treg and Tconv cells along different signaling pathways and nodes downstream of the TCR. In agreement with ref. 22, the increase in intracytoplasmic Ca++ was strongly reduced in Treg relative to Tconv (Fig. 1*F*). Reduced activation was observed in Treg cells for p-CD3ζ (Y142), indicating that lower signaling efficacy was already manifest at the apex of the signaling cascade, and for Treg p-SLP76 (Y128), p-AKT (Ser473), and p-S6 (S235) (Fig. 1*G*), indicating that dampened signals are found throughout the different signaling branches.

Correlations of Dampened TCR Signals in Treg Cells. Given this difference in TCR-induced signals in Tconv and Treg cells, we asked whether it might be tied to some of the known phenotypic differences between these lineages. Most immediately, Treg and Tconv express slightly different levels of TCR, and this quantitative difference could potentially account for the different signal intensities. This was not the case, however. When the response to anti-CD3/CD28 stimulation was parsed by analyzing cells binned across matched quantiles of surface TCR (Fig. 2*A*, *Left*), Treg cells showed lower pErk than Tconv cells, at all TCR levels (Fig. 2*A*).

Responses to TCR engagement are known to be less intense in memory than in naïve T cells (27), and this could be an important confounder, as a significant fraction of Treg cells have a memory phenotype (28–30). We used the expression of CD44 and CD62L molecules to distinguish between naïve and memory phenotypes during the response to CD3/CD28 cross-linking. As expected, naïve CD44^{lo} Tconv cells responded more than CD44^{hi} Tconv (Fig. 2*B*, *Left*), and naïve CD62L^{hi} Tconv cells responded more than CD62L^{lo} Tconv (Fig. S2). The same pattern was

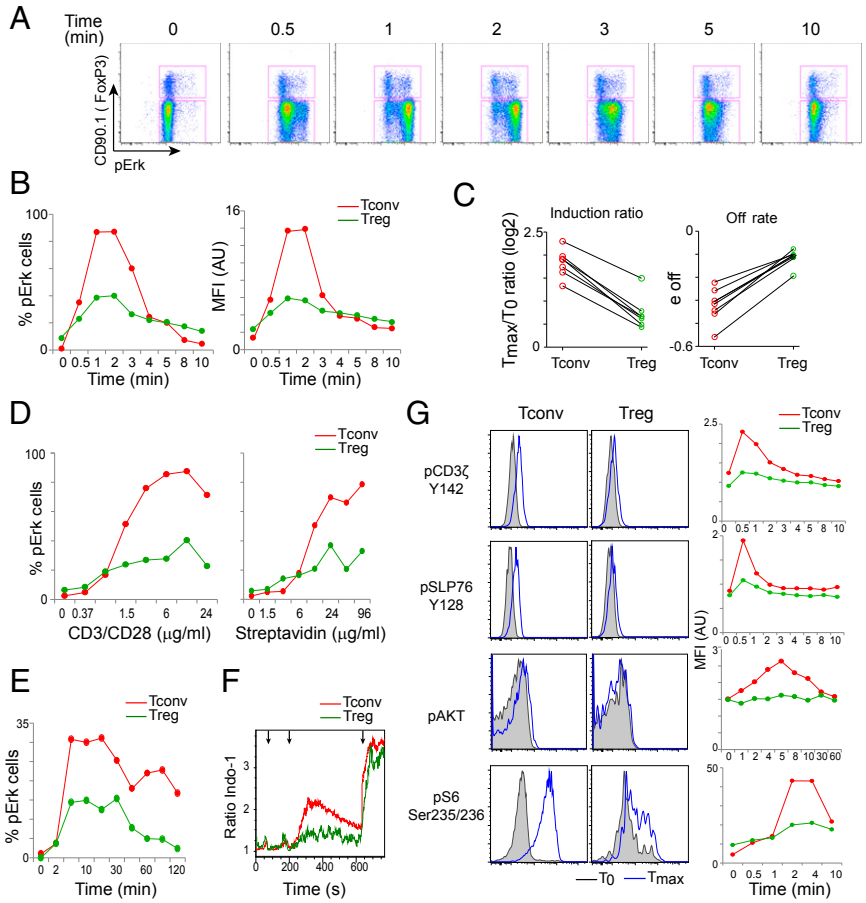

Fig. 1. Lower TCR-induced signals in Treg cells. (*A*) pErk versus CD90.1 (FoxP3 reporter) cytometry profiles at different times after streptavidin cross-linking of CD3 and CD28, gated on CD4$^+$TCRβ+ cells. (*B*) Quantitation of data from *A*, as percent pErk$^+$ cells and mean fluorescence intensity (MFI) (*A* and *B* representative of seven independent experiments). (*C*) Compilation across experiments of pErk induction (2 min MFI/baseline MFI ratio) and of the off-rate (exponent of an exponential fit). (*D*) Representative (of three experiments) pErk responses at 2 min across a titration of anti-CD3 and -CD28 antibodies (*Left*) or strepavidin (*Right*). (*E*) Representative (of four experiments) pErk responses in CD4$^+$ T cells from BDC2.5 TCR transgenic mice stimulated with mimotope peptide in the presence of antigen-presenting cells (APCs). (*F*) Calcium flux induced in CD4$^+$ T cells by biotinylated anti-CD3/CD28 antibodies (first arrow), streptavidin cross-linking (second arrow), and control ionomycin (third arrow) (representative of two independent experiments). (*G*) Changes in MFI of pCD3ζ, pSLP76, and pAKT in CD4$^+$ Tconv and Treg cells after streptavidin cross-linking of CD3 and CD28 (representative of three experiments).

