The meta-Treg signature generated in silico is centered on IL-2, members of the TNF receptor superfamily and the endogenous opioid pathway

Gilles MARODON, PhD

Sorbonne University, INSERM, CNRS, Centre d'Immunologie et de Maladies Infectieuses (CIMI-PARIS), Paris France

correspondence to gilles.marodon@upmc.fr, Sorbonne University, CIMI-PARIS, 91 Bd de l'Hopital, 75013 PARIS

Funding: no extramural financial support was obtained for this work

Abbreviations: Treg, Regulatory T cells; Tconv, Conventional T cells; TNFRSF, Tumor Necrosis Factor Receptor Super Family; MTS, Meta-Treg Signature; TF, Transcription Factors; MENK, Met-Enkephalin; PPI, Protein-Protein Interactions

Keywords: Bioinformatics, Mice, Treg, TNFRSF, IL-2, proenkephalin, met-enkephalin
Abstract

Regulatory T cells (Treg) are crucial in the proper balance of the immune system. A better knowledge of Treg-specific genes will extend our knowledge on their complex biology. However, to date there is no consensual Treg signature in the literature. Here, we extracted a list of 72 genes differentially expressed in Treg compared to CD4⁺ conventional T cells across 6 different but comparable publicly available datasets. A simple network analysis confirms that Foxp3 was a central node of the meta-Treg signature. A third of the genes from this in silico-generated signature interacted with IL-2, confirming the central role for this cytokine in Treg biology and the validity of our approach. When projected into a quantitative gene expression database, many genes of the meta-Treg signature were also expressed by other immune and non-immune cell subsets, with the noticeable exceptions of Foxp3, Ctl4, Tnfrsf4 and Tnfrsf9 and surprisingly, the pro enkephalin (Penk) gene. Subsequent bioinformatic analysis of available datasets indicated the molecular mechanisms that could explain specific Penk expression in Treg. Altogether, our results show that the meta-Treg signature concerns a very limited set of genes centered on the IL-2 family, members of the TNF receptor superfamily, and the endogenous opioid pathway.
Introduction

Proper number and function of regulatory T cells (Treg) are essential for a well-balanced immune system: too few of these cells leads to autoimmunity and too much prevents an efficient immune response, with harmful consequences for anti-tumor immunity, for instance. Treg are a subset of CD4+ T cells that express the transcription factor (TF) Foxp3 and the alpha chain of the interleukin-2 receptor CD25, both indispensable for suppressive functions and proper homeostasis. Probably the best example illustrating the crucial role for Foxp3-expressing cells in the homeostasis of the immune system is given by the lethal auto immune syndrome seen in patients bearing mutations in the FOXP3 gene, the IPEX syndrome [1]. Like most CD4+ T cells, Treg are generated in the thymus upon MHC-driven selection based on affinity of the T cell receptor for self antigens [2]. A sizable proportion of those cells are also induced in the periphery (pTreg) from CD4+ T cells precursors, but those pTreg are reported not to express Helios or Neuropilin-1, contrary to thymic-derived Treg (tTreg) that are positive for those markers [3]. Known pTreg inducers in the periphery can be byproducts of bacterial metabolism [4,5] but it is likely that inflammation per se is a main driver for pTreg differentiation [6]. Thus, finding ways of manipulating Treg for therapeutic purposes in the auto immunity field has become a major endeavor for immunologists worldwide. Moreover, more recent results linking the presence of Treg to a bad prognostic in some cancers extended their potential clinical applications from autoimmunity to cancer immunotherapy [7]. In the case of cancer, one would want to get rid of Treg to wake up a dim immune response to tumors. An example of this powerful approach has been recently illustrated by Treg-depleting CTLA-4 or CD25-specific mAb [8,9]. However, this weak response to tumors is part of a natural tolerance process, preventing the immune system to attack self-tissues. Thus, breaking immune tolerance by removing Treg is not without consequences on the integrity of healthy tissues. This is illustrated by studies showing that Ipilimumab (anti-CTLA-4), a powerful anti-cancer drug affecting Treg, indeed help the immune system to fight tumors at the expense of a generalized auto immunity in treated patients [10]. A better knowledge of Treg biology will be crucial for preserving therapeutic efficacy without severe adverse events.

