The relationship between human thymus-derived regulatory T cells, TGF-β-induced regulatory T cells and conventional CD4$^+$ T cells as revealed by proteomics

Mark Mensink[1], Ellen Schrama[1], Maartje van den Biggelaar[2],
Derk Amsen[3], Sander de Kivit[1,#] & Jannie Borst[1,#]

[1] Department of Immunology and Oncode Institute, Leiden University Medical Center, Leiden, The Netherlands

[2] Department of Molecular and Cellular Homeostasis, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[3] Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[#] These authors share senior authorship

CORRESPONDING AUTHORS

Correspondence to Dr Sander de Kivit (S.de_Kivit@lumc.nl) or Prof Jannie Borst (J.G.Borst@lumc.nl).

ABSTRACT

The CD4$^+$ regulatory T (Treg) cell lineage, as defined by FOXP3 expression, comprises thymus-derived (t)Treg cells and peripherally induced (p)Treg cells. In human, naïve tTreg cells can be purified from blood, but occur in low abundance, while effector pTreg and tTreg cell populations cannot be purified for lack of discriminating cell surface markers. Therefore, studies often employ TGF-β-induced (i)Treg cells that are generated from CD4$^+$ conventional T (Tconv) cells *in vitro*. Here, we describe the relationship of iTreg cells to tTreg and Tconv cells, as optimally purified from human blood. Global proteomic analysis revealed that iTreg, tTreg and Tconv cell populations each have a unique protein expression pattern. We next used as a benchmark a previously defined proteomic signature that discerns *ex vivo* naïve and effector phenotype Treg cells from Tconv cells and reflects unique Treg cell properties. This Treg cell core signature was largely absent from iTreg cells, while clearly present in simultaneously analyzed tTreg cells. In addition, we used a proteomic signature that distinguishes *ex vivo* effector Treg cells from Tconv cells and naïve Treg cells. This effector Treg cell signature was partially present in iTreg cells. Thus, iTreg cells are distinct from tTreg cells and largely lack the common Treg cell proteomic signature. However, they do have certain protein expression features in common with *ex vivo* effector Treg cells. These data demonstrate the utility of the core and effector Treg cell signatures as tools to define Treg cell populations and encourage the use of *ex vivo* Treg cells for functional analyses.

INTRODUCTION

Immune responses depend on a balance between the opposing activities of conventional (Tconv) and regulatory T (Treg) cells[1]. While Tconv cells are key mediators of adaptive immunity, Treg cells are specialized in maintaining immune tolerance and controlling inflammation[1]. Based on their origin, Treg cells can be classified into two main groups. Thymus-derived (t)Treg cells stably express the Treg cell master transcription factor FOXP3 and warrant against autoimmunity due to recognition of self-antigen[2, 3, 4, 5]. Peripherally induced (p)Treg cells are converted from Tconv cells in the course of a response to foreign antigen and have less stable FOXP3 expression[6]. Stable expression of FOXP3 is established epigenetically by demethylation of the Treg-specific demethylated region (TSDR) within the *FOXP3* locus[2]. Besides FOXP3, Treg cells express key molecules that are important for diverse mechanisms of suppression. For instance, Treg cells express high levels of CTLA-4, which serves to attenuate CD28 costimulation of Tconv cells by downregulating CD80/86 on antigen-presenting cells[7]. Another example is high level expression on Treg cells of CD39, which facilitates extracellular ATP hydrolysis that generates adenosine, a molecule with immunosuppressive effects on Tconv cells[8].

Amongst human effector phenotype Treg cells, tTreg and pTreg cells can currently not be discriminated for a lack of cell surface markers[9, 10]. It is possible to isolate naïve tTreg cells with high

purity, but these occur in low abundance in the blood[11, 12]. For this reason, *in vitro* Treg cell models often employ TGF-β-induced (i)Treg cells that are generated from Tconv cells[13, 14, 15, 16]. It is unclear to what extent iTreg cells resemble *ex vivo* Treg cell subsets, i.e. pTreg or tTreg cells. Moreover, iTreg cells are likely less stable than tTreg cells, since in a mouse model adoptively transferred iTreg cells lost Foxp3 expression and failed to prevent graft-versus-host disease, in contrast to tTreg cells[17].

We recently described that *ex vivo* naïve and effector Treg cells from human peripheral blood have a common proteomic Treg cell signature, which sets them apart from other $CD4^+$ T cells[12]. This Treg cell core signature reflects unique Treg cell properties regarding signal transduction, transcriptional regulation and cell metabolism. In addition, we identified a proteomic signature that distinguishes *ex vivo* effector Treg cells from both Tconv cells and naïve Treg cells. Using these proteomic signatures as benchmarks, we compared in the current study the proteomes of iTreg, tTreg and Tconv cells to determine to what extent *in vitro*-generated iTreg cells resemble *ex vivo* Treg cells.

RESULTS

Validation of Tconv, tTreg and iTreg cell populations used in this study

To study human Tconv, tTreg and iTreg cells in parallel, $CD4^+$ T cells were isolated from peripheral blood and naïve Tconv and tTreg cells were flow cytometrically sorted based on a $CD25^{lo}CD127^{hi}CD45RA^+GPA33^{int}$ or $CD25^{hi}CD127^{lo}CD45RA^+GPA33^{hi}$ phenotype, respectively[11, 18] (Supplementary Figure 1). After sorting, naïve cells were expanded using agonistic monoclonal antibodies against CD3 and CD28 in the presence of IL-2. To generate iTreg cells, TGF-β was added to Tconv cell cultures. Expanded cells had “rested” for 4 days prior to restimulation with anti-CD3/CD28 antibodies for 1 day (Figure 1A).

After expansion, Tconv cells were $FOXP3^-Helios^-$ and largely retained this phenotype after CD3/CD28-mediated restimulation (Figure 1B, C). tTreg cells showed a uniform expression of FOXP3 and Helios (IKFZ2), both before and after restimulation, validating that the method generated pure tTreg cells. The iTreg cells showed bimodal FOXP3 expression, with an upregulation of FOXP3 upon CD3/CD28-mediated restimulation, while Helios was minimally expressed, in agreement with the generation of iTreg cells from Tconv cells[19].

The TSDR of the *FOXP3* locus had the highly demethylated configuration in tTreg cells and was methylated in Tconv and iTreg cells, in agreement with published data[2] (Figure 1D). Next, we functionally characterized the cell populations in a standard assay, testing their ability to suppress CD3-induced proliferation of CellTrace Violet-labeled Tconv cells from peripheral blood. In this assay, tTreg cells were suppressive and more so than iTreg cells, while Tconv cells were not, in agreement with earlier research[20] (Figure 1E). Together, these data validate the purification and cell culture protocols to generate the appropriate tTreg, iTreg and Tconv cell populations for further characterization.

Figure 1

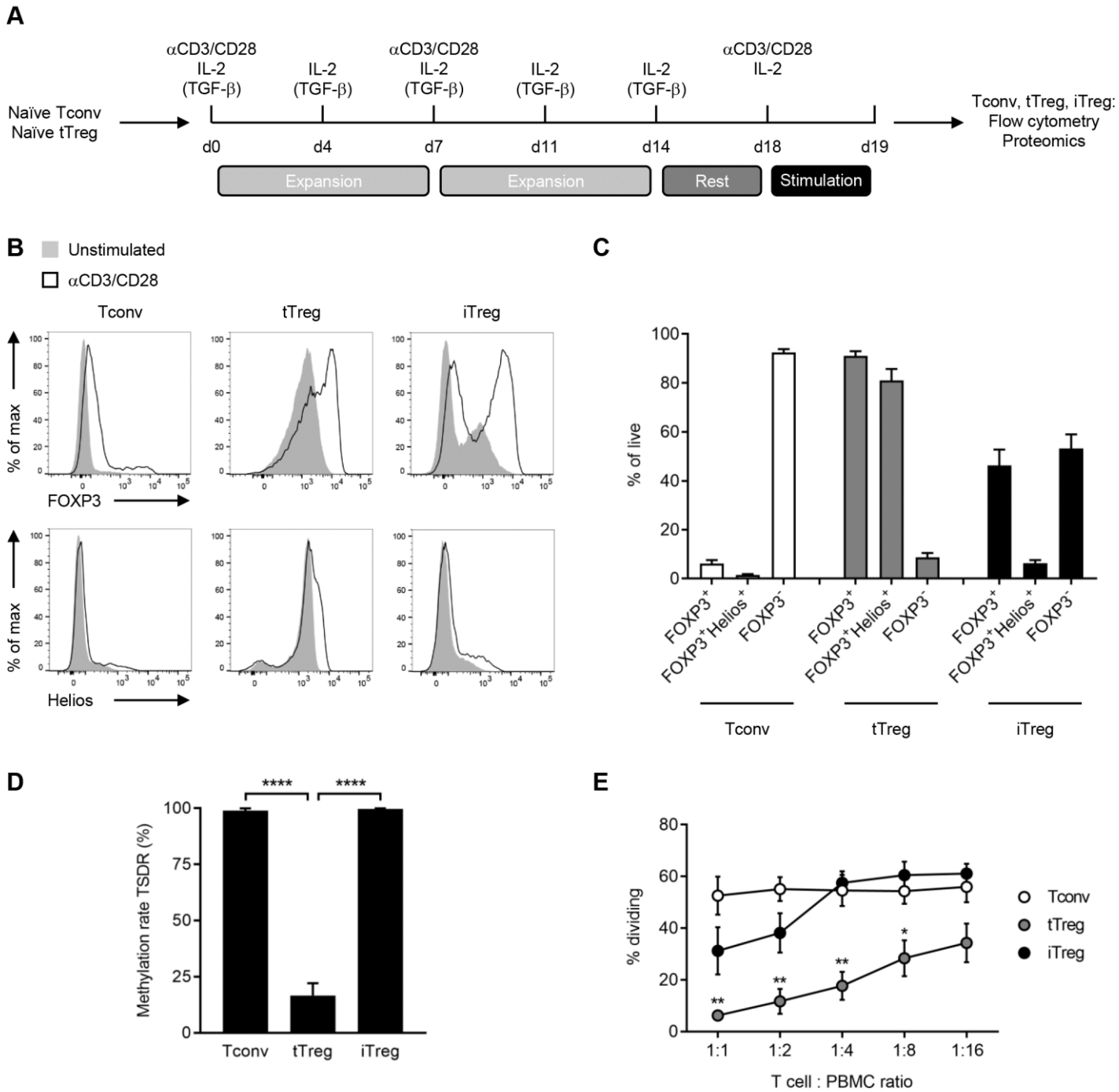


Figure 1. Characterization of Tconv, tTreg and iTreg cell populations. **(A)** Schematic overview of expansion protocols for Tconv, tTreg and iTreg cells. TGF-β was used to generate iTreg cells. Prior to analysis, expanded cells were restimulated for 24 h or not, as indicated in figure legends. **(B)** Flow cytometric analysis of FOXP3 and Helios (IKZF2) protein levels in Tconv, tTreg and iTreg cells at 24 h after restimulation with anti-CD3/CD28 mAbs or unstimulated control (representative of n = 7). **(C)** Quantification of the frequency of $FOXP3^+$, $FOXP3^+Helios^+$ and $FOXP3^-$ cells in the resting Tconv, tTreg and iTreg cell populations after expansion (n = 7). **(D)** Analysis of the methylation status of the TSDR in the *FOXP3* locus in Tconv, tTreg and iTreg cells. One-way ANOVA with Tukey's *post hoc* test was used for statistical analysis (n = 6-7). **(E)** Assessment of the suppressive capacity of Tconv, tTreg and iTreg cells that were cocultured for 4 days with CellTrace Violet-labeled responder PBMCs in the presence of agonistic mAb to CD3. The percentage of dividing PBMCs is displayed (n = 4). One-way ANOVA with Bonferroni's *post hoc* test was used for statistical analysis (n = 4). **(C-E)** Data are presented as mean ± SEM. Sample size (n) represents cells from individual donors, analyzed in independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Expression of characteristic cell surface receptors on tTreg and iTreg cells