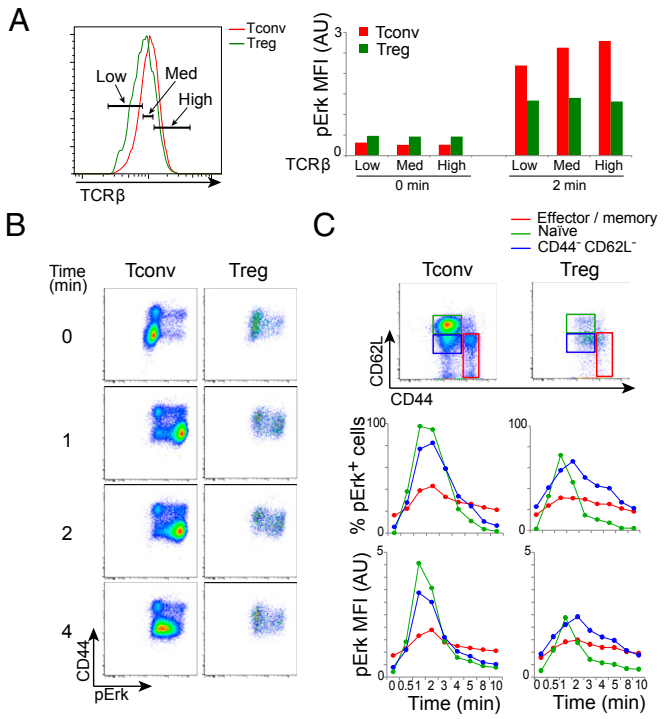

Fig. 2. Phenotypic correlates of dampened TCR signals in Treg cells. (*A*) pErk responses in Tconv and Treg cells (*Left*), in bins of cells matched for TCR surface levels (*Right*); anti-CD3/CD28 cross-linking is as in Fig. 1 and is representative of four independent experiments. (*B*) pErk induction versus CD44 level, for TCRβ^{+}CD4^{+} Tconv and Treg cells gated from the CD90.1 reporter. (*C*) Different naïve/memory states for Treg and Tconv cells were distinguished (*Top*), and the pErk responses of these gated cells were tracked over time after anti-CD3/-CD28 cross-linking (representative of three independent experiments).

observed for Treg cells, and it was thus clear that a good part of the Treg/Tconv signaling difference could be explained by the higher proportion of CD44^{hi} low responders among the Treg pool. This bias did not provide the entire explanation for the Treg/Tcov signaling difference, however, as Treg cells responded less intensely even in matched ranges of CD44 expression (Fig. 2*B*). For quantitation, we gated the three main populations defined by CD44 and CD62L. For CD44^{lo}CD62L^{hi} naïve cells that dominated among Tconv or for CD44^{lo}CD62L^{lo} that were more frequent among Treg cells (Fig. 2*C*, *Top*), responses were less intense in Treg cells than in Tconv (Fig. 2*C*, *Bottom*). They were uniformly low for CD44^{+}CD62L^{lo} effector/memory cells. Thus, although the predominant antigen-experienced phenotype of Treg cells explains part of their lower phosphorylation response to TCR engagement, this characteristic does not provide the whole explanation.

Sluggish Phosphorylation Cascades Are Not a General Treg Trait. Thus, Treg cells show a generally dampened response to activation through the TCR. We then asked whether this is a general characteristic of phosphorylation cascades in Treg cells, possibly due to high expression of inhibitory phosphatases, by testing responses to a range of cytokines (Fig. 3). Responses to cytokine stimulation in vitro were assessed by measuring the phosphorylation of different STAT transducers. In the presence of type I IFN (IFNα or IFNβ), Treg cells showed a significantly higher degree of STAT1 phosphorylation than Tconvs at all time points (Fig. 3*A*). In contrast, Tconv cells showed a stronger pSTAT1 response to IFNγ than Treg cells (Fig. 3*A*, *Right*).

For IL6, there was an interesting variation according to the dose and the signaling molecule: At low doses of IL6, Treg and Tconv showed equivalent STAT3 phosphorylation over time, but the higher level and faster appearance of pSTAT3 at higher doses of IL6 (50 ng/mL) were only manifest in Treg cells. In contrast, STAT1 phosphorylation elicited by IL6 (most convincingly at the higher dose) was equivalent in Treg and Tconv, suggesting that the STAT3 divergence at these high doses was not due to receptor saturation (Fig. 3*B*, *Right*). These subtle divergences are in line with the complexity of IL6's impact in T cells (31) and suggest that the location or configuration of the IL6 receptor, or its immediate signaling assembly, must vary between CD4+ lineages (Stat genes are equally expressed in Treg and Tconv cells; www.immgen.org).

Finally, as expected from the characteristic overexpression of CD25 (*Il2ra*) in Treg cells, Il2 elicited much stronger pSTAT5 activation in Treg than in Tconv (Fig. 3*C*), in keeping with previous reports (ref. 32 and references therein). These results indicate that there is complex variability in the sensitivity of Treg and Tconv cells to cytokine signals but that there is no overall Treg unresponsiveness, as might have been suggested by the low TCR-induced responses.