This knowledge has been mostly collected from mice due to their ease of use, their versatility and the thousands of genetic models available to answer mechanistic questions. To that end, hundreds of investigators have pursued the quest for specific Treg markers, revealing molecules and pathways potentially targetable by monoclonal antibodies or pharmacological compounds. However, there are still confusions about these specific markers, since the comparisons are often made across unrelated studies employing different technologies. Moreover, a characteristic of Treg is their ability to
adapt to the cellular environment in which they are present. This mechanism, referred as to effector class control few years ago [11], deeply affect cell surface phenotype and thus presumably, gene expression patterns as well. In addition, there are evidences that pTreg, contrary to tTreg, might be relatively unstable [12], meaning that pTreg may acquire some effector functions. One can immediately realize that providing a common definition of Treg across this diversity of phenotype and function represents a difficult challenge. Furthermore, Treg specific markers should ideally mark Treg only or should be minimally expressed by other cells of the immune system or other non-immune cells. This has been an overlooked issue since most of the so-called “Treg signatures” are established relative to CD4+ T conventional cells (Tconv), and not looking outside of Tconv (not mentioning major phenotypic differences used to define Treg and Tconv). Safety and efficacy of Treg-based therapies will surely benefit from targeting molecules and/or pathways truly specific to Treg.

In an attempt to resolve some of these issues, we reasoned that digging out a meta-Treg signature (MTS) from available datasets comparing Treg and Tconv should lead to a more robust signature than studies taken separately. Projection of this qualitative signature in quantitative databases reporting normalized gene expression level across multiple cell types should also inform us on the specificity of the MTS beyond Tconv. As a starting point, we decided to focus our investigation in Mus Musculus, because many databases and tools are available in mice. We also focused our analysis in resting Treg freshly isolated ex vivo, taken from non inflammatory settings, to avoid bias due to T cell activation. This naive approach led to the first in silico description of a “universal” Treg signature.
Methods

Hardware and software informations

All the analyses in this report were performed using a MacBook Pro mid-2012 (MACOS 10.14.4) with 8Go of RAM and a 2.5Ghz Intel Core i5. The report was written with LibreOffice v6.1.3.2 and Mendeley 1.19.4. Figures were mounted on LibreOffice from panels extracted from the indicated websites. In some instances, Prism v7 (GraphPad Inc) was used.

Extraction of the Meta-Treg signature

The datasets used were selected based on a “Treg* AND (Tconv* OR Teff*) AND Mus Musculus” search in the GEO dataset web site (https://www.ncbi.nlm.nih.gov/gds). GEO datasets were manually inspected for inclusion of studies comparing fresh Treg with fresh Tconv from lymphoid organs. Only GSE17580 [13], GSE24210 [14], GSE37532 [15,16], GSE40685 [17], GSE42021 [18], and GSE7852 [19] were selected and a list of genes significantly up or down regulated in Treg compared to Tconv was determined for each dataset using GEO2R with an adjusted p.value cutoff of 0.05 (FalseDiscoveryRate). On average, 687 (range: 338-1581) genes were isolated using this threshold. The commonality within these gene sets was determined using the online tool from the Bioinformatics Evolutionary Genomics department form the Ghent University (http://bioinformatics.psb.ugent.be/webtools/Venn/). All but one dataset (GSE37532) where the [MoGene-1_0-st] Affymetrix Mouse Gene 1.0 ST Array was used, were generated using the [Mouse430_2] Affymetrix Mouse Genome 430 2.0 Array.

Analysis of the MTS

For representing the relations between genes of the MTS, we used Cytoscape v3.7.1 [20] (https://cytoscape.org) with the Spring application and the attached Enrichment analysis for GO terms [21], establishing a network of protein-protein interactions. It should be stressed that these interactions are not necessarily physical but represent regulation or indirect association, manually curated from the literature. The Treg signature from ImmGen (http://immgen.org) was obtained using the Population Comparisons tab from the Data Browsers window. We gathered quantitative data on the expression of the MTS genes in immune and non immune cell subsets using the MyGeneSet tab. Analysis of ATAC-Seq was done using the Chromatin tab. Expression of the Oprd1 gene was assessed using the Gene Skyline tab with RNA Seq data. Expression of Penk in various studies is represented by the graphical tool embedded in GEO2R. Ingenuity Pathway Analysis (Qiagen) was used to investigate upstream cytokine regulators linked to genes of the MTS.
Results

As a starting point, we searched for a common Treg signature (that is a list of genes differentially expressed in Treg compared to conventional T cells (Tconv)) across publicly available datasets retrieved from the GEO web site. We manually selected 6 datasets based on various subjective criteria, and availability of the GEO2R analytical tool for the dataset. We deliberately limited the search to cells isolated from peripheral lymphoid tissues to avoid any bias due to potential tissue specificity of Treg or Tconv genetic profiles. Among the 6 datasets, 4 had been performed in lymph node cells, 1 in spleen and 1 in the bone marrow, 5 had been performed in C56BL/6 (females and males) and one in BALB/c mice. Various ages were present in the datasets (range: 6-36 weeks-old). Various Treg sorting strategies were also used across the datasets, from classical CD25+ sorting to isolation of GFP+ cells from Foxp3-GFP transgenic mice. So we believe that the 6 chosen datasets were representative of typical Treg profiles found in many studies.