We next examined the expression of a number of cell surface receptors that are generally related to Treg cell function, among which CTLA-4 that is a key mediator of suppression by tTreg cells. CTLA-4 recycles between the cell surface and endosomes[21]. When total (cell surface plus intracellular) CTLA-4 levels were examined, both tTreg and iTreg cells showed a higher expression than Tconv cells (Figure 2A, Supplementary Figure 2). All three cell types upregulated total CTLA-4 expression upon CD3/CD28-mediated restimulation, but levels were higher in tTreg and iTreg cells than in Tconv cells. CTLA-4 was only found at the cell surface of expanded cells after CD3/CD28-mediated restimulation, on tTreg, iTreg and Tconv cells, with tTreg cells showing the highest levels (Figure 2A, Supplementary Figure 2). The costimulatory receptor OX40 was only present on all cell types following CD3/CD28-mediated restimulation, with highest levels on tTreg cells and a similar pattern was observed for costimulatory receptor GITR (Figure 2A, Supplementary Figure 2). Both iTreg and Tconv cells but not tTreg cells upregulated the co-inhibitory receptor PD-1 upon CD3/CD28-mediated restimulation (Figure 2A, Supplementary Figure 2). CD39 (ENTPD1) protein levels were highest on iTreg cells both before and after restimulation (Figure 2A, Supplementary Figure 2). These data show that tTreg and iTreg cells have evidently different expression patterns of these characteristic cell surface molecules associated with Treg cell responses (Figure 2B).

Proteomic analysis reveals that iTreg cells are distinct from tTreg and Tconv cells

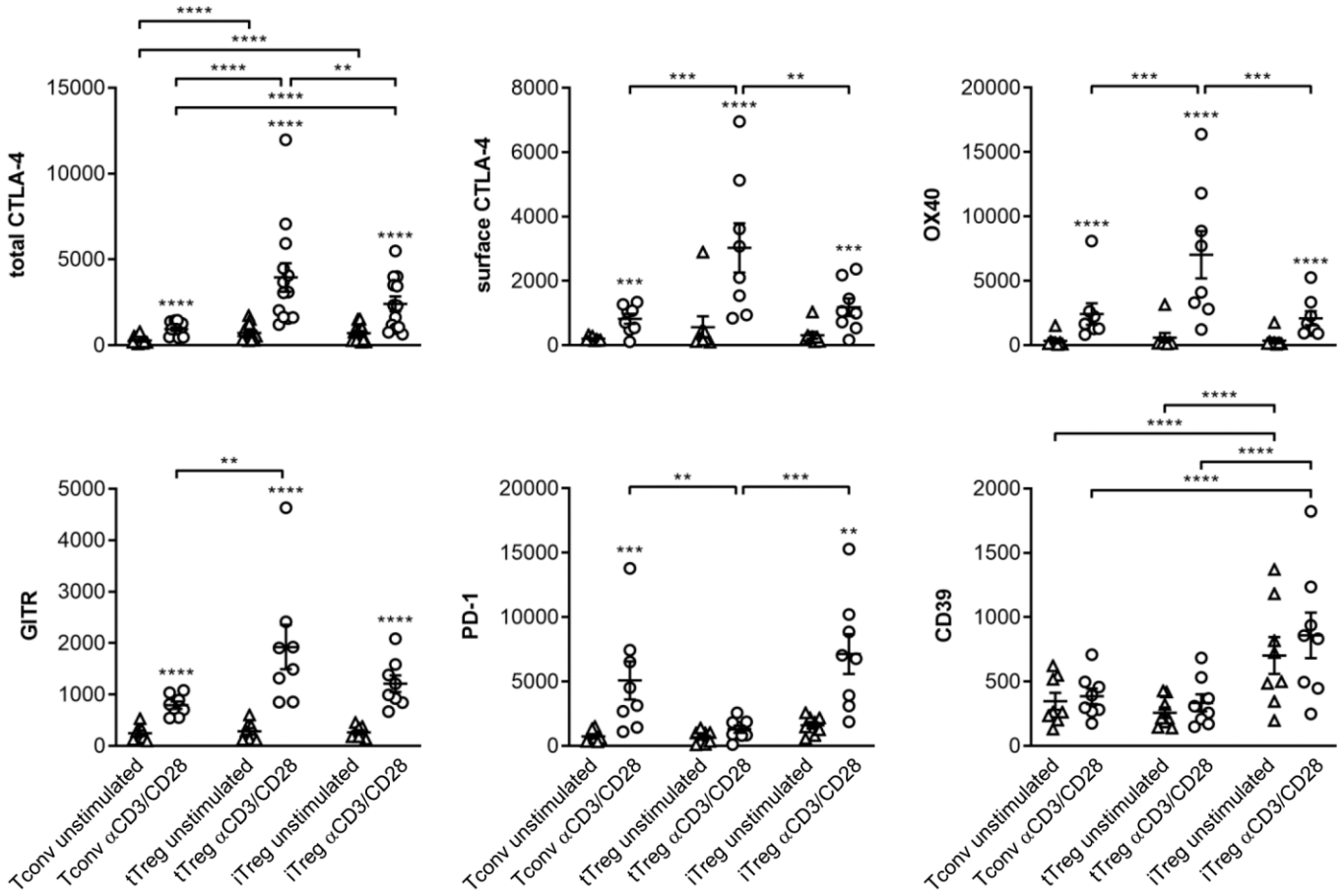
We performed label-free quantitative proteomics to compare overall protein expression in Tconv, tTreg and iTreg cells in an unbiased manner. Based on the levels of the 4224 proteins that were identified (Supplementary Table 1), a principal component analysis (PCA) readily indicated distinct grouping of expanded Tconv, tTreg and iTreg cells (Figure 3A). Unsupervised hierarchical clustering of the 1290 differentially expressed proteins among these cell types revealed clusters of proteins with high or low expression, as shown in a heat map (Figure 3B, Supplementary Table 2).

iTreg and tTreg cells shared high (cluster 1) or low (cluster 6) expression of certain proteins that set them apart from Tconv cells. Proteins from cluster 1 did not denote specific biological processes (Figure 3C), whereas proteins from cluster 6 partially formed a network connected to generic processes such as protein folding and apoptosis (Figure 3D, Supplementary Figure 3). Apart from this limited overlap in protein expression between iTreg and tTreg cells, the vast majority of proteins (clusters 2-5 and 7-10) were differentially expressed between these cells types, emphasizing their dissimilarity. Proteins in clusters 7-10 were present at higher levels in iTreg cells than in tTreg cells and were associated with cell activation, cell division, vesicle-mediated transport and cell adhesion (Figure 3E). Proteins in clusters 2-5 were expressed at lower levels in iTreg cells than in tTreg cells and were mainly associated with metabolic processes (Figure 3E).

iTreg and Tconv cells shared to a certain extent protein clusters 2-3 and 7-8 that set them apart from tTreg cells. Clusters 2-3 with low levels in iTreg and Tconv cells denoted proteins associated with multiple metabolic processes (Supplementary Figure 3), while clusters 7-8 with high levels in iTreg and Tconv cells denoted proteins involved in stress response, vesicle-mediated transport and lymphocyte

Figure 2

A



B

Molecule	Tconv unstimulated	Tconv αCD3/CD28	tTreg unstimulated	tTreg αCD3/CD28	iTreg unstimulated	iTreg αCD3/CD28
Total CTLA-4	-	+/-	+/-	+	-	+
Surface CTLA-4	-	+/-	-	+	-	+/-
OX40	-	+/-	-	+	-	+/-
GITR	-	+/-	-	+	-	+
PD-1	-	+	-	-	-	+
CD39	+/-	+/-	+/-	+/-	+	+

Figure 2. Expression of characteristic cell surface molecules associated with Treg cell responses. **(A)** Quantification of flow cytometric analysis of the protein expression of total CTLA-4 (cell surface plus intracellular) (n = 13), surface CTLA-4 (n = 8), surface OX40 (n = 8), surface GITR (n = 8), surface PD-1 (n = 8) and surface CD39 (ENTPD1) (n = 8) on indicated cell populations. Mean fluorescence intensity (MFI) is depicted on the y-axis. Two-way ANOVA with Tukey's *post hoc* test was used for statistical analysis. Data are presented as mean ± SEM. Sample size (n) represents cells from individual donors, analyzed in independent experiments. Asterisks without lines indicate comparisons between cells that were restimulated or not. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **(B)** Table summarizing the data in **A**, indicating relatively low (-), intermediate (+/-) or high (+) expression levels.