Origin of the Dampened TCR Signals in Treg Cells. We next attempted to track the root of different signaling downstream from the TCR in Tconv and Treg cells. We considered as plausible candidates

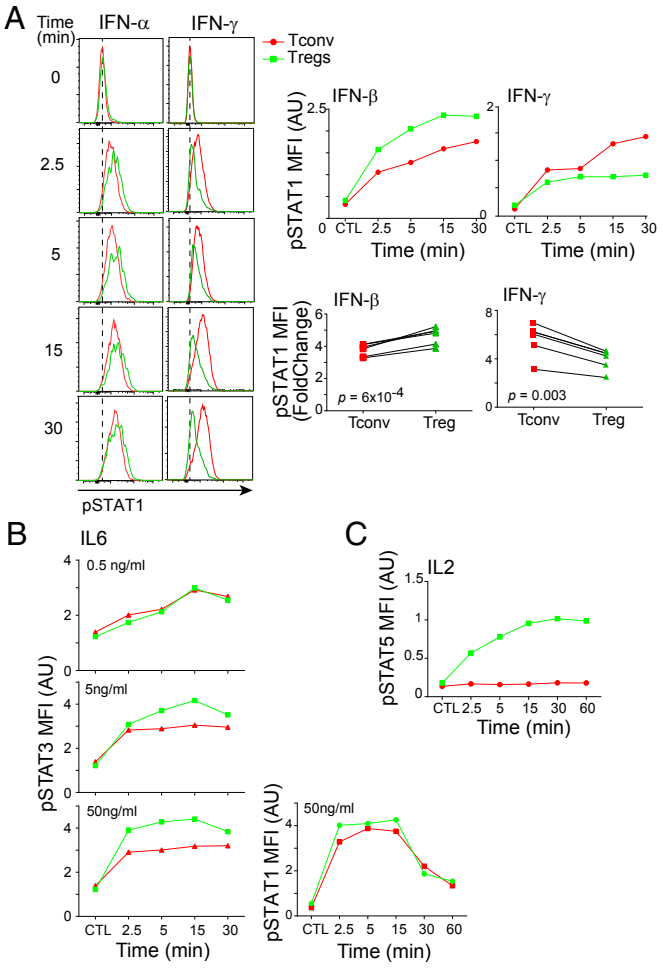

Fig. 3. Robust cytokine signaling in Treg cells. (*A*) pSTAT1 responses in Tconv and Treg cells stimulated with IFN-α (50 U/mL) or IFN-γ (50 ng/mL). (*Bottom Right*) Combined results from five independent experiments, shown as pSTAT1 fold change (MFI at 15 min/MFI at baseline). (*B*) Responses in Treg and Tconv to IL6, as pSTAT3 (*Left*) or pSTAT1 (*Right*) through different doses of IL6. (*C*) pSTAT5 response to IL2 (200 U/mL).

several inhibitory molecules known to be overexpressed in Treg cells, likely as a form of negative feedback induced by the frequent self-reactivity of their TCR. The first candidate was CD5, a cell-surface protein of the "scavenger receptor cysteine rich" family, with a large cytoplasmic domain that acts as a modulator of antigen receptor signaling in T and B lymphocytes, in part by recruiting negative regulators such as SHP1 (reviewed in ref. 33). The amount of CD5 displayed on the cell surface is proportional to the intensity of signals received through the TCR, and CD5 is robustly expressed on Treg cells (34, 35). To test whether CD5 more specifically inhibits signaling in Treg cells, we analyzed Erk phosphorylation after CD3/CD28 cross-linking across five matched ranges of surface CD5 (Fig. 4*A*), which revealed interesting cell specificity. For $CD8^+$ T cells, the relation between CD5 and Erk activation was very marked, with quasi-complete absence of pErk in cells with the most CD5. For $CD4^+$ Tconv, in contrast, CD5 seemed to have much less relevance, and strong responses were observed at essentially all levels of CD5. Treg cells were somehow intermediate, with some relation between Erk response and CD5 levels, albeit not as strong as in $CD8^+$ T cells. It was clear from this comparison, however, that the Treg/Tconv differential response could not be explained by CD5, as it was still observed between cells of matched CD5 levels (Fig. 4*A*).

CTLA-4 and PD-1 are coinhibitory receptors that negatively regulate T-cell activation, if by means that are not completely established (36–38). Both are overexpressed in Treg cells. Because their deletion or blockade leads to broad and potentially confounding T-cell activation and lymphoproliferation, we constructed mixed bone marrow chimeras by mixing equal numbers of congenically marked bone marrow from WT (CD45.1) and *Ctla4*- or *Pdcd1*-knockout mice (CD45.2) before transfer into irradiated hosts. In the resulting chimeras, the WT component effectively prevents disease. Eight weeks after transplantation, splenocytes were challenged as above. The activation profiles for p-Erk1/2 or p-CD3ζ proved to be essentially superimposable for WT or deficient cells (Fig. 4*B*), indicating that these coinhibitory receptors do not affect TCR-mediated signals in this system and that their higher expression in Treg cells is unrelated to the lower signaling intensity.