The intersection analysis of differentially expressed genes from these datasets is given as a supplemental table (Table S1): 71 genes were found to be common to all 6 datasets whereas a total of 194 genes were found in at least 5 datasets. Because we were stringent on the initial choice of our 6 datasets, we retained the list of genes common to all 6 datasets, establishing a Meta-Treg signature (MTS). The MTS genes either enriched or diminished in Treg relative to Tconv is represented in Fig. 1A). Well-known Treg markers, such as il2ra (CD25), Ikzf2 (Helios), Ikzf4 (Eos), Il2rb (CD122), Socs2, Nrp1, Ebi3 or Ctla4 were present in the MTS.

To gain insight into biological functions associated with the MTS, we generated a putative network of proteins-proteins interactions (PPI) from the list of Treg-enriched genes present in the MTS (Fig. 1B). A large number of members of the MTS were not connected to any other member of the list, suggesting that the MTS did not define a particular signaling or metabolic pathways or biological process. Those genes were thus excluded from the representation. Significant interactions above randomly expected ones (PPI enrichment p.value <10^{-15}) were observed for well established members of the Treg signature (Fig. 1B). A central node of PPI was composed of Foxp3, Ctla4, Il2ra, Il2rb, Tnfrsf4 (CD134, OX40), Tnfrsf9 (CD137, 4-1BB), Itgae (CD103), Ccr6, CD83, Klrg1, and Entpd1 (CD39) gene. A simple network analysis reveals that Foxp3 was the node most connected to other nodes of the network (degree), as depicted by the size of the node in the figure. The Foxp3 node had also the higher “betweenness” of the network, showing that most of the nodes directly connected to Foxp3. Those genes constituting the “core” of the MTS were associated with generic GO terms typical of activated cells and linked to regulatory biological processes (Fig. 1B). To establish the robustness of our MTS, we compared it to the one generated in the ImmGen database curated by the Immunological
Genome Project Consortium [22]. As expected, most of the MTS genes were found in the list of 906 DE genes provided by ImmGen using microarray v1 dataset (Fig 1D). Interestingly, 5 genes of our MTS were not present in the ImmGen Treg signature (Ybx3, Ncmap, Scamp1, Prg4, and Gucd1). To go further, we searched for possible upstream regulators of the MTS, that would be cytokines with known direct or indirect connections with genes of the MTS. Five cytokines had significant connections with many genes of the MTS (Fig. 2). Not surprisingly, IL-2 was the prime cytokine with connections to 26 genes of the MTS, a highly significant enrichment relative to a random set of genes. More surprising was that IL-4 and IFNg had also connections with genes of the MTS. IL-5 and IL-27 had fewer connections whereas TNF had connections with only 2 members of MTS, the TNFRSF, as expected. Thus, IL-2 might chiefly contribute at establishing the Treg signature but IL-4, IFNg, IL-5, IL-27 and TNF might participate as well.

Because of the good qualitative overlap with our MTS, we used the ImmGen ULI RnA Seq database to determine the expression levels of each genes of the MTS, giving the unique opportunity to quantitatively define “true” vs “shared” Treg markers across multiple immune and non-immune cell types (Fig. 3). There is two subsets of Treg in the Immgen dataset: one is labelled as 4_25hi-Sp and the other is referred as 4_FP3+_Nrplo_Co, isolated from the colon, and most likely representing pTreg. To be coherent with the MTS, mostly obtained from pTreg, we present the heatmap ordered according to expression levels of each gene of the MTS in pTreg, from high on the top to low on the bottom of the figure. Although the general hierarchy of expression was respected, we noticed several differences between the two Treg subsets for some genes of the MTS: Klrg1 expression was low in CD25+ Treg but high in pTreg whereas it was the contrary for Gpr83. Ift80 and Arhgap20 were highly expressed in CD25+ Treg but not in pTreg. However, those genes were poorly specific to Treg since similar levels of expression were observed in Tconv. A second observation was that the MTS extracted from microarrays well defined Treg when projected into the RNA Seq database: about half of the genes of the MTS positively defined Treg relative to Tconv (yellow/red in Treg, white/blue in Tconv) whereas the other half was a mix between genes negatively or not discriminating Treg from Tconv (yellow/red in Tconv, white/blue in Treg). With this representation (Fig. 3), we can then observed which genes truly defined Treg across all the subsets present in ImmGen: only few genes at the top of the list positively defined Treg when compared to the rest of the subsets. Not surprisingly, Ctla4 was a very specific one, basically expressed at higher levels in Treg than in any other subsets. More surprising was the presence of Ncmap at the top of the list but since it was not part of the overlap with the microarray data (Fig 1D), and since some expression can be seen in Tconv (Fig. 3), we cannot firmly conclude on Ncmap at this stage. The third gene of the list was Tnfrsf4 (CD134, OX40), with a pattern of expression resembling Ctla4 but with detectable expression in a subset of CD8+ dendritic
The fourth member of the list was *Penk*, a gene coding for the proenkephalin enzyme, responsible for production of Met-Enkephalins (MENK) peptides, belonging to the endogenous opioid family. However, expression of *Penk* was also detected in stromal cell types. Next was *Foxp3*, highly expressed in Treg, and not detected in any other subsets. *Tnfsrf9* (4-1BB) was next on the list, but its expression was detected in several other subsets, including NK, NKT, and γδ-T cells. The rest of the MTS was much less specific to Treg although their expressions remain enriched in Treg relative to Tconv. At the other end of the spectrum, few genes negatively defined Treg (that are weakly expressed in Treg relative to Tconv) but, as expected, low expression of those genes was not specific to Treg. Most B cells, for instance, were also negative for these markers. Thus, projection of the MTS into the ImmGen database led to expected and unexpected findings: (i) the MTS genes segregated Treg from Tconv but most of the genes were also expressed by other cells than Treg, (ii) some genes were highly specific to Treg, including *Ctla4, Foxp3*, members of the TNFRSF family and, more surprisingly *Penk*, a gene belonging to the endogenous opioid pathway.