Figure 3

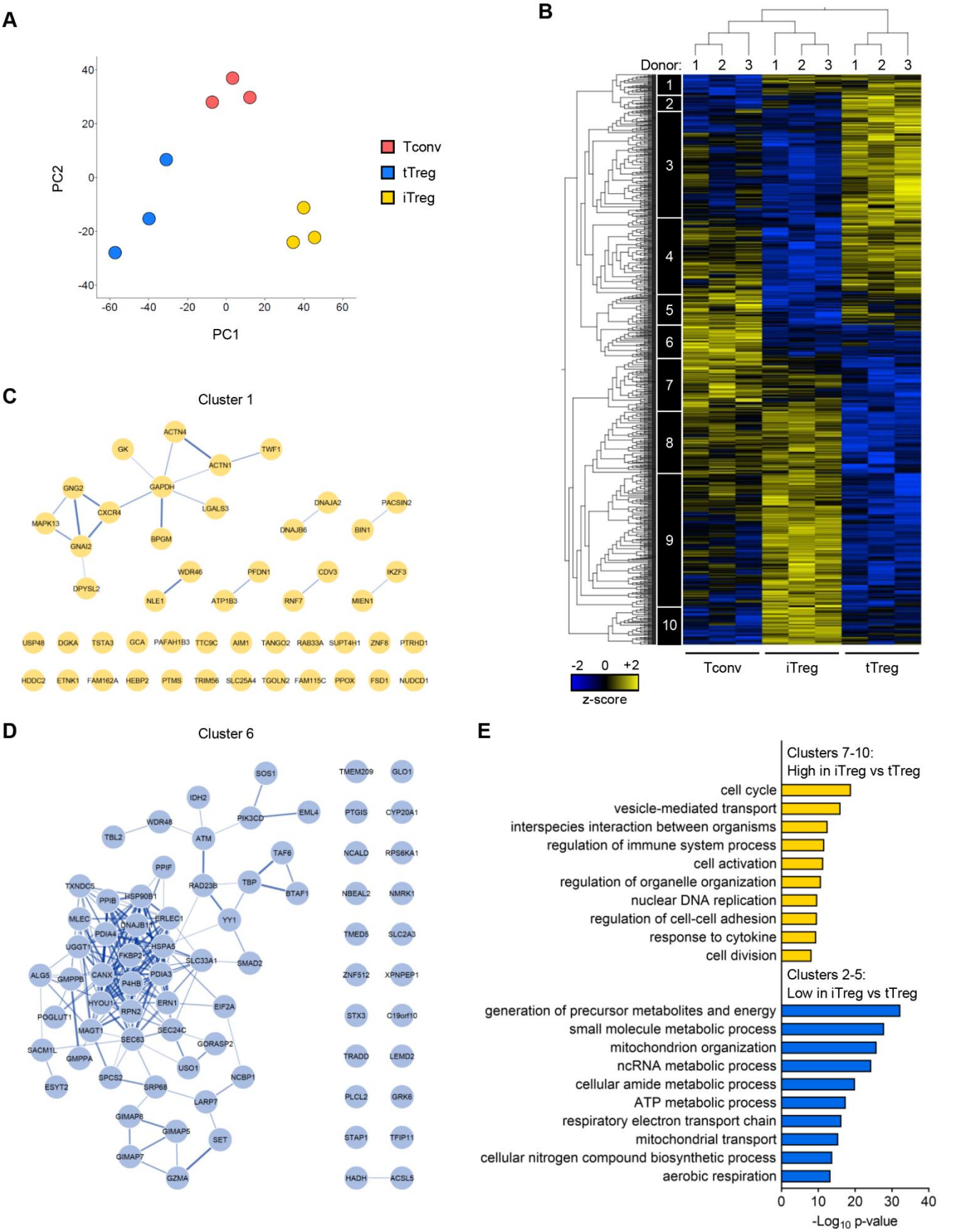


Figure 3. The iTreg, tTreg and Tconv cell proteomes are distinct. **(A)** PCA plot of the proteome of Tconv, tTreg and iTreg cells that were not restimulated before assessment (n = 3). **(B)** Heat map showing hierarchical clustering of the 1290 proteins that were differentially expressed between unstimulated Tconv, tTreg and iTreg cells (ANOVA, $p < 0.05$). Z-scores showing relative protein expression values are color-coded. Numbered boxes (1-10) indicate clusters of proteins. **(C)** STRING network of proteins from cluster 1 that have shared high expression in iTreg and tTreg cells, relative to Tconv cells. **(D)** STRING network of proteins from cluster 6 that have shared low expression in iTreg and tTreg cells, relative to Tconv cells. **(E)** GO enrichment analyses of clusters 7-10 (proteins with high expression in iTreg cells compared to tTreg cells) and clusters 2-5 (proteins with low expression in iTreg cells compared to tTreg cells), showing 10 significantly enriched GO biological processes per analysis.

activation (Supplementary Figure 3). iTreg cells also showed unique protein clusters with low (clusters 4-5) or high (clusters 9-10) levels that set them apart from both Tconv and tTreg cells. Clusters 4-5 were connected to multiple metabolic processes, while clusters 9-10 were associated with cell division (Supplementary Figure 3). Overall, this proteomic analysis indicates that the iTreg cell proteome partially overlaps with the Tconv cell proteome and to a lesser extent with the tTreg cell proteome, but also includes many unique features. It also testified to an active mitotic phenotype of iTreg cells.

iTreg and tTreg cells differentially respond to CD3/CD28 stimulation

We also analyzed the proteome of Tconv, tTreg and iTreg cells following CD3/CD28-mediated restimulation. Distinct clusters were identified in a PCA, showing that particularly Tconv and iTreg cells altered their protein expression upon activation (Figure 4A). A much smaller response was observed in tTreg cells, which clustered adjacent to each other regardless of stimulation. The three cell types had limited overlap in proteins that altered their expression levels upon restimulation, as visualized by a DiVenn diagram (Figure 4B). For many proteins, levels were uniquely altered in either Tconv, iTreg or tTreg cells. These proteins were involved in metabolism, RNA processing, biogenesis, stress and defense responses (Supplementary Figure 4A). Other proteins similarly altered expression levels upon CD3/CD28-mediated restimulation in all three cell types (Figure 4B). Linear regression analysis of the whole proteomes revealed that the response of Tconv and iTreg cells was more comparable than the response of tTreg and iTreg cells (Supplementary Figure 4B).

Next, we analyzed the proteomic response to CD3/CD28-mediated restimulation by each cell type individually. In tTreg cells, the lowest number of proteins (185) were differentially expressed (Figure 4C). Gene set enrichment analysis (GSEA) on the whole proteome indicated that upregulated proteins were associated with immune-related processes, lymphocyte differentiation and an interferon gamma response (Figure 4D). In iTreg cells, 760 proteins were differentially expressed and a multitude of biological processes was altered, including upregulation of immune-related processes, cell division and protein synthesis, and downregulation of actin filament organization, transport regulation and cell migration (Figure 4E, F). Tconv cells also responded greatly to restimulation, with 585 proteins differentially expressed (Figure 4G). GSEA revealed that processes such as ribosomal processing, DNA replication and chromatin remodeling were upregulated, whereas downregulation was observed for actin filament organization, cell adhesion and cell movement (Figure 4F). To conclude, these data indicate that iTreg cells, like Tconv cells and unlike tTreg cells, strongly respond to CD3/CD28 stimulation, including an increase of processes associated with cell proliferation.

A Treg cell core signature reflecting unique properties is present in tTreg but not iTreg cells

Recently, we established that *ex vivo* naïve and effector Treg cells from human peripheral blood have a common proteomic signature that distinguishes them from $CD4^+$ Tconv cells, which we termed the Treg cell core signature[12]. This signature contains proteins with high or low expression reflecting unique properties regarding signal transduction, transcriptional regulation, iron storage, lysosomal processes and cell metabolism (Figure 5A, B). Here, we questioned whether the Treg cell core signature is also

Figure 4

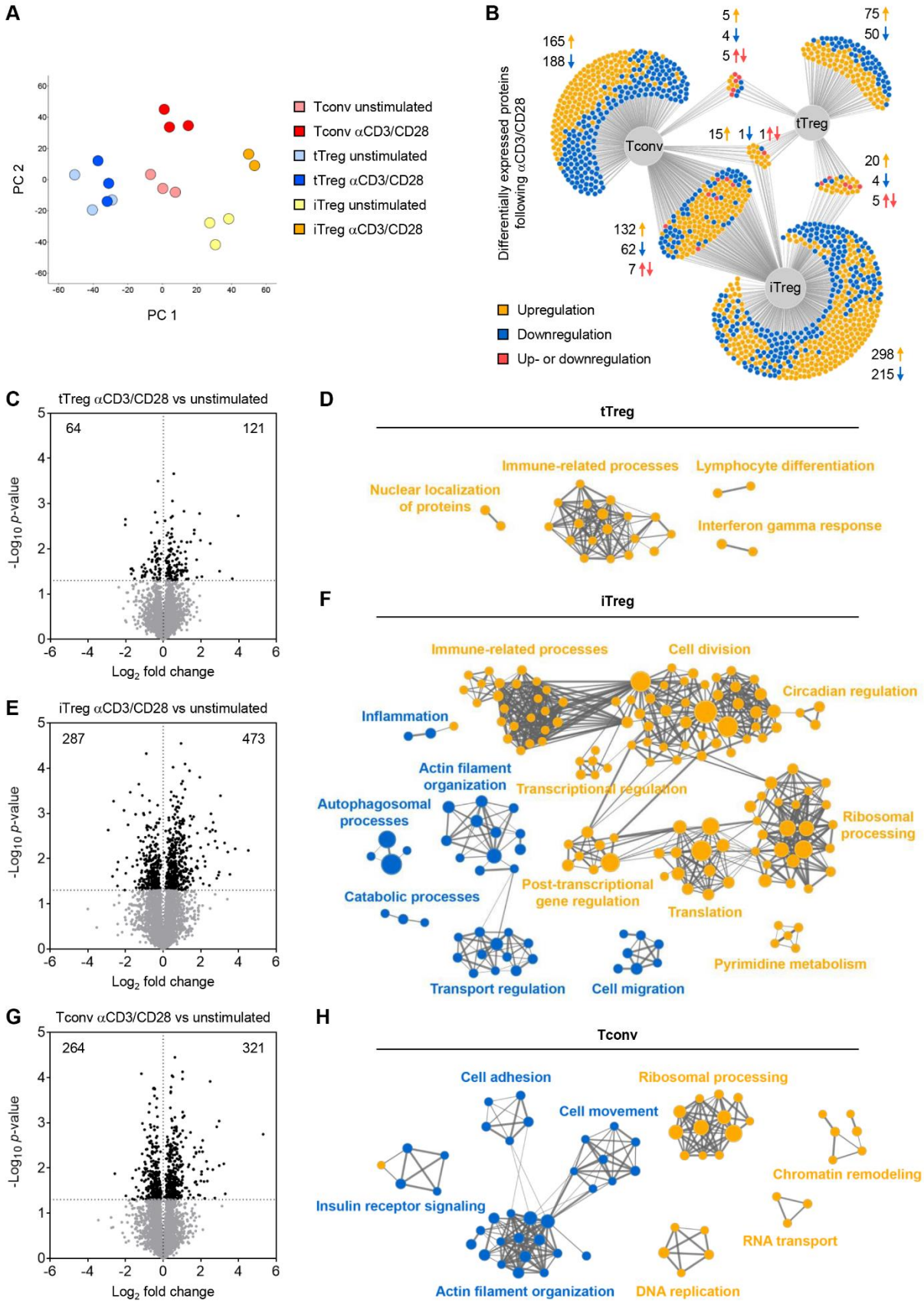


Figure 4. Proteomic analysis of CD3/CD28-stimulated Tconv, tTreg and iTreg cells. **(A)** PCA plot of the proteome of Tconv, tTreg and iTreg cells that were restimulated or not (n = 3, n = 2 only for CD3/CD28-stimulated iTreg cells). **(B)** DiVenn diagram showing shared and unique proteins responding to CD3/CD28-stimulation in Tconv, tTreg and iTreg cells (two-sided t-test, $p < 0.05$). Proteins with unique or shared upregulation (yellow), unique or shared downregulation (blue) or opposite regulation (red) are shown. **(C, E, G)** Volcano plots showing the proteomic responses of tTreg **(C)**, iTreg **(E)** or Tconv cells **(G)** to CD3/CD28-stimulation. Horizontal ($-\log_{10}$ p-value = 1.3) and vertical dotted lines ($\log_2$ fold change = 0) indicate applied cutoffs. Black and grey dots indicate proteins that are differentially expressed or not, respectively. **(D, F, H)** GSEA enrichment maps on the proteomic responses of tTreg **(D)**, iTreg **(F)** or Tconv cells **(H)** to CD3/CD28-stimulation, showing enriched gene sets (GO biological processes) in yellow (enriched upon CD3/CD28-stimulation) and blue (enriched upon no stimulation). Gene sets sharing a large number of proteins are clustered together and are connected by grey lines of which the thickness corresponds with the number of shared proteins. Clusters of similar biological processes are annotated using more general terms.