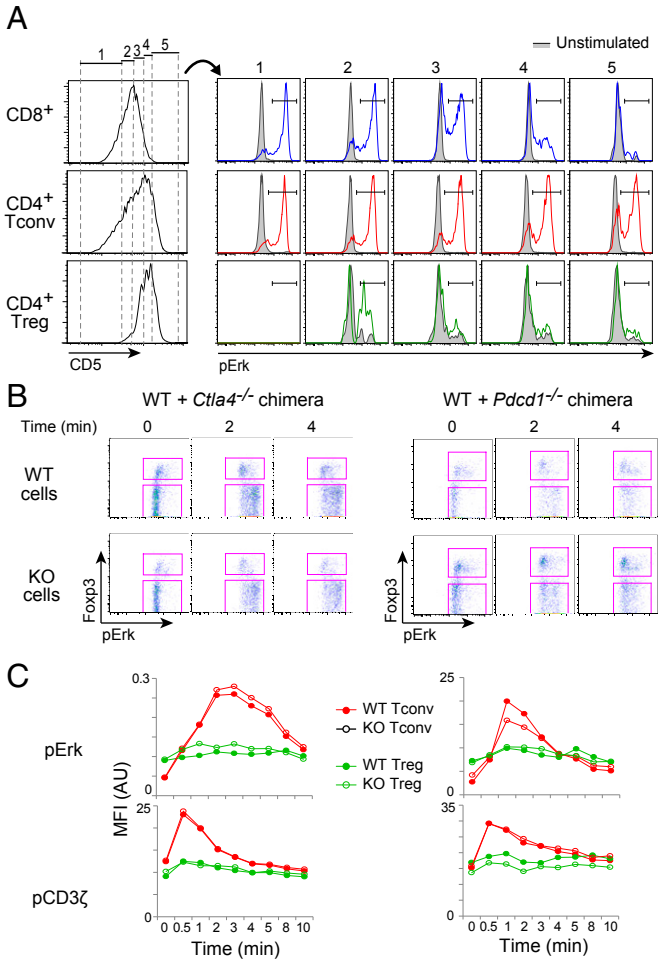

Fig. 4. Origin seeking of the dampened TCR signals in Treg cells. (*A*) pERK responses conditioned on different levels of CD5 (*Left*) in $CD8^+$ T cells and Tconv and Treg cells after anti-CD3/CD28 cross-linking (representative of three independent experiments). (*B*) Responses to anti-CD3/CD28 cross-linking in $CD4^+$ T cells (Treg cells distinguished from Tconv by anti-FoxP3 staining, *y* axis) in mixed hematopoietic chimeras generated in $Rag1^{-/-}$ recipients with an equal proportion of WT and $Ctla4^{-/-}$ (*Left*) or $Pdcd1^{-/-}$ (*Right*) bone marrow; WT cells were distinguished with the CD45.1 congenic marker (representative of two mice in two independent experiments). (*C*) Quantitation of the pErk or pCD3ζ MFI in the experiment shown in *B*.

Dusp4 Modulates Specific Facets of Treg Function and Homeostasis. The last candidate we examined as a signaling regulator in Treg cells was the Y/T dual-acting DUSP4. Its best recognized substrate is pERK (39, 40), although the full breadth of its substrates is not well charted. *Dusp4* is strongly overexpressed in Treg cells, which might plausibly account for dampened TCR-induced signals in Treg cells. We thus analyzed responses in cells from *Dusp4*-deficient mice (41), in which Treg cells are present in normal proportions and which have no autoimmune or immune dysregulation manifestations. Contrary to our hypothesis, ERK phosphorylation dynamics in *Dusp4*-deficient T cells proved essentially superimposable to those of cocultured WT cells, for both Treg and Tconv (Fig. 5*A*).

However, because the strong bias in *Dusp4* expression made it interesting to track its putative targets, we pursued this analysis by generating gene expression profiles of Treg cells purified from these *Dusp4* KO mice, in comparison with those of cohoused B6 mice. The profiles were very similar, with a distribution of Treg signature genes that was mostly in the normal range, save for a small subset of transcripts that were underexpressed in $Dusp4^{-/-}$ Treg cells (22 genes, at fold change and *t* test *P* value thresholds of 1.5 and 0.05, respectively; Table S1). These included several markers typical of activated or tissue Treg populations, such as *Klrg1* or *Il1rl1* (which encodes the Il33 receptor). Several groups have analyzed the homeostasis of Treg cells in which expression of the TCR was terminated by Tamoxifen-controlled germ-line engineering in mature Treg cells (15, 16) and identified a set of Treg signature genes whose expression is dependent on the continued presence of the TCR. Interestingly, the set of DUSP4-sensitive genes identified here showed stronger dependence on TCR signals than the average [Fig. 5*C*; Kolmogorov–Smirnov $P = 10^{-8}$ and 10^{-9}, respectively, in the data of Levine et al. and Vahl et al. (15, 16)]. This overlap indicated that DUSP4, perhaps paradoxically, positively modulates the transcriptional consequences of TCR signals in Treg cells.

To better understand the role of *Dusp4* in Treg homeostasis, we constructed mixed hematopoietic chimeras, mixing congenically marked bone marrow from WT and KO donors. In most lymphoid compartments, the proportion of Treg cells among $CD4^+$ T cells was equivalent (and the ratio of cells from both donors equivalent across Treg and Tconv compartments), with the exception of the colon, in which *Dusp4*-deficient Treg cells seemed at a competitive disadvantage (Fig. 5*E*; in unmanipulated *Dusp4*-knockout mice, this defect was not as apparent). We also assessed the impact of DUSP4 on iTreg generation, with cocultures in which naïve $CD4^+$ T cells of WT and KO origin were activated in the presence of IL2 + TGFβ. Equivalent responses were observed for WT and *Dusp4*-deficient cells in those conditions (Fig. 5*F*).