Following on the surprising observation that *Penk* was a highly specific Treg genetic marker, we wanted to confirm this observation by other means. First, we undertook a survey of studies examining gene expression in Tconv and Treg in various tissues: *Penk* was found to be over expressed by Treg in the thymus [19], in the spleen [23] and LN [15,16] (Fig. 4A) and of several tissues, including fat [15,16], muscle [23] and tumors [24] (Fig. 4B), showing that *Penk* over expression by Treg is consubstantial of their generation and independent of their localization. We also independently verified over expression of *Penk* in Treg relative to Tconv in our own set of RNA-Seq data in non-tumor bearing mice (Fig. 4C). In tumor-bearing mice, we also observed massive up regulation of *Penk* mRNA in Treg of the tumor relative to the draining LN (Fig. 4C). Thus, *Penk* over expression by Treg was verified in 12 different microarray datasets and verified in-house by RNA Seq.

We then wanted to know more about the molecular mechanisms that may explain *Penk* over expression in Treg. Using ATAC-Seq data available in ImmGen, we dig out the chromatin profile of the *Penk* regulatory region in various subsets. There was an open chromatin region (OCR) near the TSS in Treg that was not observed in naïve CD4+ T cells, nor in naïve CD8+ T cells (highlighted in Fig. 5A). In contrast, this OCR was observed in stromal cells and in CD4+ T cells stimulated in vitro (Fig. 5A) in which *Penk* expression was also detected (Fig. 3). Thus, an OCR in the *Penk* promoter region was present specifically in cells permissive for *Penk* expression. We also noticed the presence of a CpG island located upstream of the TSS (the *Penk* gene is on the negative DNA strand). To investigate the possibility that *Penk* expression might be regulated by the methylation status of this region, we looked for *Penk* expression levels in Treg deficient for the DNA-methyl-transferase Dnmt1, the main driver of
epigenetic regulation in Treg [25]. Deficiency of Dnmt1 in Treg had no effect on Penk expression (Fig. 5B), suggesting that the CpG island of Penk is demethylated in wild-type Treg (a methylated state in wild-type Treg would have been associated with an increase in Penk gene expression in the absence of Dnmt1). Moreover, deletion of DICER, a master regulator of miRNA function in Treg [26], was associated with a considerable increase in Penk expression in Treg (Fig. 5B), suggesting that miRNAs repress Penk expression in Treg. Finally, we wanted to know whether the expression of Penk by Treg could have functional consequences relevant to the immune system. Indeed, the main ligand of Penk by-products (MENK peptides), the Oprd1 gene (coding for the opioid delta receptor-1) was expressed in CD4+ Tconv, and highly expressed in CD4+ and CD8+ dendritic cells (DCs) relative to other immune and non immune cell types (Fig. 5C). Interestingly, Oprd1 was also highly expressed by Vγ2+ T cells, a subset of innate γδ-T cells, known to be regulated by Treg in the intestine [27]. Thus, the main receptor for MENK was expressed by immune subsets known to dialog with Treg.