Figure 5

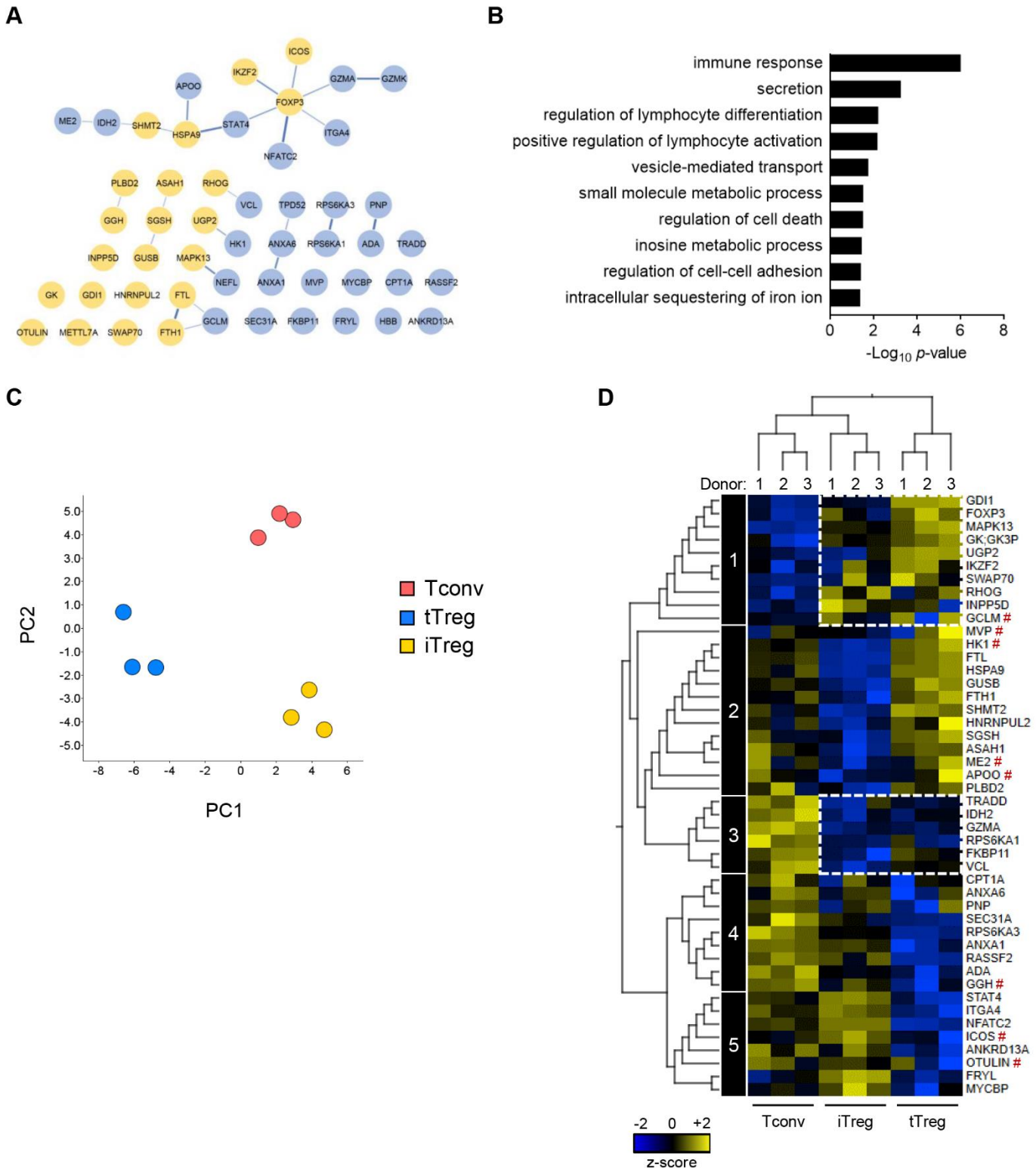


Figure 5. A Treg cell core signature is present in *in vitro*-expanded tTreg but not iTreg cells. **(A)** STRING network displaying 51 proteins of a previously defined Treg cell core proteomic signature[12]. Upregulated and downregulated proteins are indicated as yellow and blue nodes, respectively. **(B)** GO enrichment analysis of the Treg cell core signature proteins, showing 10 significantly enriched GO biological processes. **(C)** PCA plot based on expression of proteins of the Treg cell core signature in unstimulated Tconv, tTreg and iTreg cells (n = 3). **(D)** Heat map showing hierarchical clustering of 46 detected proteins of the Treg cell core signature in unstimulated Tconv, tTreg and iTreg cells. Z-scores showing relative protein expression values are color-coded. Numbered boxes (1-5) indicate clusters of proteins. The expression of indicated proteins (#) in tTreg cells deviates from the expected expression pattern according to the Treg cell core signature that was based on direct *ex vivo* analysis of cells from human blood cells.

present in iTreg cells. In our proteomics dataset, we detected 46 out of 51 proteins of the Treg cell core signature and evaluated their expression in expanded Tconv, tTreg and iTreg cells. PCA indicated distinct clustering of these three cell types based on Treg cell core signature protein levels (Figure 5C). We previously reported that the Treg cell core signature is largely preserved in tTreg cells following *in vitro* expansion[12], which was confirmed here (Figure 5D). Hierarchical clustering based on this signature also separated the three cell types. For a number of the Treg cell core signature proteins in cluster 1, characteristic high levels in tTreg cells were shared with iTreg cells. Furthermore, for part of the Treg cell core signature proteins in cluster 3 low levels were shared with iTreg cells, but not with Tconv cells. Nevertheless, the majority of the Treg cell core signature proteins was not expressed at characteristic levels in iTreg cells. For instance, iTreg cells showed high levels of the transcription factors STAT4 and NFATC2, while low levels in Treg cells as compared to Tconv cells are important for Treg cell identity[12]. These proteomic data suggest that iTreg cells share only a minor part of the unique characteristics of *ex vivo* Treg cells.

iTreg cells do not acquire the Treg cell core signature upon CD3/CD28-mediated activation

Since iTreg cells responded greatly to CD3/CD28-mediated restimulation in terms of altered protein expression, we determined whether they obtained more of the Treg cell core signature under these conditions. The core signature is present in both naïve and effector tTreg cells, indicating that core signature proteins in tTreg cells do not respond majorly to cell activation. This was indeed observed in a PCA, where levels of Treg cell core signature proteins did not alter in tTreg cells upon CD3/CD28-mediated restimulation (Figure 6A). Interestingly, the levels of these proteins also did not alter majorly in Tconv or iTreg cells upon restimulation (Figure 6A). Accordingly, a dendrogram that followed from hierarchical clustering showed grouping of core protein expression levels based on cell type, with tTreg cells being most distinct from Tconv and iTreg cells (Figure 6B). Heat map depiction clearly showed that core signature protein levels did not alter in any of the cell types upon CD3/CD28-mediated restimulation (Figure 6B). Part of the Treg cell core signature was shared between iTreg and tTreg cells, including proteins with either high or low expression levels compared to Tconv cells (clusters 2 and 3, respectively). These were largely the same proteins described before for unstimulated cells (Figure 5D). However, the major part of the Treg cell core signature proteins showed opposite expression between iTreg and tTreg cells, also after restimulation (clusters 1, 4 and 5). Differential expression of core signature proteins NFATC2, ITGA4 and ICOS between tTreg and iTreg cells was confirmed by flow cytometry (Figure 6C, Supplementary Figure 5). In conclusion, Treg cell core signature protein levels identify resting and activated tTreg cells, but not iTreg cells.

iTreg cells acquire properties of an effector Treg cell signature

Besides the Treg cell core signature, we previously observed a unique set of proteins that was expressed at high or low levels in *ex vivo* effector Treg cells alone, which sets them apart from Tconv cells and naïve Treg cells[12]. This was termed the effector Treg cell signature, of which the highly expressed proteins were involved in DNA replication (e.g. MCM proteins), apoptosis (e.g. FAS, CASP3),

