In silico characterization of a “universal” Treg signature reveals the proenkephalin gene as a novel Treg marker

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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

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Abstract

Regulatory T cells (Treg) are crucial in the proper balance of the immune system. A better knowledge of Treg-specific genes may provide novel targets for therapeutic purposes and 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. Most of the genes from this list did not connect to each other in a functional protein network analysis, but a third of those interacted somehow with IL-2, confirming the central role for this cytokine in Treg biology. 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*, *Ctla4*, *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 “universal” 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 in silico discovery that the “universal” Treg signature is relatively small, with most of the genes not belonging to any specific biological functions. Furthermore, the MTS did not defined Treg very well when cells other than $CD4^+$ Tconv were considered, apart from *Foxp3*, genes of the interleukin-2 family and some members of the TNF receptors superfamily (TNFRSF). Serendipitously, we found that the *Penk* gene, encoding the pro enkephalin protein responsible for generating Met-enkephalin peptides (MENK), is one of the few genes which expression is restricted to Treg across the whole continuum of cells of the immune system. The meta-Treg signature compiled here provide a common reference data set that might prove useful to the immunology community and provide new hypotheses to investigate Treg biology further.

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 (MTS)

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 (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. We used the Gene Ontology consortium website (http://geneontology.org) to investigate the biological functions associated with the MTS. 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). Surprisingly, *Foxp3*, the master TF of Treg, was absent from this list because it was missing from one dataset (Table S1). To be consistent with previous knowledge on Treg, we thus “forced” *Foxp3* into the MTS, establishing a list of 72 genes (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 investigate whether the genes of the MTS might encompass specific biological functions, we analyzed the 72 genes using gene ontology database. Several genes of the MTS were significantly enriched in GO terms, including but not limited to negative regulation of cytokine secretion (GO:0050710), lymphocyte proliferation (GO:0050670), lymphocyte activation (GO:0046649), and regulation of developmental growth (GO:0048638) (Fig 1B), in line with known biological functions and activation status of Treg. To gain insight into biological functions of the MTS, we generated a putative network of proteins-proteins interactions (PPI) from the MTS (Fig. 1C). 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 pathway. Those genes were thus excluded from the representation. Significant interactions above randomly expected ones (PPI enrichment p.value $<10^{-16}$) were observed for well established members of the Treg signature. A central node of PPI was composed of *Foxp3*, *Ctla4*, *Il2ra*, *il2rb*, *Tnfrsf4* (CD134, OX40), *Tnfrsf9* (CD137, 4-1BB), *Tnfrsf1b* (TNFR2), *Itgae* (CD103), *CD83*, *Klrg1*, *Nrp1* and *Entpd1* (CD39). Surprisingly, a smaller node of proteins more related to the nervous system was observed and

composed of *Penk* (pro enkephalin), *App* (Amyloid beta-A4protein) and *Prnp* (Prion protein). Thus, expected and unexpected Treg specific markers were observed in the MTS and some clustered together in the PPI network around Foxp3. Those genes constituting the “core” of the MTS were mostly responsible for the GO match reported in figure 1B. librarTo 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 1000 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 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 cells. 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 $\gamma\delta$-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 localisation. 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 analyzed the chromatin profile of the *Penk* regulatory region in Treg. 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. 4C). In contrast, this OCR was observed in stromal cells and in $CD4^+$ T cells stimulated in vitro (Fig. 5C) 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. 5A), 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. 5A), 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. 5B), 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 Mucosal Associated Invariant T cells (MAIT) (not present in the ImmGene subsets) and in activated Treg (Fig. 5C), confirming our observations in mice. An interesting difference with the mouse model was the high expression of the OPDR1 gene in activated Treg, contrary to its almost complete absence in mice Treg (Fig 5B), suggesting that an autocrine loop of MENK/OPDR1 interactions may exist in humans and not in mice.

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). Nevertheless, our analysis puts forward the idea that targeting IL-2 and TNFRSF family members together might represent the most effective therapeutic strategy to affect Treg preferentially over Tconv. But what about other cell types? 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. 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 separated from Treg identity (29)) is mostly dictated at the epigenetic rather than the gene expression level (30). Nevertheless, a few genes stand out to be very specific to Treg beyond Treg/Tconv comparison. These were *Ctla4*, *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.