Finally, we wanted to investigate whether Penk over expression by murine Treg would also be observed in humans. The recent availability of the immune cell atlas (http://immunecellatlas.net) focusing on human cells provided a mean to that end although only few cell types and only from the blood are available for now. Remarkably, PENK expression was detected in activated Treg at much higher level than in Tconv, confirming our observation in total mouse Treg. However, Mucosal Associated Invariant T cells (MAIT) also expressed high levels of Penk (Fig. 5D). Similar to mice, high expression of the OPRD1 gene was observed in cells susceptible to interact with Treg (Fig. 5D). Thus, over expression of Penk mRNA in Treg relative to Tconv is apparently conserved in mice and humans
Discussion

To our knowledge, the present report is the first attempt to define a “universal” Treg signature in mice by a meta analysis of published and available datasets and provide a more solid appreciation of Treg-specific genes. Our unbiased approach confirm that the genes central to Treg identity revolves around well established family of genes belonging to the interleukin-2 family and emphasize the contribution of members of the TNF receptor super-family. Our gene ontology enrichment analysis confirms that the core set of genes of the MTS belongs to biological processes important for Treg function but also show that most of the genes of the MTS did not belong to any particular biological function. One point of caution for the interpretation of this latter observation is that mRNA expression may or may not be correlated with protein expression, and thus with biological functions. For instance, a recent study has found strong discrepancies between mRNA and protein levels in human Treg [28].

Our primary objective was to evaluate whether a true core set of genes really defined Treg across the whole diversity of cell types in the immune system and beyond. However, we found that most of the genes of the MTS were also expressed by other cells of the immune system. Thus, a “universal” Treg signature, reflecting hard wiring of Treg lineage at the gene expression level might not exist at all, a view supported by functional plasticity of Treg (effector class control), and possible acquisition of effector functions by Treg in some inflammatory conditions. Analysis of the MTS extend the notion that Treg lineage specification (that must be distinguished from Treg identity [29]) is mostly dictated at the epigenetic rather than at the gene expression level [30]. Nevertheless, a few genes stand out to be highly enriched in Treg beyond the comparison with Tconv. These were Cita4, Tnfrsf4 and Tnfrsf9 and obviously Foxp3, all confirmed to be highly expressed in Treg at the protein level. Thus, these molecules should probably concentrate the efforts for therapeutic targeting of Treg. The former is already in the clinics (Ipilimumab) with great efficacy to affect Treg but with possible severe adverse events whereas the others have been the subject of many preclinical studies and are currently being tested in clinical trials. The TF Foxp3 is to our understanding not “druggable”, given the possible catastrophic consequences that this might have on immune tolerance. Based on our observations, we suggest that targeting IL-2 and TNFRSF family members together might represent the most effective strategy to affect Treg preferentially over Tconv.

A surprising and serendipitous finding in the quest for a “universal” Treg signature was that the Penk gene was highly enriched in Treg. Enriched Penk expression by Treg has been reported before in Treg clones derived from TCR-transgenic mice [31]. Our analysis significantly extends this earlier
observation by showing that Penk over expression in Treg is seen in normal cells across multiple datasets, in multiples organs and in multiple mouse strains. Thus, we believe that Penk over expression by Treg should be considered to understand their function further. As stated above, enriched gene expression may or may not translate into enriched protein levels. We investigated whether the enrichment in Penk at the genetic level would also be seen at the protein level.

Deceptively, Penk was not listed in 2 different databases reporting the proteomes of murine Treg or Tconv [32,33]. We believe this observation reflects technical issues related to mass spectrometry rather than a “real” negative result contradicting our analysis. As an evidence for such technical issues, the Tnfrsf1b (TNFR2) protein, a member of the MTS, was also not found in the databases whereas it is clearly expressed by Treg at the cell surface and plays important role in Treg biology [34]. This observation confirms that proteomic analyses are not yet exhaustive.

Additional bioinformatic analysis gave some clues on the molecular mechanisms that might be responsible for Penk expression in Treg, namely an OCR close to the Penk TSS and a possible demethylated CpG island in the promoter region. We also report a massive up regulation of Penk expression in Treg deprived of DICER, the master regulator of miRNA processing and function and crucial for Treg identity and function [26]. This suggest that specific Penk expression in Treg may originate from multiple mechanisms, at the epigenetic and post transcriptional levels. Obviously, “wet” experiments are needed to confirm and extend these bioinformatics-generated hypotheses.