Discussion

We have investigated the differential activation of TCR signaling cascades in Tconv and Treg cells and sought the origin of the difference. The results indicate that, relative to Tconv counterparts,

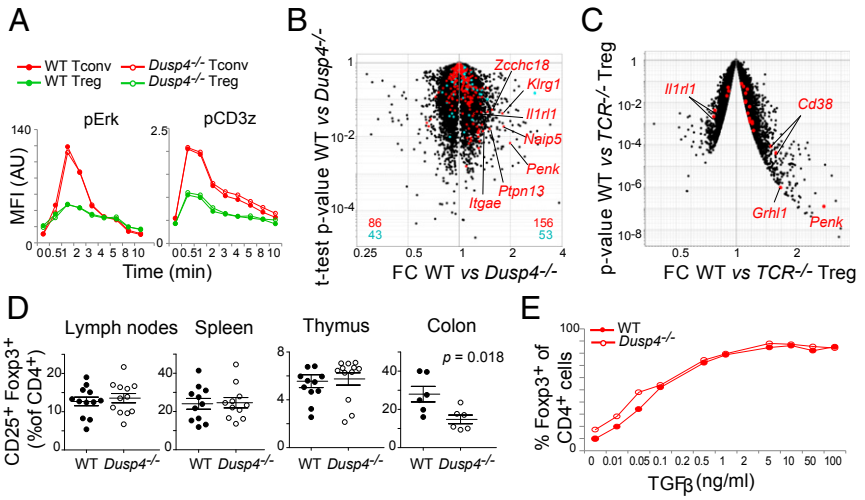

Fig. 5. Treg phenotypes in *Dusp4*-deficient mice. (*A*) pERK and pCD3ζ responses to anti-CD3/CD28 cross-linking in Tconv and Treg cells from *Dusp4*$^{-/-}$ or WT control mice, mixed in the same reaction tube (representative of three independent experiments). (*B*) Volcano plots (fold change vs. *t* test *P* value) comparing gene expression in WT Treg versus *Dusp4*$^{-/-}$ Treg cells. Treg signature genes are highlighted in red (induced) or blue (repressed). (*C*) Volcano plot comparing gene expression in TCR+ and TCR-deleted Treg cells [data from Levine et al. (16)], where red highlights are Dusp4-sensitive transcripts in the present data (defined as fold change > 1.5 and *P* value < 0.01, per *B*) (*D*) Frequencies of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in different organs of mixed hematopoietic chimeras from mixed WT and *Dusp4*$^{-/-}$ bone marrow. Data are pooled from two to four independent experiments; each dot is an individual mouse. (*E*) Naive CD4$^+$ T cells from spleens of WT and *Dusp4*$^{-/-}$ mice were sorted and stimulated with anti-CD3/CD28 beads in the presence of 20 U/mL IL2, with graded concentrations of TGF-β for 5 d (representative of two independent experiments).

Treg cells have a broadly dampened activation of several pathways and signaling nodes upon TCR-mediated activation. In contrast, Treg cells were very responsive to several cytokines, painting a picture of a cell population more attuned to generic intercellular communication cues than to antigen-driven signals, in line with the relative dispensability of their TCR (15, 16).

The difference in signaling efficacy appears to start from the apex of the signaling chain, manifest at the level of CD3ζ phosphorylation, and radiate from there along several signaling pathways. In a previous study, we showed ineffective activation of activation modules after TCR triggering in Tconv cells from NOD mice, with computational analysis highlighting an amplification of small initial differences as the signal propagates down the signaling cascades (24). Here, the more robust differences were also detected at downstream nodes (pS6, pErk, Ca++ flux), suggesting that, in this respect, Treg cells resemble NOD Tconv cells. One caveat, related to the functional consequences of these observations, is that it is not clearly known how the differences in protein phosphorylation actually translate into transcriptional or other cell activation readouts, whether the signaling dynamics are linear or whether increased proportions of phosphorylated intermediates are immaterial once a threshold has been reached. Indeed, the range and intensity of early transcriptional responses to anti-CD3/CD28 cross-linking are not grossly different in Treg and Tconv cells (42).

Although their repertoire is in part molded by interaction with self, Treg cells also partake strongly in modulating responses to microbial and environmental nonself, as during responses to infection (43). In this context, one might speculate that the signaling imbalance observed here allows initial responses to develop more effectively, as naïve Tconv would be more sensitive to TCR-mediated activation by foreign antigens, which Treg cells would comparatively ignore. Additionally, the continuous activation of Treg cells could be harmful to the host.

Much, albeit not all, of the dampened signaling in Treg cells could be ascribed to their higher proportion of CD44^{hi} effector/memory cells (28–30). Signaling cascades are less readily activated via the TCR in such cells (27), and this distinction also applies to Treg cells. The likeliest interpretation is that prior TCR engagement, by cognate antigen for effector/memory cells or by self-antigen for Treg cells, engages one or more of the negative feedback loops that tune TCR signals. We attempted to identify the molecular nature of this putative negative regulator. It seemed unlikely to be THEMIS (44), because it strongly underexpressed in Treg cells. SOCS1 also seemed an unlikely candidate, even though it is required for Foxp3 expression and Treg specification (45), because SOCS1 overactivity would dampen responses to IFN, which was clearly not the case. The coinhibitory receptors PD1 and CTLA4 are overexpressed in Treg cells, and some reports have suggested an effect of CTLA4 on signal transduction (46), but this was not observed in our experimental system, where adventitious effects of generalized inflammation due to the mutations were avoided by the mixed chimera context. Similarly, the inhibitory phosphatase DUSP4, known to dampen Erk and other kinases, could not account for Treg hyporesponsiveness, although this exploration did allow us to define an interesting set of Treg signature genes whose expression was enhanced by DUSP4.