Finally, a surprising and serendipitous finding in the search for a “universal” Treg signature was that the *Penk* gene was highly specific to Treg in the immune system, at least at the gene level. Actually, 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 (31,32). 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 (33). 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. Nevertheless, 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 contrary to humans (34). 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 (35). Although *Penk* expression by Treg has not been reported before, an influence of MENK peptides on Treg and DC biology, with possible functional impact on tumor growth, has been reported by the group of F. Shan (36,37). 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 specific *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.

Several crucial questions remain unanswered: is *Penk* over expression at the genetic level confirmed at the protein level? Is PENK over expression also observed in human Treg and in which tissue? What is the role of MENK peptides in immuno regulation? Clearly, more “wet” lab experiments are needed but depending on the answers, these might lead to novel strategies to manipulate immuno regulation for therapeutic purposes.

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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) **27**:20–1. doi:10.1038/83713

2. Hsieh CS, Lee HM, Lio CWJ. Selection of regulatory T cells in the thymus. *Nat Rev Immunol* (2012) doi:10.1038/nri3155

3. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, Anthony BA, Sverdrup FM, Head R, Kuster DJ, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* (2012) doi:10.1084/jem.20120822

4. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* (2013) doi:10.1038/nature12331

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) **341**:569–73. doi:10.1126/science.1241165

6. Gagliani N, Amezcua Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, De Zoete MR, Licona-Limón P, Paiva RS, Ching T, et al. TH17 cells transdifferentiate into regulatory T cells uring resolution of inflammation. *Nature* (2015) doi:10.1038/nature14452

7. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res* (2017) doi:10.1038/cr.2016.151

8. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* (2013) **210**:1695–1710. doi:10.1084/jem.20130579

9. Arce Vargas F, Furness AJS, Solomon I, Joshi K, Mekkaoui L, Lesko MH, Miranda Rota E, Dahan R, Georgiou A, Sledzinska A, et al. Fc-Optimized Anti-CD25 Depletes Tumor-Infiltrating Regulatory T Cells and Synergizes with PD-1 Blockade to Eradicate Established Tumors. *Immunity* (2017) **46**:577–586. doi:10.1016/j.immuni.2017.03.013

10. Friedman CF, Proverbs-Singh TA, Postow MA. Treatment of the Immune-Related Adverse Effects of Immune Checkpoint Inhibitors: A Review. *JAMA Oncol* (2016) doi:10.1001/jamaoncol.2016.1051

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) **326**:986–91. doi:10.1126/science.1172702

12. Hori S. Regulatory T cell plasticity: beyond the controversies. *Trends Immunol* (2011) **32**:295–300. doi:S1471-4906(11)00059-7 [pii]10.1016/j.it.2011.04.004

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**:713–724. doi:10.4049/jimmunol.0901435

14. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol* (2010) **11**:1093–1101. doi:ni.1952 [pii]10.1038/ni.1952

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) doi:10.1038/nature11132

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) doi:10.1073/pnas.1423486112

17. Samstein RM, Arvey A, Josefowicz SZ, Peng X, Reynolds A, Sandstrom R, Neph S, Sabo P, Kim JM, Liao W, et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* (2012) **151**:153–166. doi:10.1016/j.cell.2012.06.053

18. Toker A, Engelbert D, Garg G, Polansky JK, Floess S, Miyao T, Baron U, D√ºber S, Geffers R, Giehr P, et al. Active Demethylation of the Foxp3 Locus Leads to the Generation of Stable Regulatory T Cells within the Thymus. *J Immunol* (2013) **190**:3180–3188. doi:10.4049/jimmunol.1203473

19. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* (2009) **15**:930–939. doi:10.1038/nm.2002

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) **13**:2498–504. doi:10.1101/gr.1239303

21. Doncheva NT, Morris JH, Gorodkin J, Jensen LJ. Cytoscape StringApp: Network Analysis and Visualization of Proteomics Data. *J Proteome Res* (2019) **18**:623–632. doi:10.1021/acs.jproteome.8b00702

22. Heng TSP, Painter MW, Elpek K, Lukacs-Kornek V, Mauermann N, Turley SJ, Koller D, Kim FS, Wagers AJ, Asinovski N, et al. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* (2008) doi:10.1038/ni1008-1091

23. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, Sefik E, Tan TG, Wagers AJ, Benoist C, et al. A special population of regulatory T cells potentiates muscle repair. *Cell* (2013) **155**:1282–95. doi:10.1016/j.cell.2013.10.054

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) **15**:580–587. doi:10.1038/ni.2868

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) doi:10.1182/blood-2012-08-451765

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) **205**:1993–2004. doi:jem.20081062 [pii]10.1084/jem.20081062

27. Okeke EB, Uzonna JE. The Pivotal Role of Regulatory T Cells in the Regulation of Innate Immune Cells. *Front Immunol* (2019) **10**:680. doi:10.3389/fimmu.2019.00680

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) **48**:1046-1059.e6. doi:10.1016/j.immuni.2018.04.008

29. Hori S. Lineage stability and phenotypic plasticity of Foxp3+ regulatory T cells. *Immunol Rev* (2014) doi:10.1111/imr.12175

30. Morikawa H, Ohkura N, Vandenbon A, Itoh M, Nagao-Sato S, Kawaji H, Lassmann T, Carninci P, Hayashizaki Y, Forrest ARR, et al. Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc Natl Acad Sci* (2014) **111**:5289–5294. doi:10.1073/pnas.1312717110

31. 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**:3058–3070. doi:10.4049/jimmunol.1500821

32. Duguet F, Locard-Paulet M, Marcellin M, Chaoui K, Bernard I, Andreoletti O, Lesourne 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**:1416–1432. doi:10.1074/mcp.M116.062745

33. 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**:1–12. doi:10.3389/fimmu.2018.00444

34. Plein LM, Rittner HL. Opioids and the immune system – friend or foe. *Br J Pharmacol* (2018) **175**:2717–2725. doi:10.1111/bph.13750

35. 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) **13**:132. doi:10.1186/s12974-016-0591-x

36. 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**:450–459. doi:10.1080/15384047.2014.1003006

37. 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. doi:10.1016/j.intimp.2017.01.004

Figure legends

Figure 1: Identification and analysis of the Meta-Treg Signature. **(A)** the list of genes differentially expressed in Treg relative to Tconv common to 6 datasets (see Methods) defines the Meta-treg Signature (MTS). **(B)** Gene Ontology enrichment analysis genes from the MTS and the associated FalseDiscoveryRate. Negative regulation of cytokine secretion (GO:0050710), lymphocyte proliferation (GO:0050670), lymphocyte activation (GO:0046649), and regulation of developmental growth (GO:0048638). **(C)** Putative Protein-to-Protein Interaction network of the genes from the MTS. **(D)** Intersection of the genes from the MTS and a list of 1000 differentially expressed genes from ImmGen (SigImmGen).