Our preliminary observations in human cells confirmed that PENK was over expressed in Treg relative to CD4+ Tconv. However, this was restricted to activated Treg and not seen with resting Treg. A recent report described the over expression of PENK in activated brain Treg [35]. Our analysis extend this notion to blood Treg, a first indication that PENK might be enriched in Treg from various tissues in humans. In addition, PENK was also expressed by MAIT cells of the blood, a subset not part of the ImmGen database, preventing the comparison with murine MAIT cells. This result raises the possibility that high levels of Penk expression seen in Treg may extend to other subsets currently not present in the murine databases. Also, the OPRD1 receptor for MENK peptides was expressed by naïve Tconv, and NKT cells, all subsets susceptible to Treg-mediated suppression, similar to our observations in mice. Thus, we believe that the endogenous opioid pathway should be considered to understand Treg biology further. The impact of opioid peptides produced by innate and adaptive immune cells on immune suppression and pain relief is of little doubts in mice [36]. Moreover, recent studies using Penk knock-out mice have unambiguously identified opioid peptides produced by CD4+ T cells as key players in a model of pain control in vivo [37]. An effect of MENK peptides on Treg and DC biology, with possible impact on tumor growth, has been reported by the group of F. Shan
However, the impact of MENK on Treg was rather dull, in contrast to the effects of DC that were more pronounced, in agreement with the high expression of the *Oprd1* gene on DC that we report here. Based on enriched *Penk* expression in Treg and the expression pattern of the MENK peptides receptor that we report here, we would like to speculate that peripheral nociception (the neuronal pain sensing pathway) might be intermingled with immune regulation at the Treg level, an hypothesis testable beyond bioinformatics analyses. It will be particularly crucial to analyze mice specifically deficient for *Penk* in Treg to determine the direct vs indirect impact of *Penk* expression on the immune response and how that relates to peripheral nociception.
Acknowledgements

The author wish to thank Dr B Combadière for access to IPA, all members of the ITAC team and all the members of the CIMI-PARIS research center for their encouragements and support, the anonymous curators of GEO and ImmGen for maintaining up-to-date databases.
References

1. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001 Jan 1;27(1):20–1.

2. Hsieh CS, Lee HM, Lio CWJ. Selection of regulatory T cells in the thymus. Nature Reviews Immunology. 2012.

3. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, Anthony BA, Sverdrup FM, Head R, Kuster DJ, Ruminski P, Weiss D, Von Schack D, Bluestone JA. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J Exp Med. 2012;

4. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz J V., Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013 Jul 10;

5. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013 Aug 2;341(6145):569–73.

6. Gagliani N, Amezcua Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, De Zoete MR, Licona-Limón P, Paiva RS, Ching T, Weaver C, Zi X, Pan X, Fan R, Garmire LX, Cotton MJ, Drier Y, Bernstein B, Geginat J, Stockinger B, Esplugues E, Huber S, Flavell RA. TH17 cells transdifferentiate into regulatory T cells uring resolution of inflammation. Nature. 2015;

7. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Cell Research. 2017.

8. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Woloch SJ, Massey KS, Ravetch J V, Allison JP, Quezada SA a. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med. 2013 Jul 29;210(9):1695–710.

9. Arce Vargas F, Furness AJ, Solomon I, Joshi K, Mekkaoui L, Lesko MH, Miranda Rota E, Dahan R, Georgiou A, Sledzinska A, Ben Aissa A, Franz D, Werner Sunderland M, Wong YNS, Henry JY, O’Brien T, Nicol D, Challacombe B, Beers SA, Melanoma TRACERx Consortium, Renal TRACERx Consortium, Lung TRACERx Consortium, Turajlic S, Gore M, Larkin J, Swanton C, Chester KA, Pule M, Ravetch J V, Marafioti T, Peggs KS, Quezada SA. Fc-Optimized Anti-CD25 Depletes Tumor-Infiltrating Regulatory T Cells and Synergizes with PD-1 Blockade to Eradicate Established Tumors. Immunity. 2017;46(4):577–86.
10. Friedman CF, Proverbs-Singh TA, Postow MA. Treatment of the Immune-Related Adverse Effects of Immune Checkpoint Inhibitors: A Review. JAMA oncology. 2016.

11. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, Rudensky AY. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science. 2009 Nov 13;326(5955):986–91.

12. Hori S. Regulatory T cell plasticity: beyond the controversies. Trends Immunol. 2011/06/04. 2011;32(7):295–300.

13. Layland LE, Mages J, Loddenkemper C, Hoerauf A, Wagner H, Lang R, Prazeres da Costa CU. Pronounced Phenotype in Activated Regulatory T Cells during a Chronic Helminth Infection. J Immunol. 2010;184(2):713–24.

14. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, Rehg JE, Jones ML, Ni HT, Artis D, Turk MJ, Vignali DA. IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol. 2010/10/19. 2010;11(12):1093–101.

15. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, Benoist C, Mathis D. PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue T reg cells. Nature. 2012;

16. Cipolletta D, Cohen P, Spiegelman BM, Benoist C, Mathis D. Appearance and disappearance of the mRNA signature characteristic of T reg cells in visceral adipose tissue: Age, diet, and PPARγ effects. Proc Natl Acad Sci. 2015;

17. Samstein RM, Arvey A, Josefowicz SZ, Peng X, Reynolds A, Sandstrom R, Neph S, Sabo P, Kim JM, Liao W, Li MO, Leslie C, Stamatoyannopoulos J a., Rudensky AY. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. Cell. 2012;151(1):153–66.