Consistent with the correlation between CD5 expression and TCR signal strength, expression of CD5 is comparatively high in Treg cells (35, 44). A recent study showed that CD5 facilitates the extrathymic differentiation of pTreg cells by blocking mTOR-dependent signals induced by effector-differentiating cytokines that otherwise inhibit Treg cell induction (35). However, our results showed that Treg/Tconv differential response was not due to CD5 expression. On the other hand, we noted an interesting diversity in the relationship between CD5 levels and signaling strength. Most sensitive were CD8$^+$ T cells, whose degree of Erk activation seemed highly dependent on CD5 levels, whereas signals in CD4$^+$ Tconv cells seemed far less dependent. This divergence may denote differential recruitment of CD5 to the signaling synapse in these lineages or the association between CD5 and Lck, perhaps more impactful in CD8$^+$ T cells as CD8 does not associate directly with Lck, as CD4 does.

In conclusion, we have demonstrated that Tconv and Treg cells respond with different balances to TCR and cytokine stimulation, with Treg cells being more attuned to cytokine- than to TCR-driven cues.

Materials and Methods

Mice and Flow Cytometry. Animal experiments were performed under Harvard Medical School Institutional Animal Care and Use Committee-reviewed protocol 02954. C57BL/6J, B6.CD45.1 congenic, and B6;129-*Dusp4*^{tm1Jmol}/J mice (stock 23671) were obtained from the Jackson Laboratory. B6.*Foxp3-Cd90.1* mice were

kindly provided by Alexander Y. Rudensky, Memorial Sloan Kettering Cancer Center, New York (25). Mice were maintained in specific pathogen-free facilities at Harvard Medical School (Institutional Animal Care and Use Committee protocol 02954). *Ctla4*- and *Pdcd1*-deficient mice (B6 background) were a kind gift from P. Sage and A. Sharpe, Harvard Medical School. For Treg analysis, single-cell suspensions from lymphoid organs were stained with antibodies against TCR-β, CD4, CD8 (Biolegend), and FoxP3 (eBioscience) and analyzed on BD LSRII, and data analysis was performed with FlowJo software (Tree Star).

TCR Activation and Phospho-Flow Cytometry. Spleen cell suspensions from Foxp3-Thy1.1 or chimeric mice were stimulated in medium containing 6 μg/mL biotinylated anti-CD3ε and anti-CD28 stimulatory antibodies and incubated for 2 min at 37 °C before the addition of 24 μg/mL streptavidin. At various times after cross-linking, the stimulation was stopped by the addition of paraformaldehyde to 2% (wt/vol) (room temperature for 20 min). For stimulation of BDC2.5 T cells, BDC2.5 mimotope peptide (Peptide 2.0 Inc.) was added to 100 ng/mL PFA. Fixed cell suspensions were permeabilized by adding ice-cold 100% methanol slowly to prechilled cells, while gently vortexing (1 mL per sample). After overnight incubation at –20 °C, cells were washed with staining buffer and stained with antibodies against TCR-β, CD4, FoxP3, p-Erk1/2 (Y204), p-S6 (Ser235/236) (Cell Signaling Technology), p-CD3ζ (Y142), or p-SLP76 (Y128) (BD Biosciences).

For cytokine responses, cells were incubated at 37 °C with recombinant murine INFγ (0.5–50 ng/mL; R&D Biosciences), INFα (50 U/mL; PBL), INFβ (50 U/mL; PBL), IL6 (0.5–50 ng/mL; Biolegend), and IL2 (200 U/mL; Peprotech). After stimulation, cells were immediately fixed with paraformaldhyde (2% final concentration) for 20 min at room temperature, permeabilized with cold 90% methanol (14 h, –20 °C), washed, and stained with phospho-specific antibodies to p-STAT5 (pY694; BD), pSTAT3 (pY705; BD), and p-STAT1 (pY701; BD).

In Vitro Conversion Assay. Naïve T cells were activated with anti-CD3/CD28–coated beads (Invitrogen) at a concentration of one bead per cell in the presence of 20 U/mL of recombinant human IL2 (Proleukin; Chiron) and graded doses of recombinant TGF-β (PeproTech).

Mixed Bone Marrow Transfers. Total bone marrow cells (1×10^6) were isolated from WT (CD45.1), *Ctla4*, or *Pdcd1*-deficient mice (CD45.2) and depleted of mature T cells by negative selection of Thy1.2$^+$ cells (Miltenyi Biotech). The two populations were mixed at a 1:1 ratio and were i.v.-transferred into irradiated (1,000 rad) RAG-1$^{-/-}$ mice (6–8 wk of age). Four weeks after irradiation, antibiotic treatment was stopped until flow cytometric analysis at 6–8 wk of age.

Gene Expression Analysis. RNA was prepared (in biological duplicates) as described (47) from WT-*Dusp4* chimera Treg cells. For microarray analysis, RNA was labeled and hybridized to GeneChip Mouse Genome M1.0 ST chip arrays. Raw reads were processed with Affymetrix software, and data were normalized using the robust multipoint averaging algorithm in GenePattern software package. Data were analyzed with the "Multiplot" module from GenePattern.