Figure 6

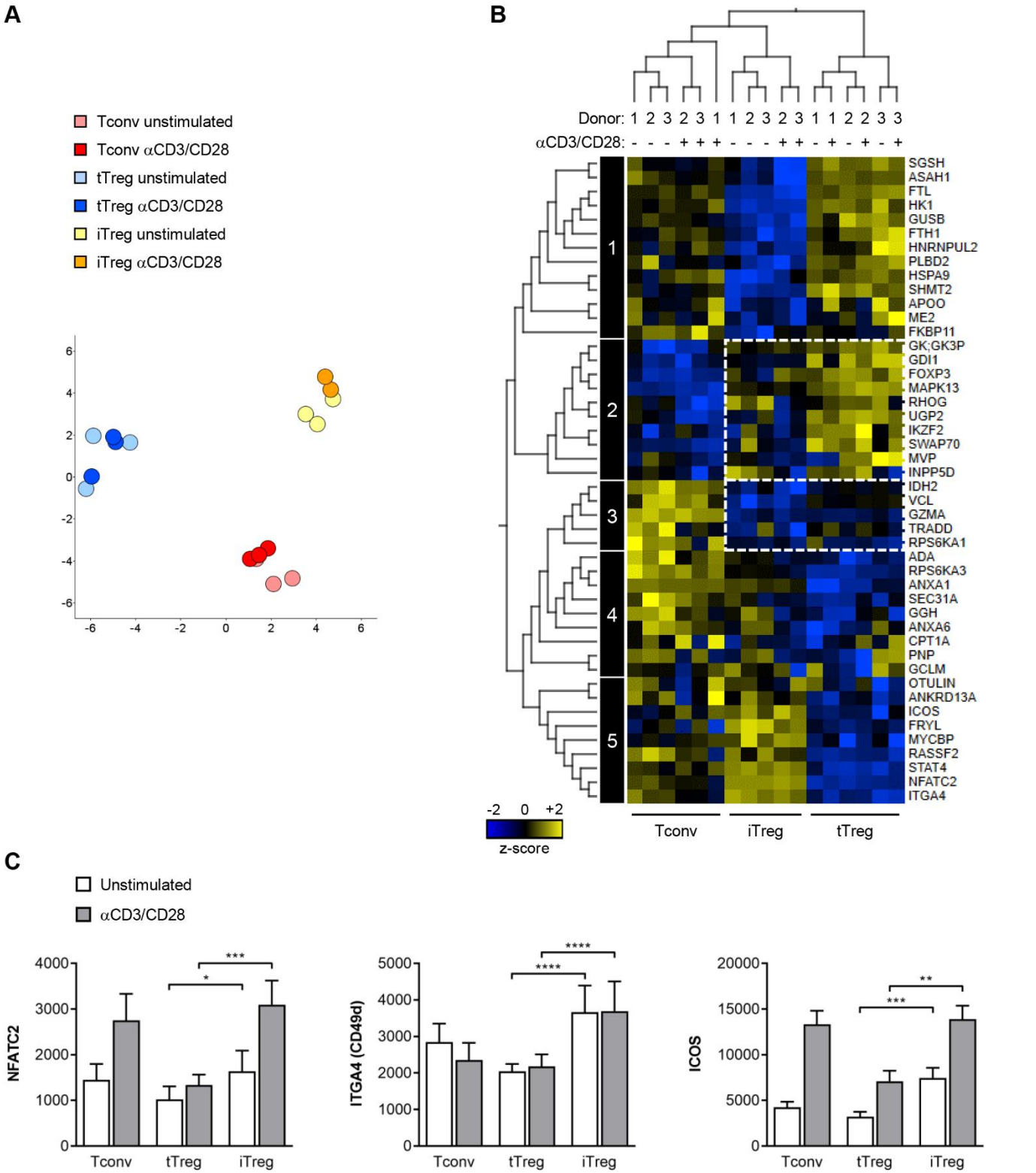


Figure 6. Expression levels of Treg cell core signature proteins do not change in any cell type upon CD3/CD28-mediated restimulation. **(A)** PCA plot based on expression of proteins of the Treg cell core signature in Tconv, tTreg and iTreg cells that were restimulated or not (n = 3, n = 2 only for CD3/CD28-stimulated iTreg cells). **(B)** Heat map showing hierarchical clustering of 46 detected proteins of the Treg cell core signature in Tconv, tTreg and iTreg cells that were CD3/CD28-stimulated or not. Z-scores showing relative protein expression values are color-coded. Numbered boxes (1-5) indicate clusters of proteins. **(C)** Quantification of flow cytometric analysis of the protein expression of total NFATC2 (n = 4), surface ITGA4 (CD49d) (n = 5) and surface ICOS (n = 5) on indicated cell populations. Mean fluorescence intensity (MFI) is depicted on the y-axis. Two-way ANOVA with Tukey's *post hoc* test was used for statistical analysis. Data are presented as mean ± SEM. Sample size (n) represents cells from individual donors, analyzed in independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

vesicular trafficking (e.g. STXBP2, NSF) and cytoskeletal reorganization (e.g. TUBA1B, TUBB) (Figure 7A). Lowly expressed proteins belonging to the effector Treg cell signature were associated with NF-κB signaling (e.g. NFKB1, NFKB2), JAK-STAT signaling (e.g. STAT3, IL7R), chemokine signaling (e.g. VAV1), TCR signaling (e.g. THEMIS, TRAT1) and phosphatidylinositol signaling (e.g. INPP4A, INPP4B) and also included members of the GIMAP family (GIMAP5, GIMAP7, GIMAP8) (Figure 7B). Here, we examined the effector Treg cell signature in unstimulated and restimulated Tconv, tTreg and iTreg cells and questioned whether iTreg cells possess effector Treg cell-like properties.

We detected all 41 effector Treg cell signature proteins with high expression levels. Interestingly, a large proportion of these proteins was highly expressed in iTreg cells, but not in tTreg cells, either before or after CD3/CD28-mediated stimulation, showing that iTreg cells have effector Treg cell-like properties that are absent in tTreg cells (clusters 1-2) (Figure 7C, Supplementary Table 3). Clusters 1 and 2 included proteins involved in DNA replication (e.g. MCM proteins) and cytoskeletal processes such as cytokinesis (e.g. MYH9, CORO1C), suggesting that iTreg cells are highly proliferative. Furthermore, we observed that among cell surface molecules, iTreg cells highly expressed the surface molecules CCR4 and HLA-DRA, while tTreg cells highly expressed FAS (CD95). By flow cytometry, we confirmed that CCR4 and HLA-DR had highest expression on iTreg cells, whereas FAS was indeed highly expressed on tTreg cells (Figure 7D, Supplementary Figure 6).

We detected 33 out of 39 effector Treg cell signature proteins with low expression levels (Figure 7E, Supplementary Table 3). Cluster 1 included proteins that play a role in signaling downstream of immune receptors, such as NFKB1, NFKB2, CARD11, STAT3, VAV1, STAM2 and INPP4B. In contrast to low expression of these proteins in tTreg cells, iTreg cells shared high protein expression with Tconv cells. Cluster 2 proteins included INPP4A, THEMIS and members of the GIMAP family that are involved in immune regulation. These were expressed at low levels in both tTreg and iTreg cells. Cluster 3 comprises miscellaneous proteins that were mainly expressed at low levels in iTreg cells, but not in tTreg cells. These data show that the effector Treg cell signature is partially present in iTreg cells, which indicates that iTreg cells resemble *ex vivo* effector Treg cells to some extent.

DISCUSSION

In this study, we used recently published proteomic signatures of *ex vivo* Treg cells as benchmarks to determine to what extent *ex vivo* Treg cells are resembled by iTreg and tTreg cells[12]. Using Tconv, tTreg and iTreg cells, we first analyzed the expression of a number of cell surface molecules that are commonly associated with Treg cells, including costimulatory and co-inhibitory receptors that regulate cell survival, cell metabolism and proliferation. The expression of CTLA-4 by Treg cells is an important mediator of their suppressive function, as it results in downregulation of CD80 and CD86 on antigen-presenting cells such as dendritic cells, rendering them incapable of costimulating Tconv cells[7]. While we observed that total (cell surface plus intracellular) CTLA-4 expression was upregulated in iTreg cells compared to Tconv cells, both total and surface CTLA-4 expression were highest in tTreg cells.

Figure 7

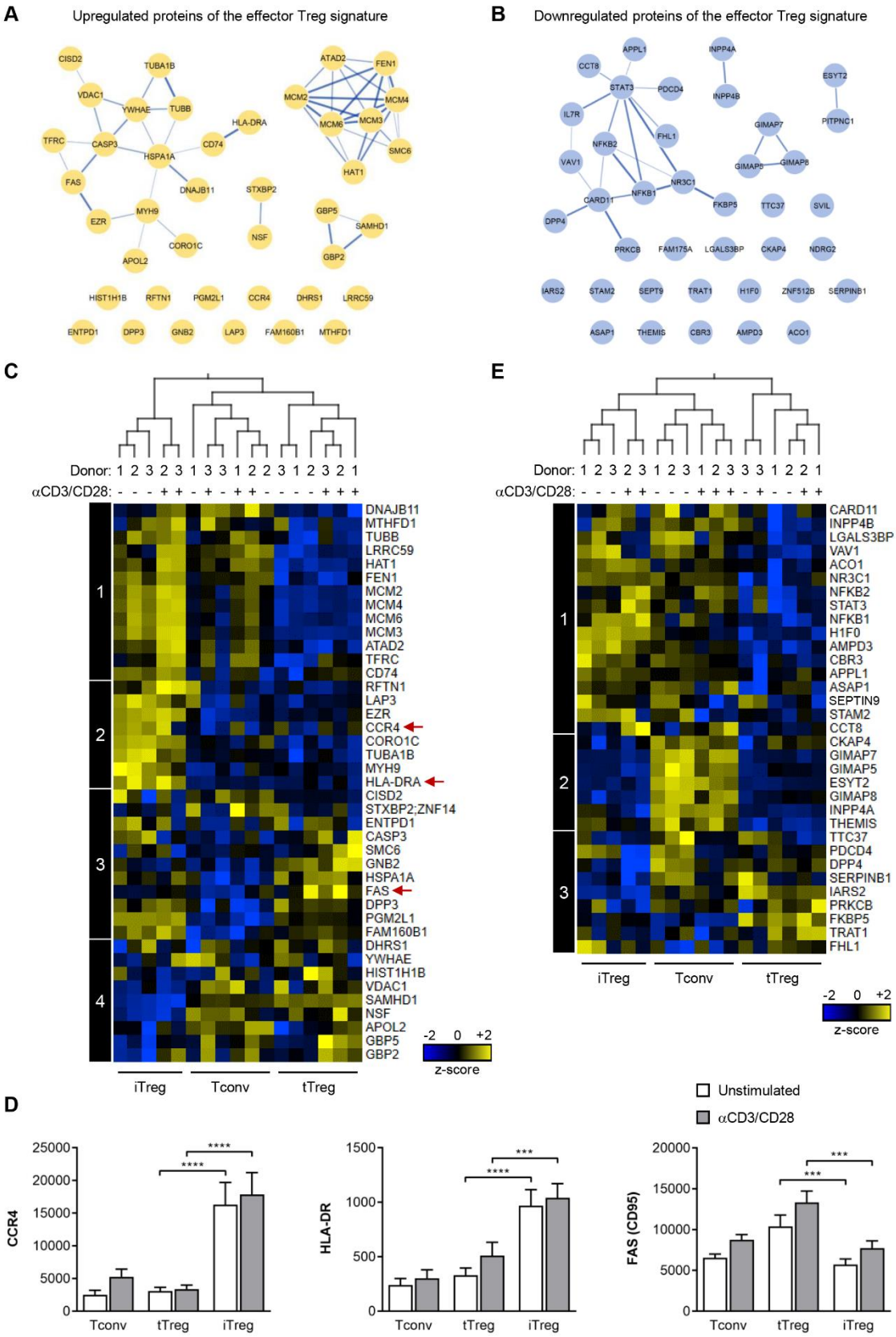


Figure 7. Properties of an effector Treg cell signature are present in iTreg cells. **(A, B)** STRING networks showing 41 upregulated **(A)** or 39 downregulated proteins **(B)** of a previously defined effector Treg cell proteomic signature[12] as yellow and blue nodes, respectively. **(C)** Heat map showing hierarchical clustering of 41 detected proteins of the upregulated part of the effector Treg cell signature in Tconv, tTreg and iTreg cells that were restimulated or not (n = 3, n = 2 for CD3/CD28-stimulated iTreg cells). Z-scores showing relative protein expression values are color-coded. Numbered boxes (1-4) indicate clusters of proteins. **(D)** Quantification of flow cytometric analysis of the protein expression of surface CCR4 (n = 4), surface HLA-DR (n = 4) and surface FAS (CD95) (n = 4) on indicated cell populations. Mean fluorescence intensity (MFI) is depicted on the y-axis. Two-way ANOVA with Tukey's *post hoc* test was used for statistical analysis. Data are presented as mean ± SEM. Sample size (n) represents cells from individual donors, analyzed in independent experiments. ***p < 0.001, ****p < 0.0001. **(E)** Heat map showing hierarchical clustering of 33 detected proteins of the downregulated part of the effector Treg cell signature in Tconv, tTreg and iTreg cells that were restimulated or not (n = 3, n = 2 for CD3/CD28-stimulated iTreg cells). Z-scores showing relative protein expression values are color-coded. Numbered boxes (1-3) indicate clusters of proteins.