18. Toker A, Engelbert D, Garg G, Polansky JK, Floess S, Miyao T, Baron U, D√öber S, Geffers R, Giehr P, Schallenberg S, Kretschmer K, Olek S, Walter J, Weiss S, Hori S, Hamann A, Huehn J. Active Demethylation of the Foxp3 Locus Leads to the Generation of Stable Regulatory T Cells within the Thymus. J Immunol. 2013;190(7):3180–8.

19. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, Mathis D. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 2009;15(8):930–9.

20. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003 Nov;13(11):2498–504.

21. Doncheva NT, Morris JH, Gorodkin J, Jensen LJ. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. J Proteome Res. 2019 Feb 1;18(2):623–32.

22. Heng TSP, Painter MW, Elpek K, Lukacs-Kornek V, Mauermann N, Turley SJ, Koller D, Kim FS, Wagers AJ, Asinovski N, Davis S, Fassett M, Feuerer M, Gray DHD, Haxhinasto S, Hill JA, Hyatt G, Laplace C, Leatherbee K, Mathis D, Benoist C, Jianu R, Laidlaw DH, Best JA, Knell J, Goldrath AW, Jarjoura J, Sun JC, Zhu Y, Lanier LL, Ergun A, Li Z, Collins JJ, Shinton SA, Hardy RR, Friedline R,
Sylvia K, Kang J. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol. 2008;

23. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, Sefik E, Tan TG, Wagers AJ, Benoist C, Mathis D. A special population of regulatory T cells potentiates muscle repair. Cell. 2013 Dec 5;155(6):1282–95.

24. 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 Jun 13;15(6):580–7.

25. Wang L, Liu Y, Beier UH, Han R, Bhatti TR, Akimova T, Hancock WW. Foxp3+ T-regulatory cells require DNA methyltransferase 1 expression to prevent development of lethal autoimmunity. Blood. 2013;

26. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. J Exp Med. 2008/08/30. 2008;205(9):1993–2004.

27. Okeke EB, Uzonna JE. The Pivotal Role of Regulatory T Cells in the Regulation of Innate Immune Cells. Front Immunol. 2019 Apr 9;10:680.

28. Cuadrado E, van den Biggelaar M, de Kivit S, Chen Y-Y, Slot M, Doubal I, Meijer A, van Lier RAW, Borst J, Amsen D. Proteomic Analyses of Human Regulatory T Cells Reveal Adaptations in Signaling Pathways that Protect Cellular Identity. Immunity. 2018 May 15;48(5):1046-1059.e6.

29. Hori S. Lineage stability and phenotypic plasticity of Foxp3+ regulatory T cells. Immunol Rev. 2014;

30. Morikawa H, Ohkura N, Vandenbon A, Itoh M, Nagao-Sato S, Kawaji H, Lassmann T, Carninci P, Hayashizaki Y, Forrest ARR, Standley DM, Date H, Sakaguchi S, FANTOM consortium. Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. Proc Natl Acad Sci. 2014;111(14):5289–94.

31. Zelenika D, Adams E, Humm S, Graca L, Thompson S, Cobbold SP, Waldmann H. Regulatory T cells overexpress a subset of Th2 gene transcripts. J Immunol. 2002 Feb 1;168(3):1069–79.

32. Barra MM, Richards DM, Hansson J, Hofer A-C, Delacher M, Hettinger J, Krijgsveld J, Feuerer M. Transcription Factor 7 Limits Regulatory T Cell Generation in the Thymus. J Immunol. 2015;195(7):3058–70.

33. Duguet F, Locard-Paulet M, Marcellin M, Chaoui K, Bernard I, Andreoletti O, Lesbourne R, Burlet-Schiltz O, Gonzalez de Peredo A, Saoudi A. Proteomic Analysis of Regulatory T Cells Reveals the Importance of Themis1 in the Control of Their Suppressive Function. Mol Cell Proteomics. 2017;16(8):1416–32.

34. Salomon BL, Leclerc M, Tosello J, Ronin E, Piaggio E, Cohen JL. Tumor necrosis factor α and regulatory T cells in oncoimmunology. Front Immunol. 2018;9(MAR):1–12.

35. Ito M, Komai K, Mise-Omata S, Iizuka-Koga M, Noguchi Y, Kondo T, Sakai R, Matsuo K, Nakayama T, Yoshie O, Nakatsukasa H, Chikuma S, Shichita T, Yoshimura A. Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery. Nature. 2019;565(7738):246–50.
36. Plein LM, Rittner HL. Opioids and the immune system – friend or foe. Br J Pharmacol. 2018;175(14):2717–25.