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Supporting Information

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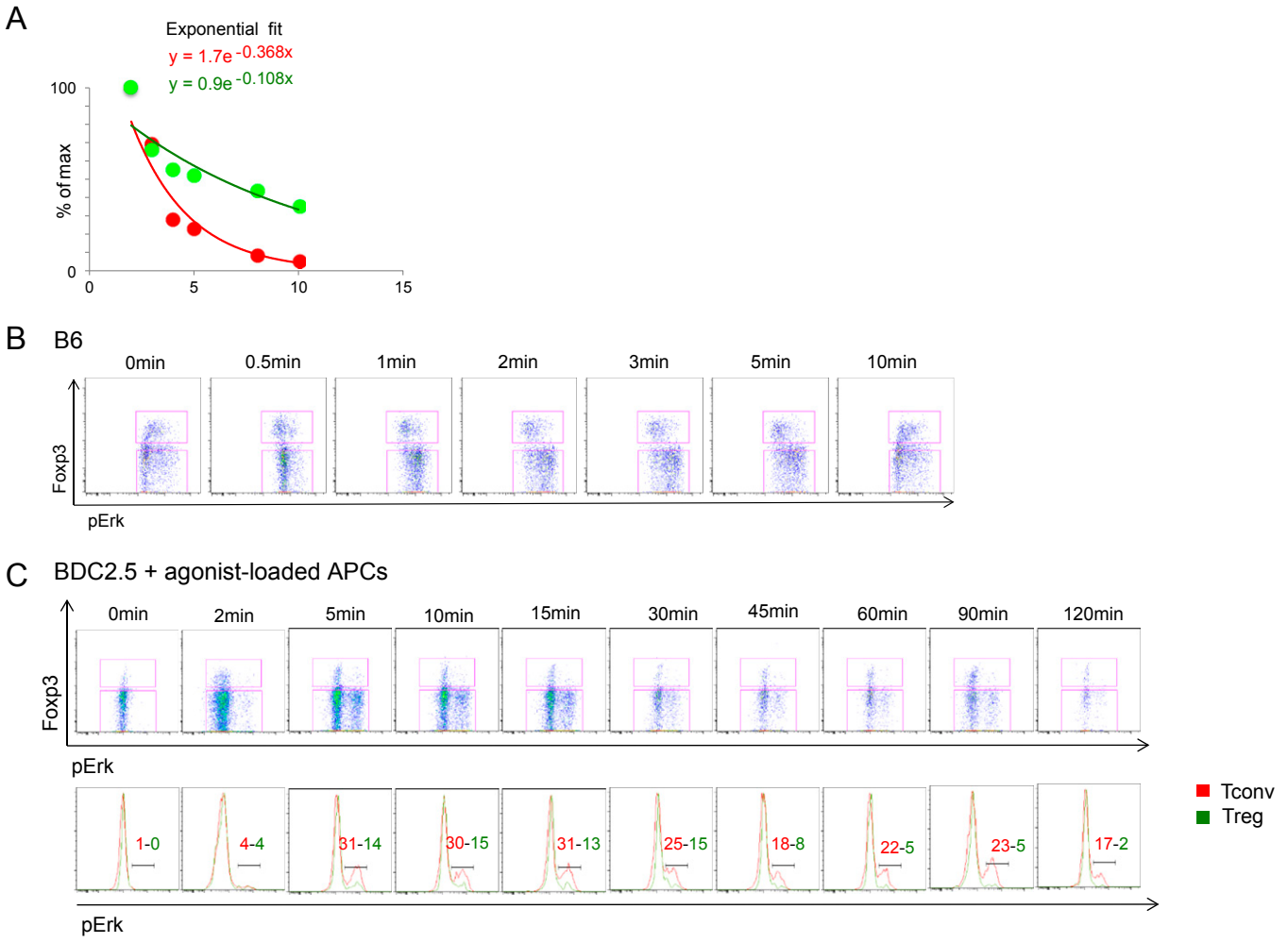

Fig. S1. (*A*) Computing the pErk signal off-rates (Fig. 1*C*) via an exponential fit. (*B*) Flow cytometry data for induced pErk at different time points after cross-linking of CD3 and CD28 in Tconv and Treg cells from B6 mice (representative of five independent experiments). (*C*) Plot and histogram of CD4+ T cells at different time points after BDC2.5 mimotope agonist peptide stimulation from BDC2.5 TCR transgenic mice (representative of four independent experiments).

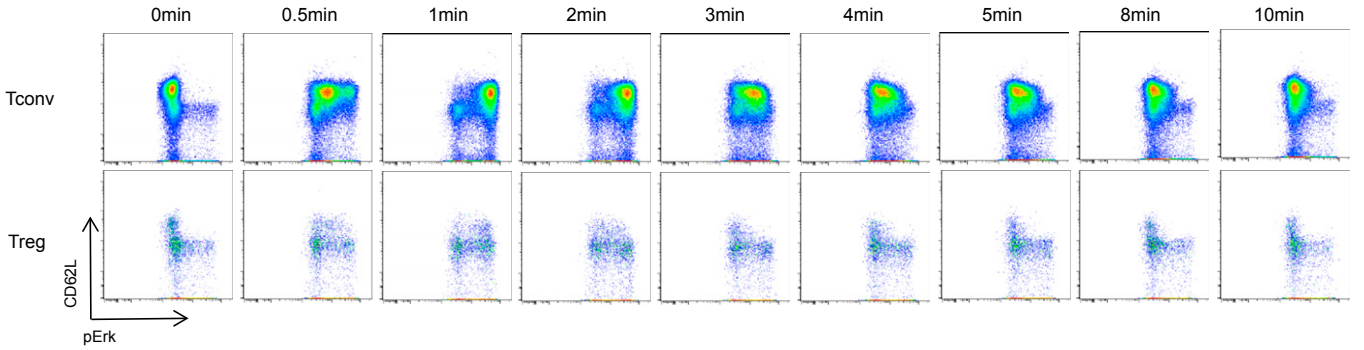

Fig. S2. Flow cytometry data for induced pErk at different time points after cross-linking of CD3 and CD28 in Tconv and Treg cells versus CD62L level (representative of three independent experiments).