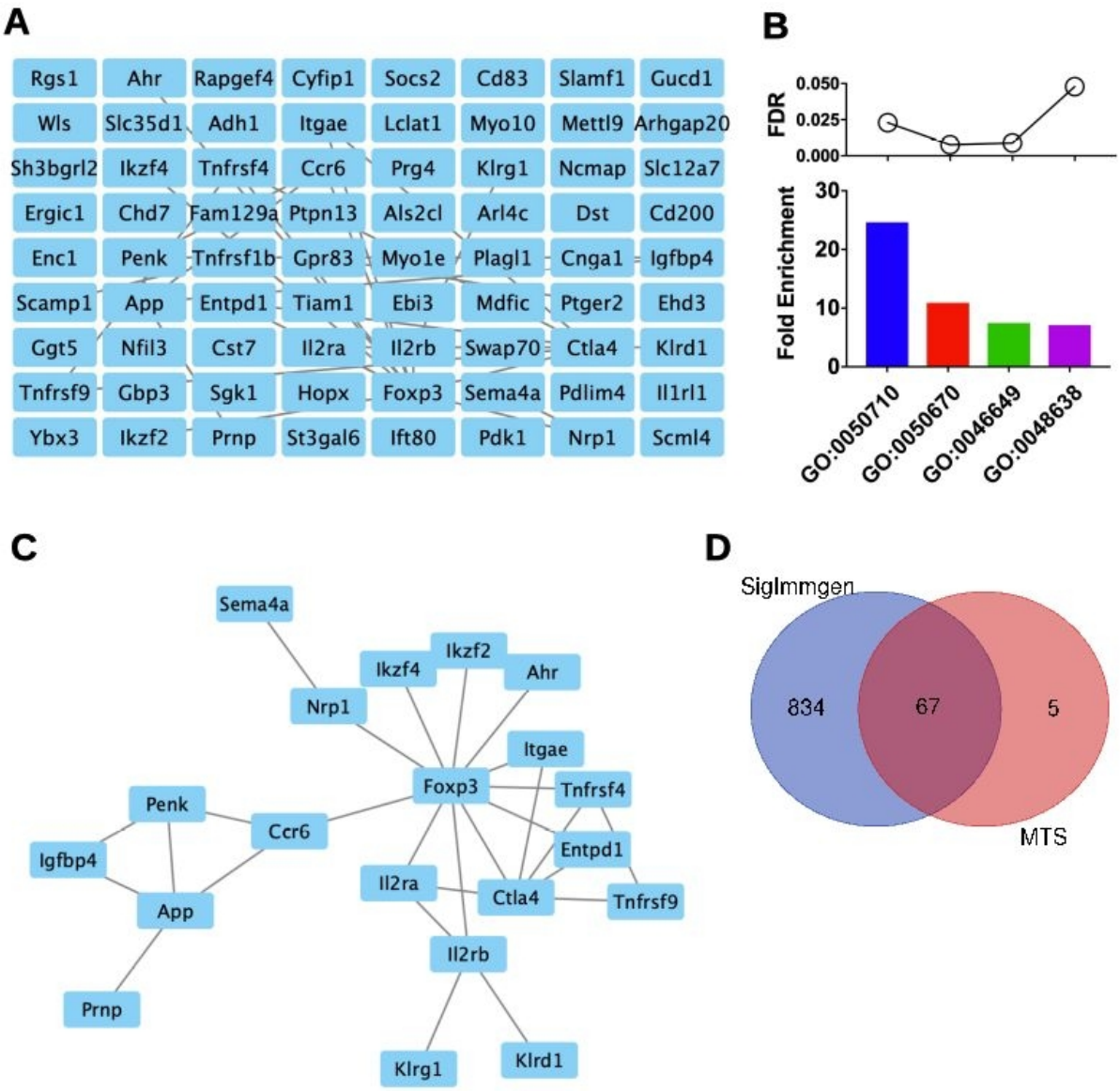
Figure 2: Putative cytokines regulators of the MTS according to their cellular localization. Arrows indicate experimentally verified or manually curated interactions from the literature between genes and/or molecules extracted from the IPA databases.

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)** 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 Naive 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.

Figure 5: **(A)** *Penk* expression in wild type relative to Dnmt-1-deficient Treg (left panel) or to DICER-deficient Treg (right panel) in the indicated GSE datasets. Wild type Treg are highlighted in light blue,

