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

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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 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*, *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 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 $<10^{-16}$) 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

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 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 *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. 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

[38,39]. 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.

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**Figure legends**

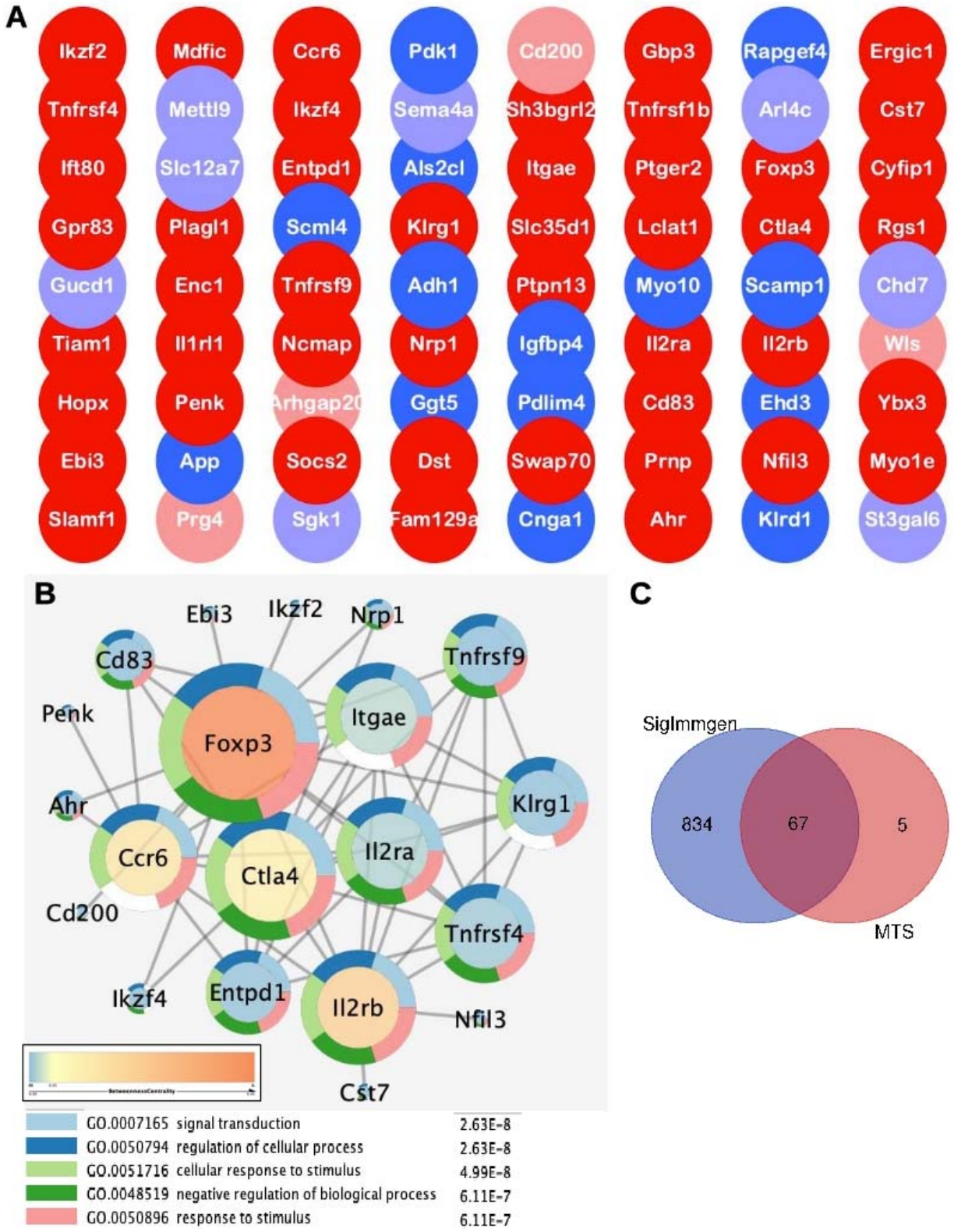