Table S1. Dusp4-induced and -suppressed genes in Treg cells

Probe set ID	Gene symbol	WT no. 1	WT no. 2	Dusp4 KO no. 1	Dusp4 KO no. 2	Fold change WT/Dusp4 KO	*P* value	Fold change Treg/Tconv
Induced*								
10353450	Gm4956	1,306	1,151	60	65	19.65	0.00062	9.30
10425321	Apobec3	2,176	1,938	386	363	5.49	0.00149	1.70
10357986	Rab6b	638	468	104	97	5.44	0.00871	1.50
10345436	LOC280487	270	279	71	72	3.86	0.00017	0.99
10459066	Gm4841	302	219	82	69	3.42	0.02095	1.91
10430649	Cbx7	1,629	1,686	555	559	2.97	0.00026	1.48
10357488	Cd55	541	514	168	197	2.90	0.00615	0.52
10565994	Art2b	3,326	3,209	1,291	1,006	2.87	0.01406	1.37
10430647	D730005E14Rik	507	427	157	174	2.81	0.00909	1.59
10578361	Asah1	406	421	141	176	2.63	0.01339	1.02
10366546	Cpm	669	557	284	231	2.39	0.02459	0.94
10444821	H2-Q8	2,656	2,138	1,002	1,090	2.28	0.01937	1.44
10363070	Gp49a	475	382	183	200	2.23	0.02090	2.59
10511258	Fam132a	350	336	150	159	2.22	0.00200	1.42
10579347	Ifi30	380	371	185	161	2.18	0.00842	0.96
10491952	Mgst2	340	288	148	154	2.07	0.01353	1.24
10476939	Gm4979	670	690	338	322	2.06	0.00165	1.35
10523595	Ptprv	343	276	160	149	2.00	0.02619	1.02
10511363	Penk	3,368	3,533	1,648	1,825	1.99	0.00665	12.97
10573430	Gadd45gip1	776	770	461	344	1.94	0.04499	1.21
10565735	A630091E08Rik	168	190	92	100	1.86	0.01460	4.20
10411611	Naip5	185	218	112	108	1.83	0.01858	4.77
10572235	Lpar2	228	195	123	112	1.80	0.02298	1.18
10547590	Klrg1	1,988	1,719	1,140	933	1.79	0.04234	13.30
10455954	Gm4951	299	276	163	159	1.78	0.00530	2.36
10490246	Gm14326	1,019	1,051	563	606	1.77	0.00462	1.34
10440131	Gpr15	335	318	206	174	1.73	0.02476	1.06
10565775	Dgat2	121	138	83	68	1.72	0.04539	1.44
10450682	H2-T23	2,564	2,413	1,523	1,391	1.71	0.01015	1.37
10363082	Lilrb4	560	507	318	305	1.71	0.00994	2.87
10515690	Wrn	301	283	176	169	1.69	0.00472	1.08
10444824	H2-Q6	4,753	4,475	2,664	2,842	1.68	0.00724	1.49
10574023	Mt2	244	265	144	161	1.67	0.01785	1.49
10450675	H2-T24	692	648	439	369	1.66	0.03201	0.91
10573483	Prdx2	1,319	1,338	843	792	1.63	0.00432	1.78
10572449	Lsm4	1,482	1,491	931	909	1.62	0.00070	1.20
10519857	Hgf	102	103	63	66	1.59	0.00318	1.89
10406614	Mtx3	148	165	104	93	1.59	0.02722	0.76
10507137	Pdzk1ip1	305	283	189	182	1.59	0.00811	1.29
10534253	Gtf2ird1	212	182	128	120	1.58	0.03121	1.00
10588283	Szt2	127	105	73	74	1.57	0.04060	0.97
10355246	Acadl	377	389	247	242	1.57	0.00161	0.98
10555041	Alg8	409	380	257	247	1.56	0.00903	0.98
10360373	E030037K03Rik	636	550	401	363	1.55	0.03868	0.71
10384458	Plek	173	170	116	106	1.54	0.00942	1.10
10580160	Mri1	393	402	253	263	1.54	0.00250	1.09
10430626	Npcd	186	200	123	129	1.53	0.01069	1.45
10579602	B3gnt3	127	150	88	94	1.52	0.04199	1.19
10356461	Hjurp	488	455	334	290	1.52	0.03421	0.92
10450694	H2-T22	1,008	947	705	591	1.51	0.04697	1.31
10356999	Ptpn13	379	415	257	270	1.51	0.01602	2.88
Suppressed†								
10572741	Olfr372	112	120	286	418	0.33	0.0296	1.51
10560719	2210010C17Rik	353	296	656	759	0.46	0.0205	1.17
10458589	Prelid2	182	164	307	282	0.59	0.0155	1.36
10458033	Stard4	184	207	343	334	0.58	0.0119	1.08
10518364	Rps19-ps3	139	151	249	243	0.59	0.0068	0.99
10450723	H2-T10	493	451	1,134	1,027	0.44	0.0064	1.11
10362674	Rnu3a	536	581	1,034	1,019	0.54	0.0045	1.43
10572591	Ocel1	309	290	678	624	0.46	0.0043	1.12
10367744	Ust	170	153	1,409	1,304	0.12	0.0010	0.33
10571274	Gsr	372	366	648	655	0.57	0.0003	1.13
10579070	Zfp868	170	169	304	303	0.56	0.0000	0.90

*Expression values and fold change of transcripts that are differentially represented in WT versus Dusp4 KO Treg cells (at a fold change > 1.5 and *P* value < 0.05).
†Expression values and fold change of transcripts that are differentially represented in WT versus Dusp4 KO Treg cells (at a fold change < 0.6 and *P* value < 0.05).