deficient Treg in light red. **(B)** Expression of the *Opdr1* gene (coding for the receptor of MENK peptides produced by Penk) in a variety of cell immune cell types. High expressing cells are labelled in the figure. **(C)** Expression of PENK and OPRD1 genes in human T cells 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. Act.=activated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Lclat1
Myo10
Mettl9
Arhgap20
Sh3bgrl2
Ikzf4
Tnfrsf4
Ccr6
Prg4
Klrg1
Ncmap
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Ergic1
Chd7
Fam129a
Ptpn13
Als2cl
Arl4c
Dst
Cd200
Enc1
Penk
Tnfrsf1b
Gpr83
Myo1e
Plagl1
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Nfil3
Cst7
Il2ra
Il2rb
Swap70
Ctla4
Klrd1
Tnfrsf9
Gbp3
Sgk1
Hopx
Foxp3
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Pdlim4
Il1rl1
Ybx3
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Prnp
St3gal6
Ift80
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Nrp1
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FDR
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0.025
0.000
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10
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Ahr
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Itgae
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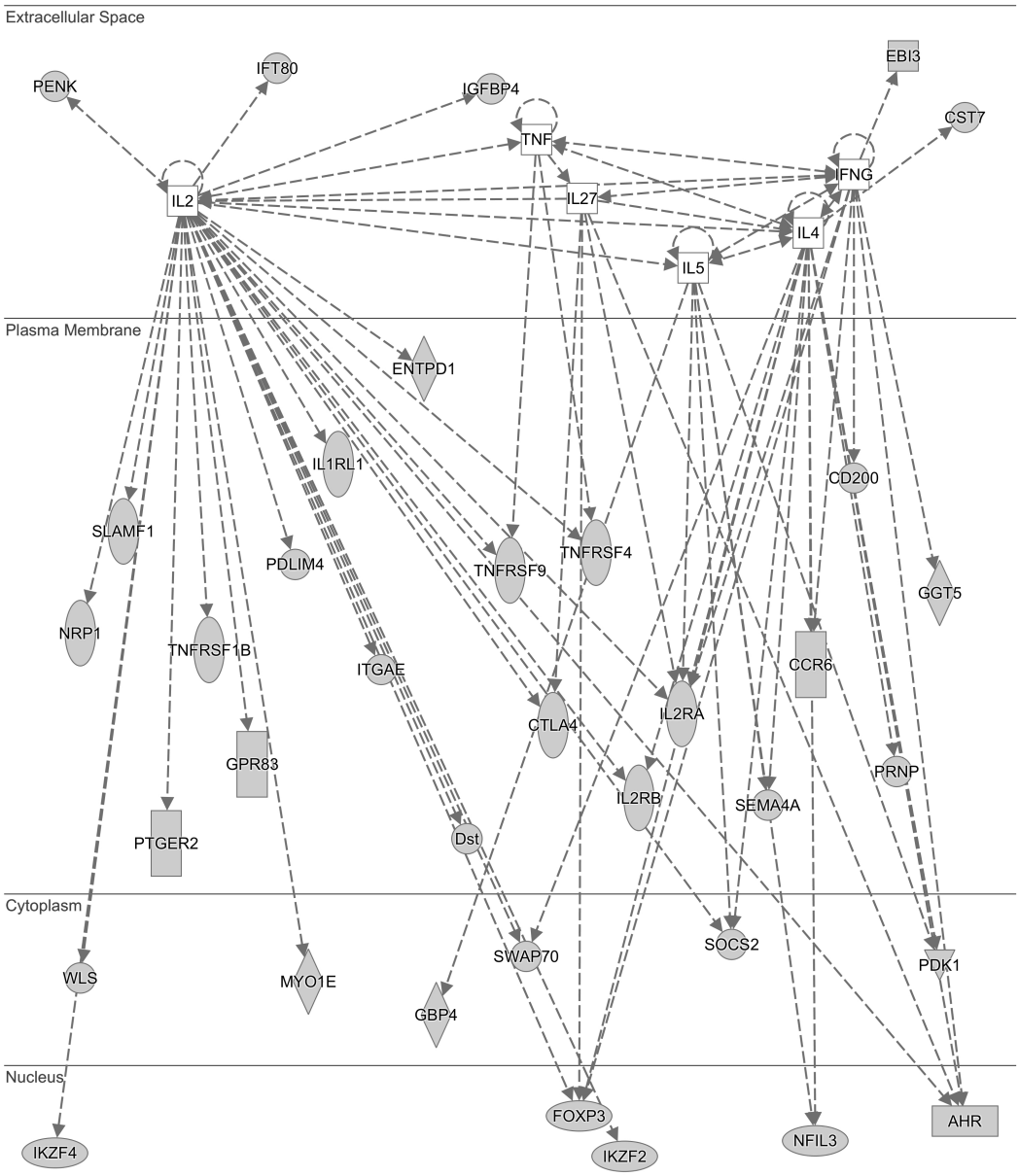
67
5
MTS