Likewise, costimulatory receptors OX40 and GITR had highest expression on tTreg cells. On the other hand, co-inhibitory receptor PD-1 was upregulated on iTreg and Tconv cells, while minimally present on tTreg cells. In mice that are genetically deficient for PD-1 in Foxp3-expressing cells, it has recently been shown that PD-1 inhibits Treg cell activation, proliferation and suppressive function[22]. Since we observed that CD3/CD28-stimulated iTreg and Tconv cells express PD-1 as opposed to tTreg cells, iTreg and Tconv cells may be more susceptible to PD-1 targeting than tTreg cells. Lastly, we observed that only iTreg cells expressed high levels of the ectonucleotidase CD39 (ENTPD1), which plays a role in immune suppression by hydrolyzing extracellular ATP, a pro-inflammatory molecule, to ADP and AMP[8]. These data indicate that iTreg and tTreg cells differentially express surface molecules characteristic to Treg cell responses, suggesting that tTreg and iTreg cells utilize different suppressive mechanisms.

We performed proteomics to globally compare protein expression in Tconv, tTreg and iTreg cells. First, we analyzed unstimulated cells and observed distinct protein expression profiles between the cell types. iTreg cells showed partial overlap with Tconv cells, which was not unexpected as iTreg cells were generated from Tconv cells. Strikingly, the iTreg cell proteome showed only minimal overlap with the tTreg cell proteome and had many unique features instead, which were associated with an active mitotic phenotype and also involved many proteins related to cell metabolism. Following CD3/CD28-mediated restimulation, strong proteomic responses were observed for iTreg and Tconv cells but not for tTreg cells, which resulted in multiple biological processes predicted to be upregulated or downregulated, such as an increase of cell division. Together, these data indicate that the generation of iTreg cells does not alter their proteome towards the phenotype of tTreg cells, but instead creates cells with a distinct phenotype.

In physiology, Treg cells are challenged to operate in pro-inflammatory conditions without compromising their suppressive identity. We previously defined a Treg cell core proteomic signature that distinguishes *ex vivo* naïve and effector Treg cells from $CD4^+$ Tconv cells in human peripheral blood[12]. This signature strongly suggests differential wiring of signaling pathways, given the constitutive differential expression of key signal transduction components between Treg and Tconv cells. For example, the Treg cell core signature includes low expression of STAT4 and NFATC2, which are major regulators of inflammatory cytokine expression[23, 24]. Low expression of these molecules may aid Treg cells to protect their identity in an inflammatory environment by preventing them from producing effector cytokines, while they can still respond to inflammatory cytokines in other ways. Indeed, it has been demonstrated that deliberate overexpression of STAT4 in Treg cells increases their susceptibility to inflammatory cytokines, as IL-12 or type I IFN provoked the production of effector cytokines and loss of FOXP3 expression[12]. We evaluated proteins of the Treg cell core signature in the proteomes of Tconv, tTreg and iTreg cells and observed that iTreg cells only share a minor part of the characteristics that *ex vivo* Treg cells have, while the signature was present in tTreg cells. Importantly, both STAT4 and NFATC2 were highly expressed in iTreg cells compared to tTreg cells, suggesting that iTreg cells are more susceptible to respond to inflammatory cues in a pro-inflammatory manner, whereas tTreg cells can maintain their suppressive identity. When cells were subjected to CD3/CD28-mediated

restimulation, the levels of Treg cell core signature proteins did not alter majorly, indicating that the Treg cell core signature identifies resting and activated tTreg cells, but not iTreg cells.

We also examined protein levels from a previously defined effector Treg cell proteomic signature in Tconv, tTreg and iTreg cells[12]. Whereas the Treg cell core signature was hardly present in iTreg cells, these cells did show properties of the effector Treg cell signature, indicating that iTreg cells resemble *ex vivo* effector Treg cells to some extent. Multiple MCM proteins, which are involved in DNA replication, were highly expressed in iTreg cells as compared to tTreg cells, showing a more proliferative character that was also identified in our global proteome analysis. *Ex vivo* Treg cells from human peripheral blood have been reported to be even more proliferative than Tconv cells[25]. Our findings suggest that this *ex vivo* Treg cell population could be composed of a combination of pTreg and tTreg cells, as we did not observe highly proliferative features in purified tTreg cells.

In conclusion, our proteomic analysis revealed that iTreg cells are distinct from both tTreg and Tconv cells. Therefore, we propose that experiments in which iTreg cells are employed as a model for studying Treg cell differentiation, function or metabolism should be interpreted with caution. We also found that a previously defined Treg cell core signature[12] was largely absent in iTreg cells, while clearly present in simultaneously analyzed tTreg cells. However, a previously identified effector Treg cell signature[12] was partially present in iTreg cells, indicating that iTreg cells show some resemblance to *ex vivo* effector Treg cells. These findings demonstrate that the core and effector Treg cell signatures are important reference tools to define Treg cell populations.

METHODS

Cell isolation and flow cytometric sorting

Human materials were obtained in compliance with the Declaration of Helsinki and the Dutch rules regarding the use of human materials from voluntary donors. Buffy coats from healthy anonymized male donors were obtained after their written informed consent, as approved by the internal ethical board of Sanquin, the Netherlands. Total $CD4^+$ T cells were collected from buffy coats by first performing a Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC) and subsequently using CD4 magnetic MicroBeads (Miltenyi Biotec) according to the manufacturers' protocols. Alternatively, total $CD4^+$ T cells were directly isolated from buffy coats using the StraightFrom Buffy Coat CD4 MicroBead kit (Miltenyi Biotec) according to the manufacturer's instructions. To sort naïve Tconv and tTreg cells, $CD4^+$ T cells were stained with CD4-PE-Cy7, CD127-BV421 (BioLegend), CD25-PE (BD Biosciences), CD45RA-FITC (Immunotools) and GPA33-AF647 (ref. [26]) monoclonal antibodies (mAbs) as indicated (Supplementary Figure 1). Cells were sorted on a MoFlo Astrios using Summit software version 6.2 (Beckman Coulter) or BD FACS Aria II using FACSDiva software version 8 (BD Biosciences). Either propidium iodide (Sigma) or near-IR dead cell stain kit (Invitrogen) was used as a live/dead marker.

T cell expansion cultures and restimulation

Following cell sorting, human naïve Tconv and tTreg cells were cultured in 96-well round bottom plates (Greiner) at 1 × 10^4 cells per well using IMDM (Gibco, Life Technologies) supplemented with 8% FCS (Sigma), penicillin/streptomycin (Roche) and 300 IU/ml IL-2 (DuPont Medical), hereinafter referred to as T cell medium, at 37°C and 5% CO_2. Cells were expanded using agonistic mAbs against CD3 (clone CLB-T3/4.E, IgE isotype, Sanquin, 0.1 µg/ml) and CD28 (clone CLB-CD28/1, Sanquin, 0.2 µg/ml) in solution. After 4 days, fresh T cell medium was added. After 7 days from the start of culture, cells were cultured in 6-well plates (Corning) at 1 × 10^6 cells per ml for 4 days using fresh T cell medium in the absence of agonistic mAbs. To generate iTreg cells, naïve Tconv cells were cultured as described above in the presence of TGF-β (Peprotech, 10 ng/ml). Prior to restimulation experiments, dead cells were removed by Ficoll-Paque Plus density gradient centrifugation. T cells were restimulated as indicated using T cell medium in the presence of agonistic mAbs against CD3 (0.1 µg/ml) and CD28 (0.2 µg/ml), but without TGF-β.

Flow cytometry

For analysis of the expression of cell surface molecules, T cells were washed in PBS/1% FCS and stained in the presence of the following mAbs in suitable combinations: anti-OX40-PE-Cy7 (BioLegend), anti-GITR-BV421 (BioLegend), anti-PD-1-APC-Cy7 (BioLegend), anti-CD39-BV510 (BioLegend), anti-ITGA4-PE (CD49d) (BD Biosciences), anti-ICOS-PerCP-Cy5.5 (BioLegend), anti-CCR4-PE-Cy7 (BioLegend), anti-HLA-DR-APC-eFluor 780 (Invitrogen), anti-HLA-DR-BV605 (BD Biosciences) and anti-FAS-BB700 (CD95) (BD Biosciences). Cell surface expression of CTLA-4 was measured by adding anti-CTLA-4-PE-Dazzle594 mAb (BioLegend) in culture during restimulation for 24 h. Near-IR dead cell stain kit (Invitrogen) was used as a live/dead marker. For intracellular staining, cells were fixed and permeabilized using the FOXP3 transcription factor staining buffer set (Invitrogen) according to the manufacturer's instructions. Next, cells were stained with combinations of anti-FOXP3-APC (Invitrogen), anti-Helios-PE-Cy7, anti-CTLA-4-PE-Dazzle594 (BioLegend) and anti-NFATC2-AF488 (Cell Signaling Technology) mAbs. Flow cytometry was performed using a BD LSR Fortessa or BD LSR II cell analyzer (BD Biosciences). Sample acquisition was performed using FACSDiva software version 8 and data were analyzed using FlowJo software version 10.6.0.

Methylation assay of the TSDR

The methylation status of the TSDR within the *FOXP3* locus was determined in Tconv, tTreg and iTreg cells as described previously[18]. In brief, expanded T cells were resuspended in PBS for proteinase K digestion, followed by bisulfite conversion of DNA using the EZ DNA Methylation-Direct kit (Zymo Research) and methylation-specific quantitative PCR using iQTM SYBR Green Supermix (Bio-Rad)[27]. Methylation-specific primers were 5'-CGATAGGGTAGTTAGTTTTCGGAAC-3' and 5'-CATTAACGTCATAACGACCGAA-3'. Demethylation-specific primers were 5'-TAGGGTAGTTAGTTTTTGGAATGA-3' and 5'-CCATTAACATCATAACAACCAAA-3'. Melt curve analysis was performed on a LightCycler® 480-II (Roche). Methylation of the TSDR (%) was calculated

using the following formula: $100/(1+2^{Ct[CG]-Ct[TG]})$, where Ct[CG] is defined as Ct values obtained using methylation-specific primers and Ct[TG] is defined as Ct values obtained using demethylation-specific primers.

Suppression assay

Total human PBMCs were washed and resuspended in PBS, followed by incubation for 8 min using 5 µM CellTrace™ Violet dye (Invitrogen). Following fluorescent labeling, an equal volume of FCS was added and cells were subsequently washed twice in IMDM supplemented with 8% FCS. CellTrace Violet-labeled PBMCs were cocultured with expanded Tconv, tTreg or iTreg cells for 96 h at indicated ratios, in the presence of anti-CD3 mAb (0.05 µg/ml). Proliferation of PBMCs was examined by tracing CellTrace Violet dye dilution by flow cytometry on a BD LSR Fortessa or BD LSR II cell analyzer. Data were analyzed using FlowJo software (version 10.6.0).