**Figure 1**: Identification and analysis of the Meta-Treg Signature. **(A)** a list of genes differentially expressed in Treg relative to Tconv common to 6 datasets (see Methods) defines the Meta-treg Signature (MTS)( red= genes up in Treg, blue = genes down in Treg). *Foxp3* was manually added to this 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 "betweeness" 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 pnale) 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 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 **(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.  Act.=activated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Sh3bgrl2
Tnfrsf1b
Arl4c
Cst7
Ift80
Slc12a7
Entpd1
Als2cl
Itgae
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Foxp3
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Plagl1
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Ctla4
Rgs1
Gucd1
Enc1
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Myo10
Scamp1
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Cd83
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Ebi3
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Ebi3
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SigImmgen
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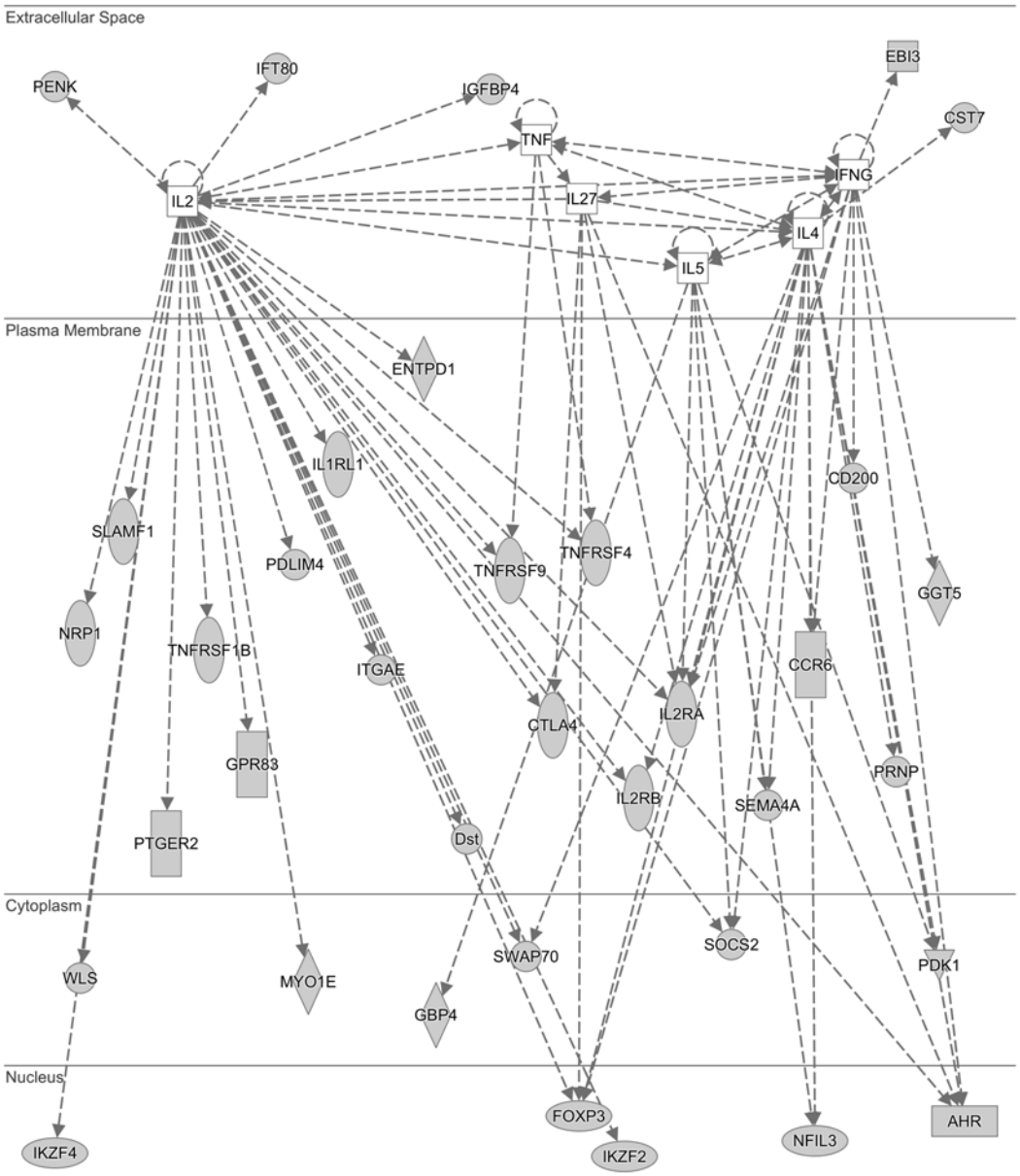
67
5
MTS

**Figure 1**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IL1RL1
CD200
SLAMF1
PDLIM4
TNFRSF9
TNFRSF4
GGT5
NRP1
TNFRSF1B
ITGAE
CCR6
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Dst
Cytoplasm
SWAP70
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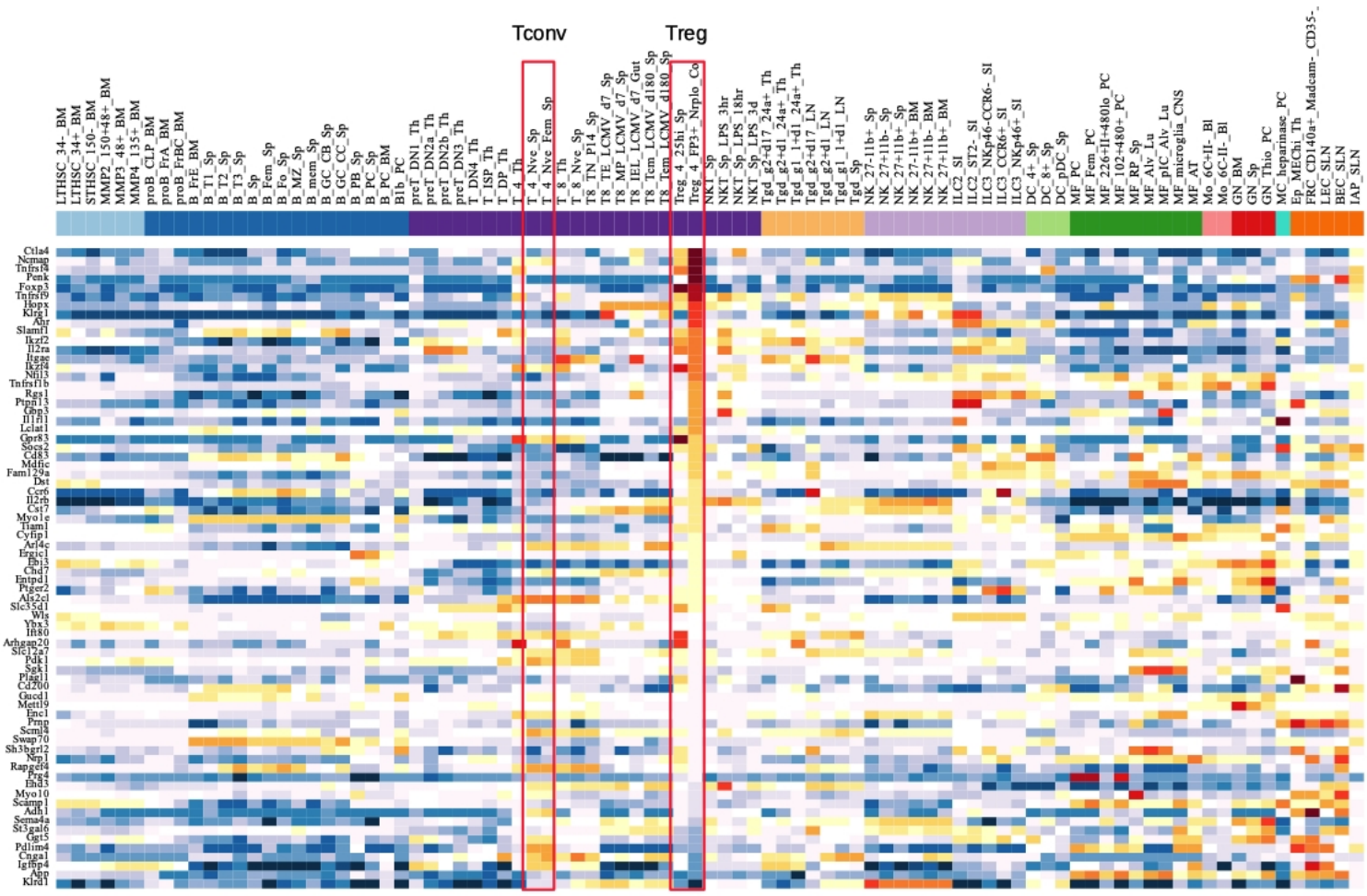
**Figure 2**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proB_FrA_BM
proB_FrBC_BM
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B_T2_Sp
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preT_DN2a_Th
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preT_DN3_Th
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T_DP_Th