37. Basso L, Boué J, Mahiddine K, Blanpied C, Robiou-du-Pont S, Vergnolle N, Deraison C, Dietrich G. Endogenous analgesia mediated by CD4(+) T lymphocytes is dependent on enkephalins in mice. J Neuroinflammation. 2016 Jun 1;13(1):132.

38. Li X, Meng Y, Plotnikoff NP, Youkilis G, Griffin N, Wang E, Lu C, Shan F. Methionine Enkephalin (MENK) inhibits tumor growth through regulating CD4+Foxp3+ regulatory T cells (Tregs) in mice. Cancer Biol Ther. 2015;16(3):450–9.

39. Gao X, Chen W, Plotnikoff NP, Griffin N, Zhang G, Shan F, Meng Y. Methionine enkephalin (MENK) mounts antitumor effect via regulating dendritic cells (DCs). Int Immunopharmacol. 2017;44:61–71.
Figure legends

**Figure 1:** Identification and analysis of the Meta-Treg Signature. (A) a list of genes differentially expressed in Treg relative to Tconv common to 6 datasets (see Methods) defines the Meta-treg Signature (MTS)( red= genes up in Treg, blue = genes down in Treg). *Foxp3* was manually added to this list because it failed to fulfill our filters (FDR<0.05) in only one dataset (Table S1) (B) Putative Protein-to-Protein Interaction network of the Treg-enriched genes from the MTS. The size of the node is proportional to the degree (number of connections to other nodes) of each node. The “betweenness” of each node (shortest path to this node) is indicated by a color code (scale is shown in the legend). The GO terms of each node is represented by a color donut around each node (color coded in the legend with the associated FalseDiscoveryRate). (C) Intersection of the genes from the MTS and a list of 906 name-matched out of 1000 differentially expressed genes derived from ImmGen (SigImmGen) .

**Figure 2:** Putative cytokines regulators of the MTS according to their cellular localization. Arrows indicate experimentally verified or manually curated interactions from the literature between genes and/or molecules extracted from the IPA databases.

**Figure 3:** A quantitative assessment of gene expression levels of the MTS across a variety of cell types. Gene list of the MTS was projected into the ImmGen RNA-Seq database and ranked according to gene expression levels in Treg (CD4+FoxP3+Nrp1lo) from the Colon of C57Bl/6 mice. Genes upregulated in Treg relative to Tconv are at the top of the figure (dark red) whereas those down regulated are at the bottom (light blue). Each column is a subset color coded according to its nature (light blue, stem cells; blue, B cells; violet, T cells; light orange, γδ-T cells; purple, NK and Innate Lymphoid cells (ILCs); light green, DCs; green, Macrophages (MF); pink, Monocytes (Mo); red, Granulocytes (GN); orange, stromal cells) and each line is a gene. For sake of clarity, Tconv and Treg columns are highlighted.

**Figure 4:** (A) *Penk* expression in Treg relative to Tconv in lymphoid organs (from left to right: thymus, spleen and lymph nodes) in the indicated GSE datasets. (B) Similar analysis in non-lymphoid organs (from left to right: Visceral Adipose Tissue, muscle and B16F10 tumor). Treg are highlighted in light blue, Tconv in light red (C) RNA Seq analysis of *Penk* mRNA expression in non tumor (left panel) or tumor-bearing mice (right panel) in the indicated subsets purified from 3 mice in one experiment. RNA Seq was performed with Illumina xxx
**Figure 5:** (A) ATAC-Seq profile at the *Penk* locus. Each peak indicates a region accessible to a mutated retrotransposase, revealing an Open Chromatin Region (OCR, highlighted in blue) in activated CD4+ T cells (Act. CD4+), Treg and in Medullary Thymic Epithelial cells (Ep.MEChi) but not in Naïve CD4+ T cells (males and females), nor naïve CD8+ T cells. The transcription start site of *Penk* (TSS) is indicated with an arrow, the CpG island is indicated with a green box. (B) *Penk* expression in wild type relative to Dnmt-1-deficient Treg (upper panel) or to DICER-deficient Treg (lower panel) in the indicated GSE datasets. Wild type Treg are highlighted in light blue, deficient Treg in light red. (C) Expression of the *Oprd1* gene in a variety of cell immune cell types (extracted from the ImmGen database). High expressing cells are labelled in the figure. (D) Expression of PENK and OPRD1 genes in human T cell subsets from the blood. Normalized expression value were extracted and represented as columns according to the indicated subsets. For phenotypic definition of the subsets, see [http://immunecellatlas.net](http://immunecellatlas.net). Act.=activated
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5