Proteomics

Proteomics data were generated as described before by Cuadrado et al.[12], including analysis of expanded Tconv, tTreg and iTreg cells. In short, at least 1×10^6 expanded T cells were restimulated for 24 h using T cell medium in the presence of agonistic mAbs against CD3 (0.1 µg/ml) and CD28 (0.2 µg/ml) or not. Unstimulated and restimulated cells were washed, lysed and processed for mass spectrometry.

RAW mass spectrometry files were processed previously according to Cuadrado et al.[12], followed by the removal of potential contaminants and reverse hits using Perseus (version 1.6.12). Label-free quantification (LFQ) values were $\log_2$-transformed and the biological replicates were grouped based on cell type. Proteins with a minimum of 5 valid values in at least one cell type were included for further analysis. For analysis of the effector Treg cell signature, a minimum of 4 valid values in at least one cell type was used. Missing value imputation was performed by replacing missing values by random numbers drawn from the lower part of the normal distribution (width = 0.3, shift = 1.8). Next, proteomics data were analyzed by PCA, ANOVA and Student's *t*-tests using Qlucore Omics Explorer (version 3.7). After z-score normalization, clusters of proteins with similar expression patterns were identified using unsupervised hierarchical clustering, visualized in heat maps. Protein clusters were subjected to overrepresentation analysis to assess enrichment of GO biological processes using the STRING app (version 1.6.0) in Cytoscape (version 3.8.2). To refine the analysis of biological processes, a redundancy cutoff was applied when the top 10 significant GO terms contained large overlap based on enriched genes. The proteomic response to restimulation was analyzed for each cell type using GO biological processes gene sets in GSEA software (version 4.1.0). GSEA was performed using default parameters and gene set permutations. To identify related groups of biological processes that were differentially regulated following restimulation of T cells, the EnrichmentMap app (version 3.3.2) was used in Cytoscape. The largest gene set clusters were displayed using manually assigned annotations that describe the general biological processes represented by the clusters. To assess commonalities and differences of the proteomic response of each cell type, the DiVenn tool[28] was used.

Statistical analysis

Data were analyzed using GraphPad Prism (version 9.0.1), except for proteomics data. Statistical analyses were performed as indicated in the figure legends. Log_2 transformation was performed when data were not normally distributed. Data are presented as mean ± SEM and a two-sided $p < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTIONS

M.M., S.d.K. and J.B. designed the study and wrote the manuscript; M.M. and S.d.K. performed experiments and analyzed the data; E.S. provided technical assistance; M.v.d.B. performed mass spectrometry measurements; D.A. provided conceptual advise in the study design and contributed to the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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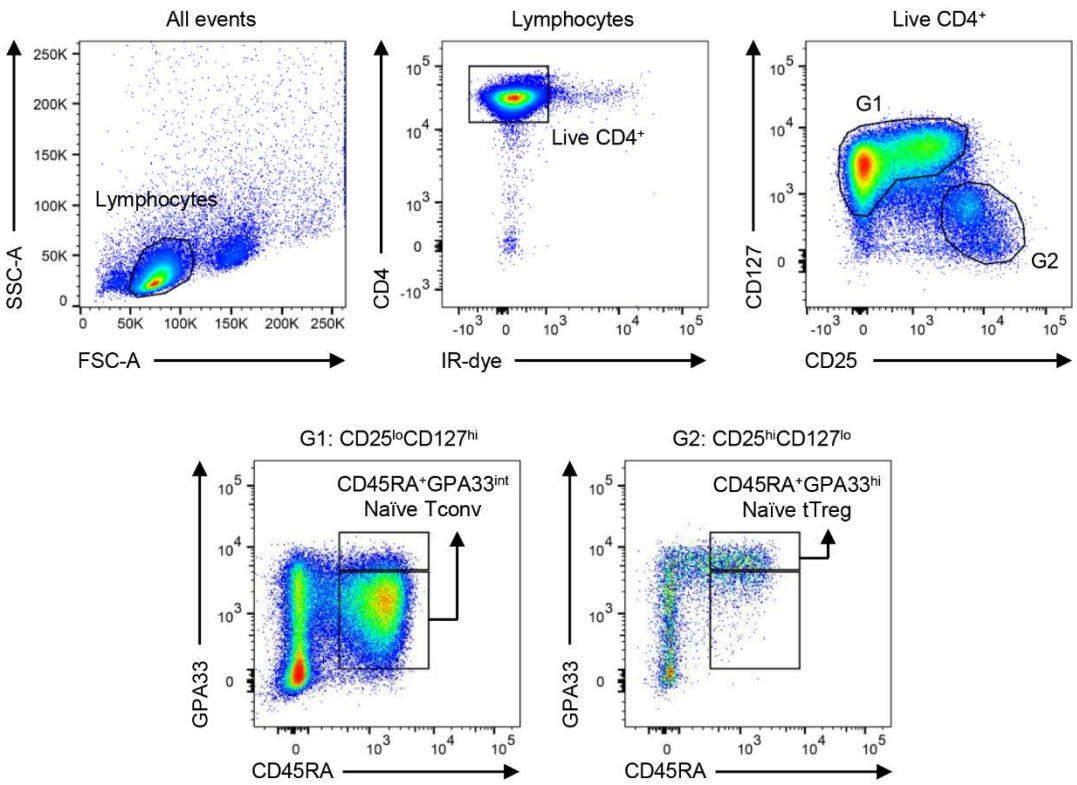
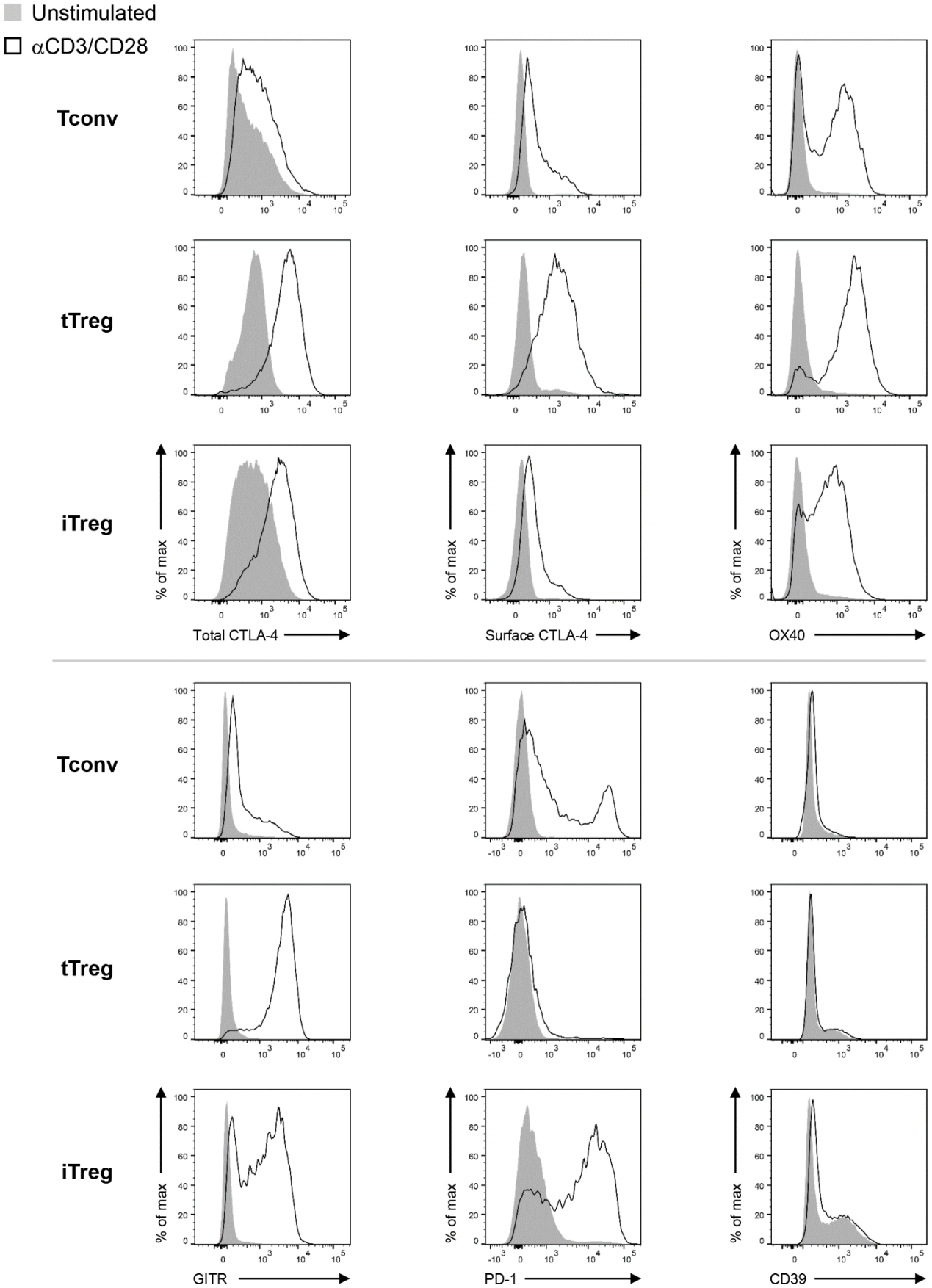
Supplementary Figure 1