T_4_Th
T_4_Nve_Sp
T_4_Nve_Fem_Sp
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T_8_Nve_Sp
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T8_MP_LCMV_d7_Sp
T8_IEL_LCMV_d7_Gut
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Treg_4_25hi_Sp
Treg_4_FP3+_Nrplo_Co
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NK_27+11b-_BM
NK_27+11b+_BM
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ILC2_ST2-_SI
ILC3_NKp46-CCR6-_SI
ILC3_CCR6+_SI
ILC3_NKp46+_SI
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DC_8+_Sp
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MF_AT
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Mo_6C-II-_Bl
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GN_Sp
GN_Thio_PC
MC_heparinase_PC
Ep_MEChi_Th
FRC_CD140a+_Madcam-_CD35-_
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BEC_SLN
IAP_SLN
Ctla4
Nemap
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Penk
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Slamf1
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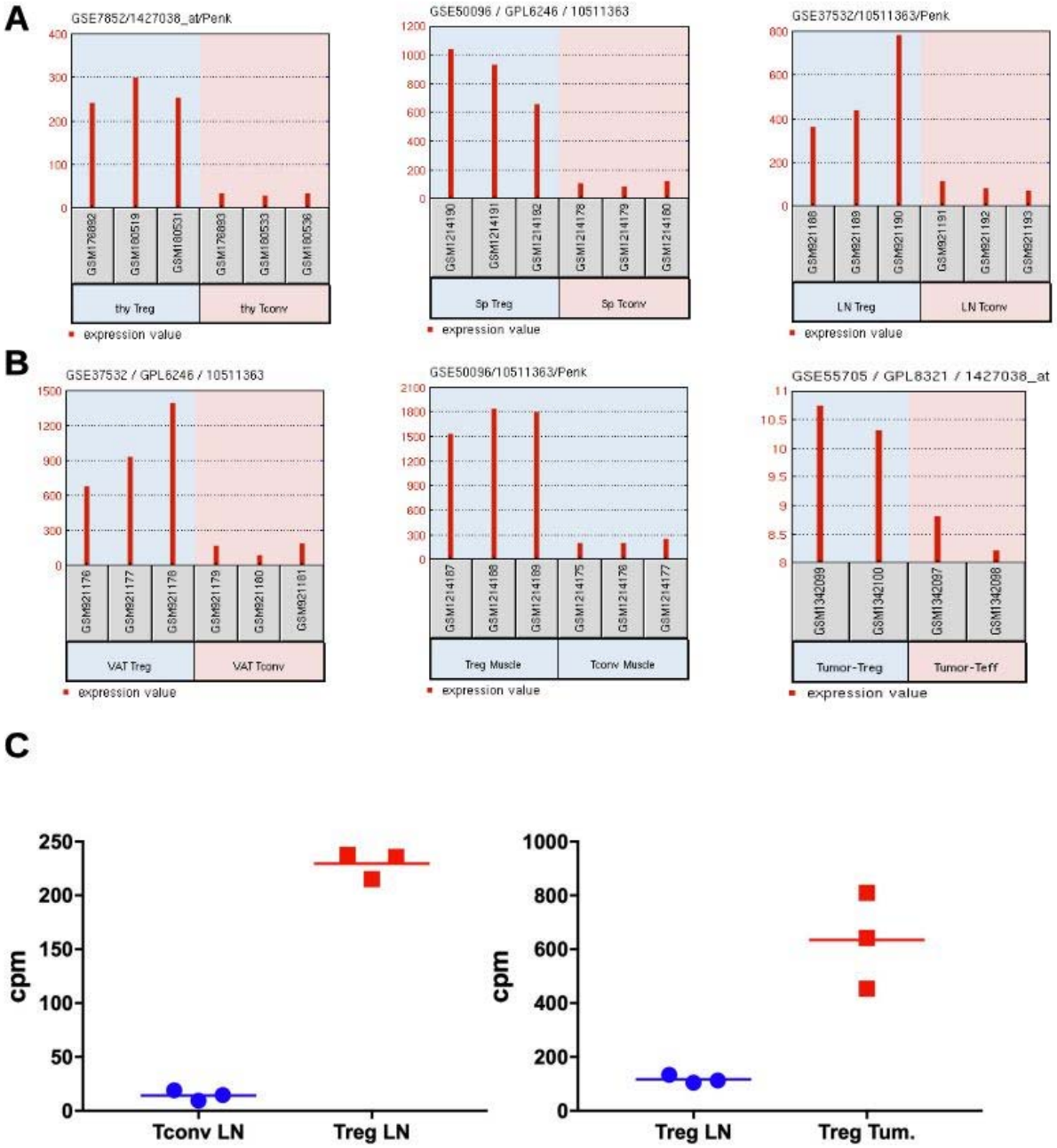
**Figure 3**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VAT Tconv
expression value
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Tconv Muscle
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Tumor-Teff
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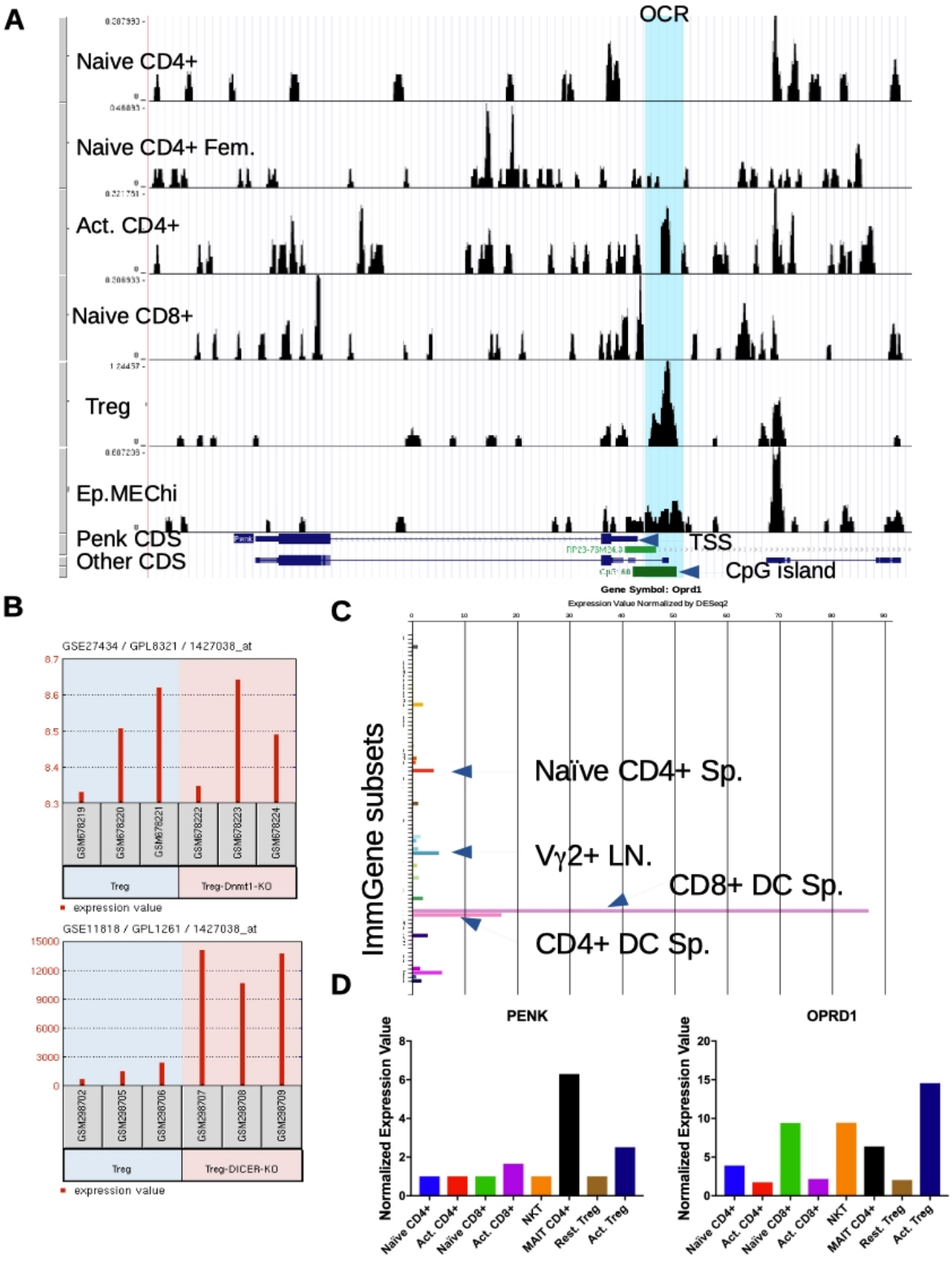
cpm
Treg LN
Treg Tum.

**Figure 4**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Expression Value Normalized by DESeq2
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Vγ2+ LN.
CD8+ DC Sp.
CD4+ DC Sp.
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PENK
OPRD1
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Act. CD4+
Naïve CD8+
Act. CD8+
NKT
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Rest. Treg
Act. Treg

**Figure 5**