Figure 1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PDLIM4
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TNFRSF4
GGT5
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TNFRSF1B
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CCR6
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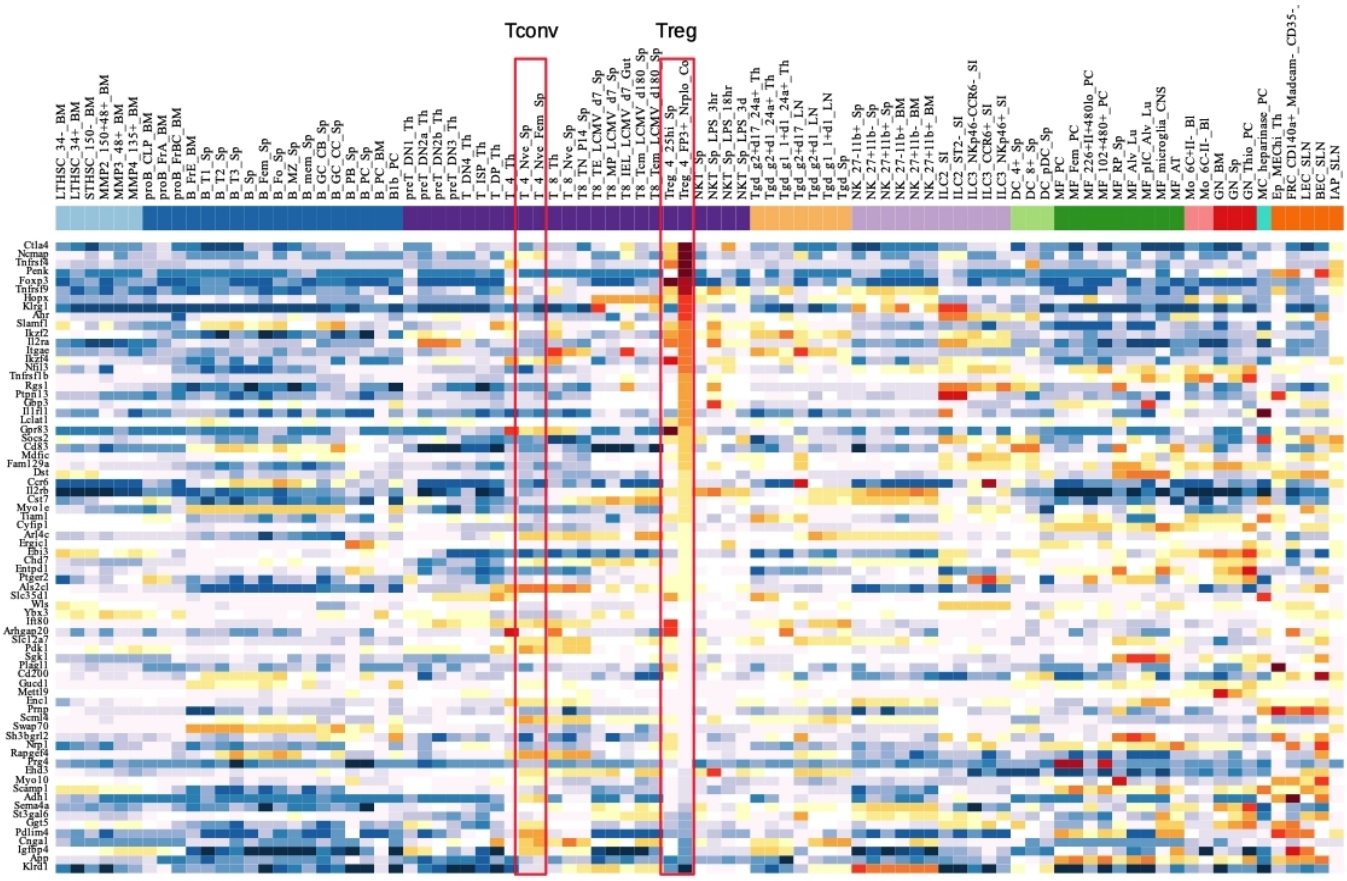
Figure 2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proB_FrBC_BM
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B_T3_Sp
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B_GC_CB_Sp
B_GC_CC_Sp
B_PB_Sp
B_PC_Sp
B_PC_BM
B1b_PC
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preT_DN2a_Th
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preT_DN3_Th
T_DN4_Th
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T_DP_Th
T_4_Th
T_4_Nve_Sp
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T8_MP_LCMV_d7_Sp
T8_IEL_LCMV_d7_Gut
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ILC3_CCR6+_SI
ILC3_NKp46+_SI
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DC_8+_Sp
DC_pDC_Sp
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MF_RP_Sp
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MF_pIC_Alv_Lu
MF_microglia_CNS
MF_AT
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Mo_6C-II-_Bl
GN_BM
GN_Sp
GN_Thio_PC
MC_heparinase_PC
Ep_MEChi_Th
FRC_CD140a+_Madcam-_CD35-
LEC_SLN
BEC_SLN
IAP_SLN
Ctla4
Ncmap
Tnfrsf8
Penk
Foxp3
Tnfrsf9
Hopx
Klrg1
Ahr
Slamf1
Ikzf2
Il2ra
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Ptpn13
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Lclat1
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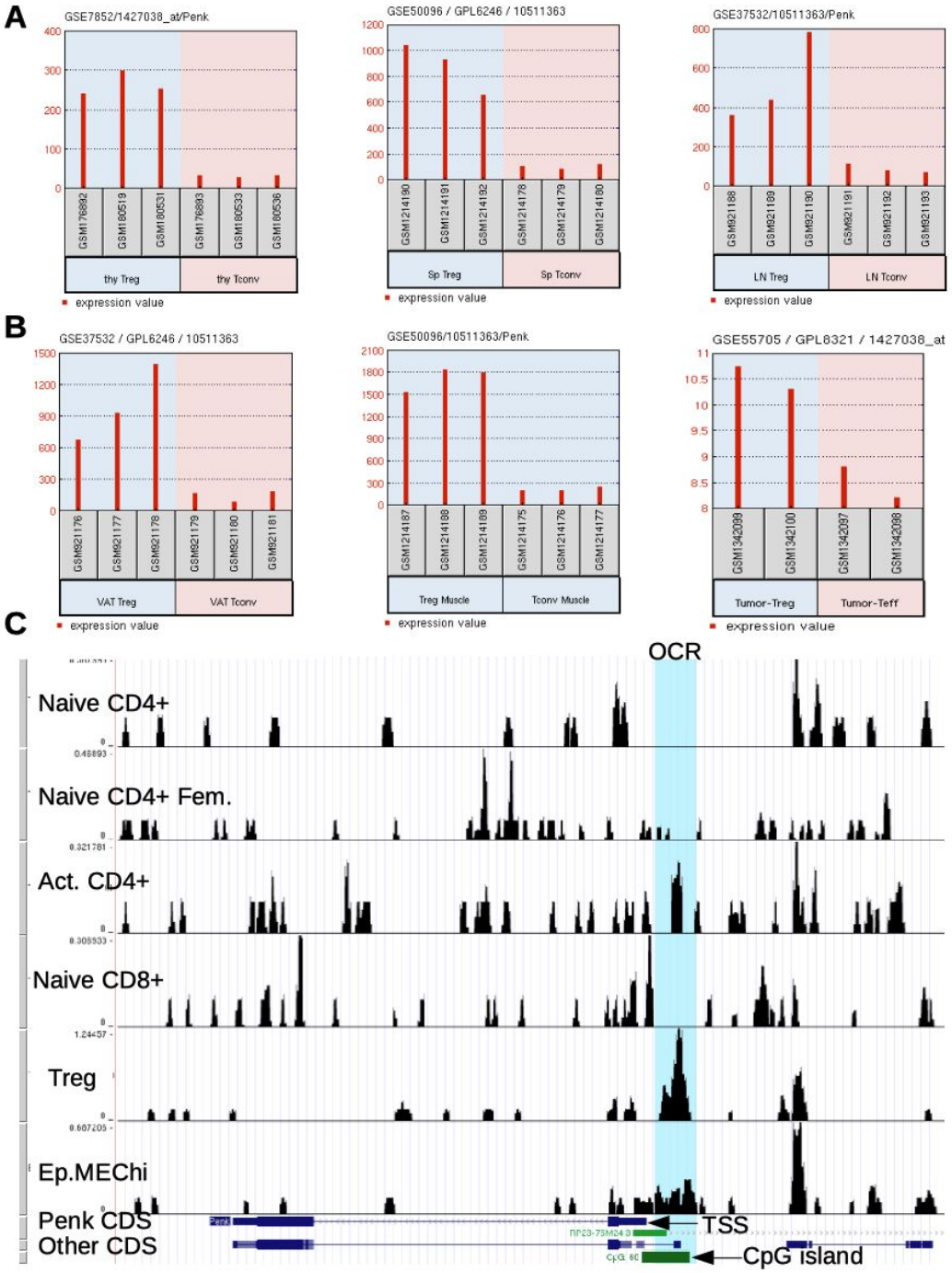
Figure 3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thy Tconv
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VAT Tconv
expression value
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GSM1214176
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Tconv Muscle
expression value
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Tumor-Treg
Tumor-Teff
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OCR
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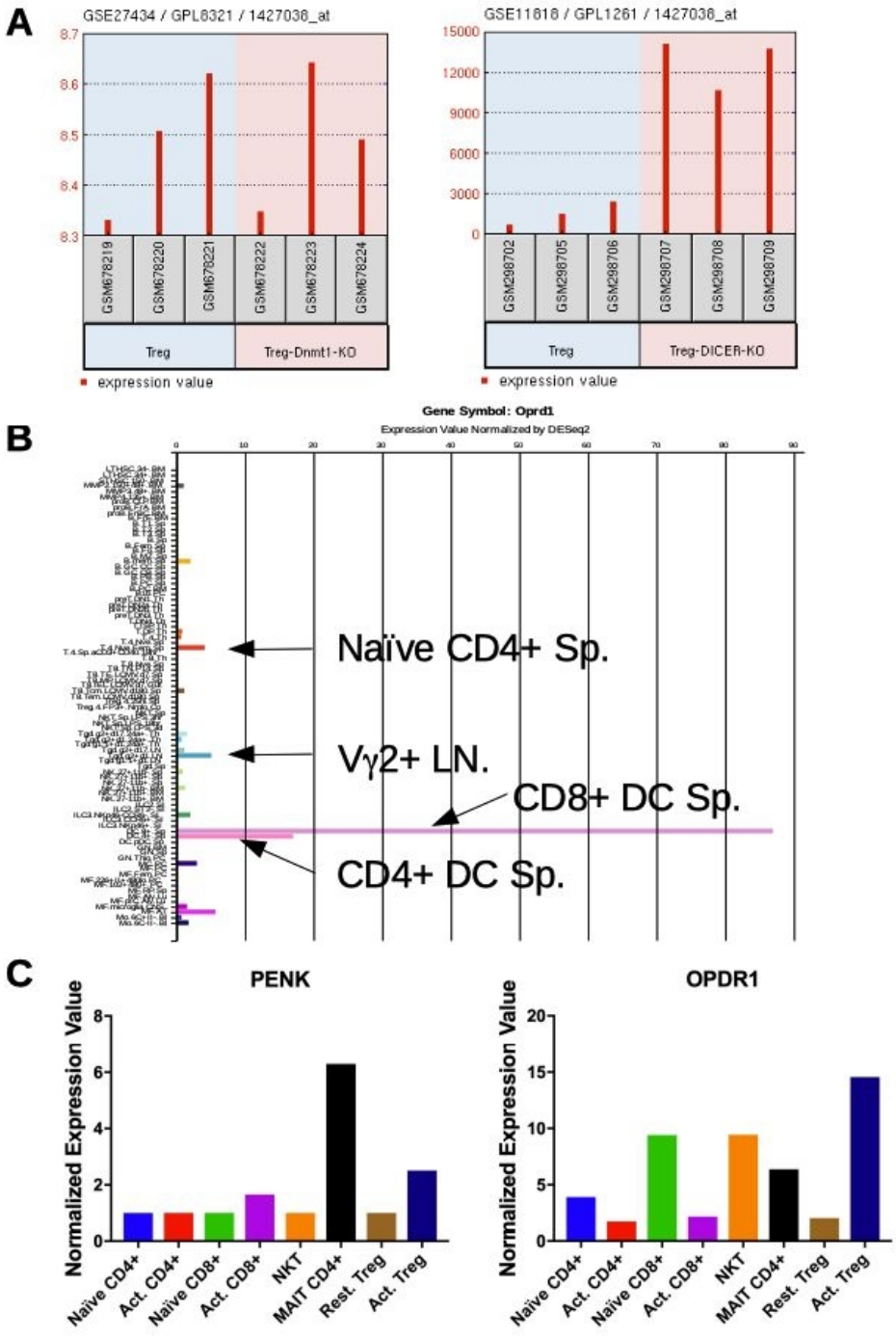
Other CDS
TSS
CpG Island

Figure 4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Vγ2+ LN.
CD8+ DC Sp.
CD4+ DC Sp.
C
PENK
OPDR1
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Act. CD4+
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Act. CD8+
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Rest. Treg
Act. Treg

Figure 5