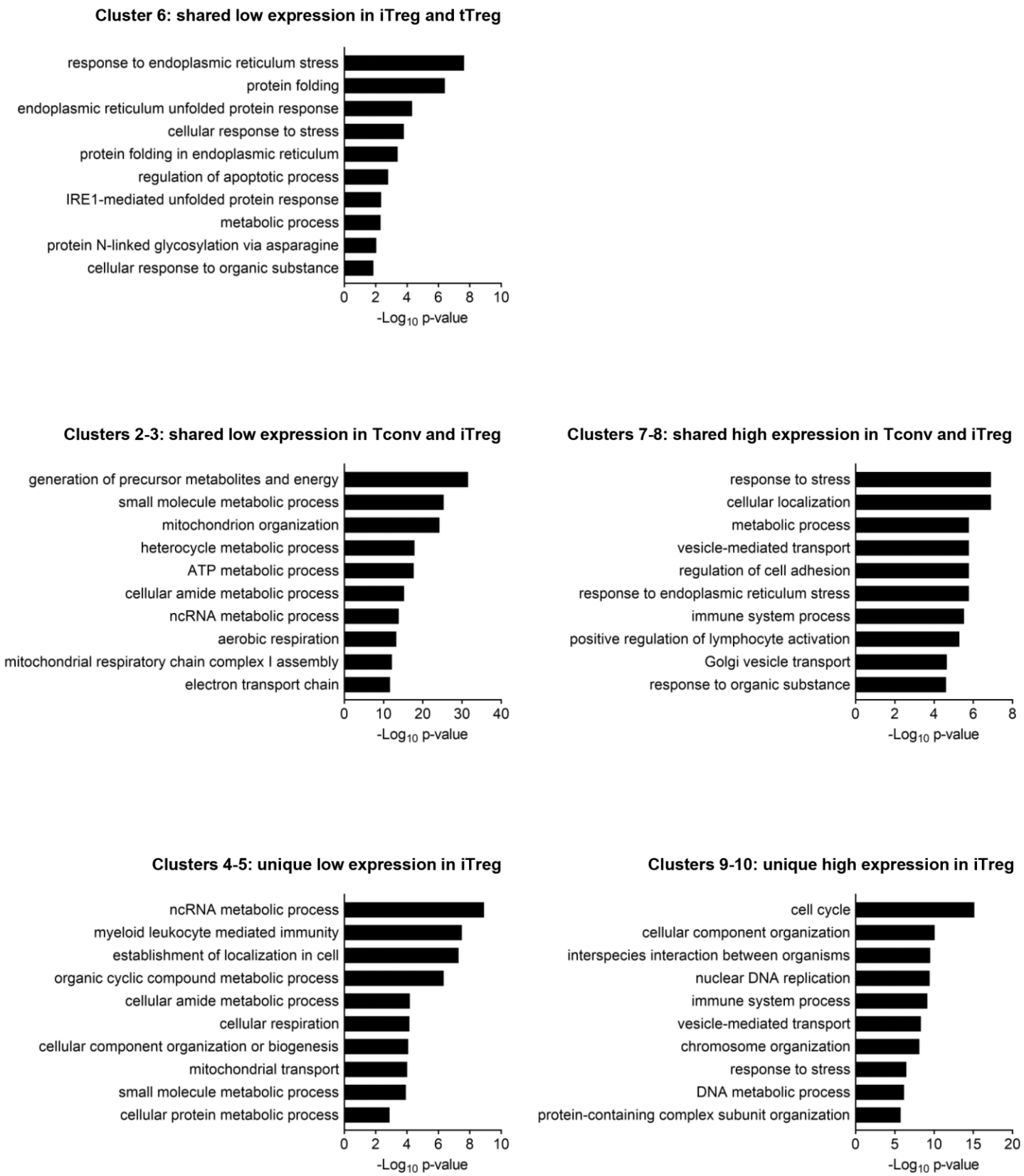
Supplementary Figure 1. Gating strategy to sort naïve Tconv and naïve tTreg cells from human peripheral blood by flow cytometry. Pre-enriched total $CD4^+$ T cells from human peripheral blood were first separated into live $CD4^+CD25^{lo}CD127^{hi}$ (Gate 1) and $CD4^+CD25^{hi}CD127^{lo}$ (Gate 2) cells. Subsequently, $CD45RA^+GPA33^{int}$ naïve Tconv and $CD45RA^+GPA33^{hi}$ naïve tTreg cells were selected within Gate 1 and Gate 2, respectively.

Supplementary Figure 2



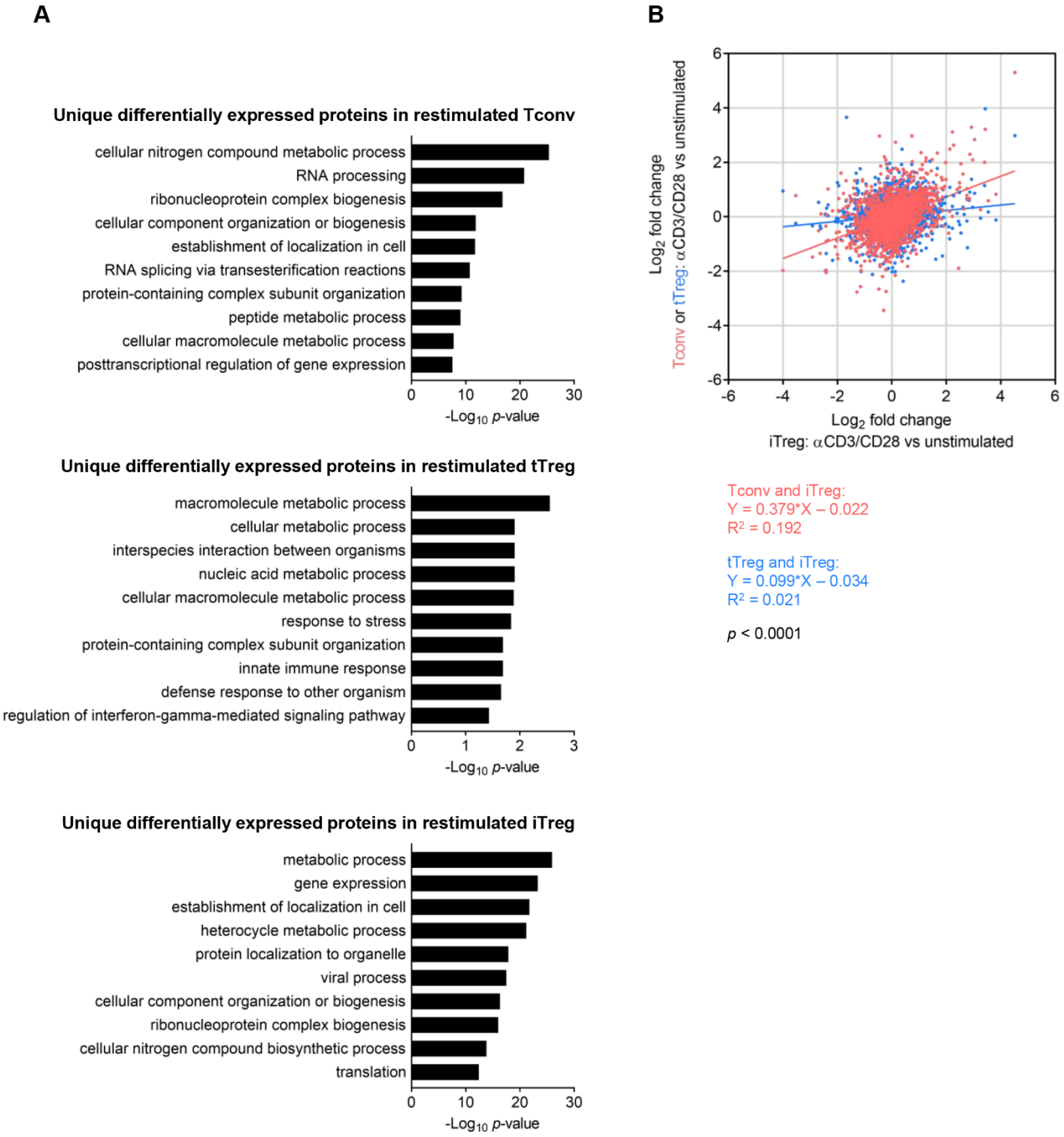
Supplementary Figure 2. Representative flow cytometry histograms showing protein expression of total CTLA-4 (*n* = 13), surface CTLA-4 (*n* = 8), surface OX40 (*n* = 8), surface GITR (*n* = 8), surface PD-1 (*n* = 8) and surface CD39 (*n* = 8) on Tconv, tTreg and iTreg cells that were restimulated via CD3/CD28 or not.

Supplementary Figure 3



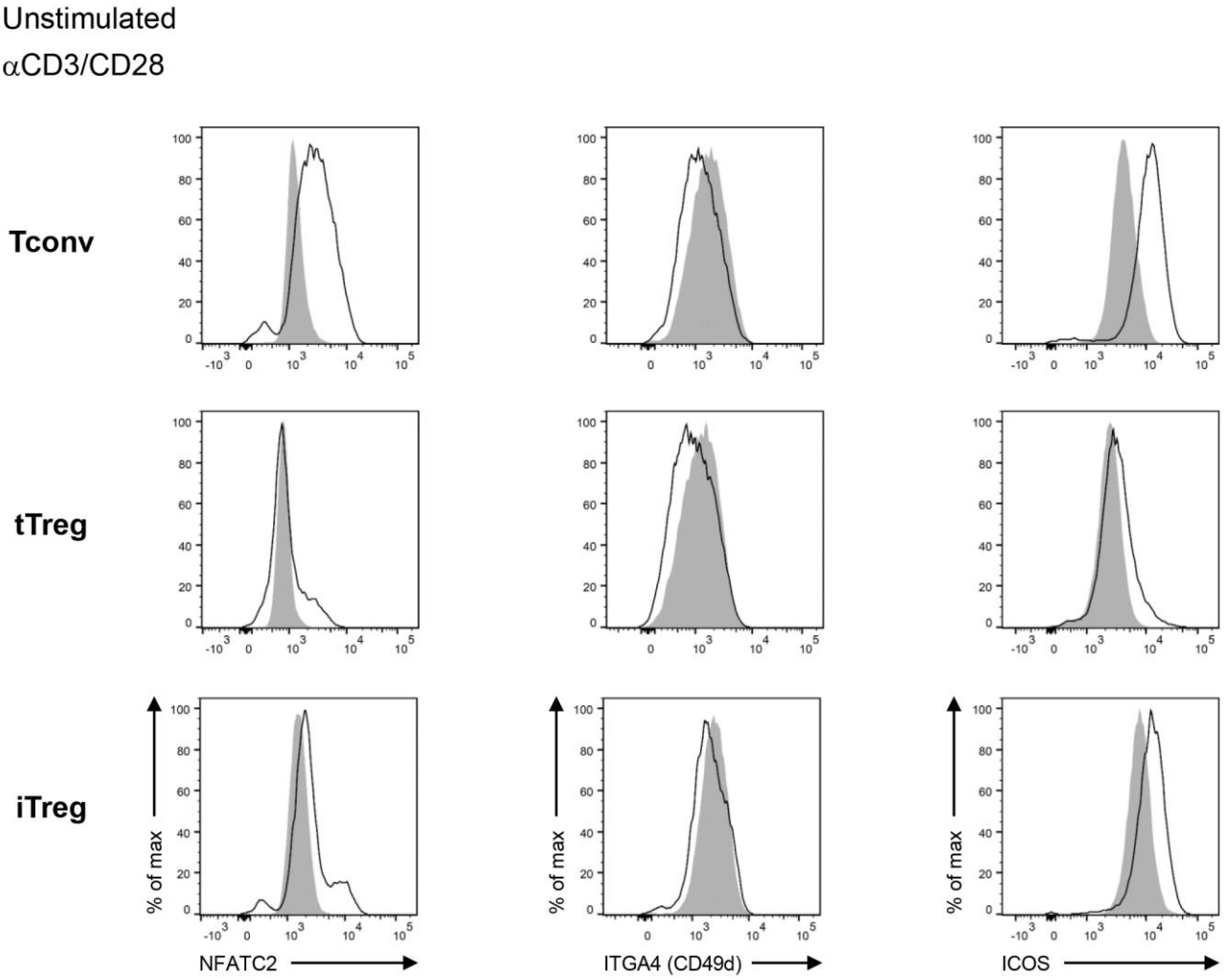
Supplementary Figure 3. GO (biological processes) enrichment analyses of indicated clusters of proteins that were differentially expressed between unstimulated Tconv, tTreg and iTreg cells ($p < 0.05$).

Supplementary Figure 4



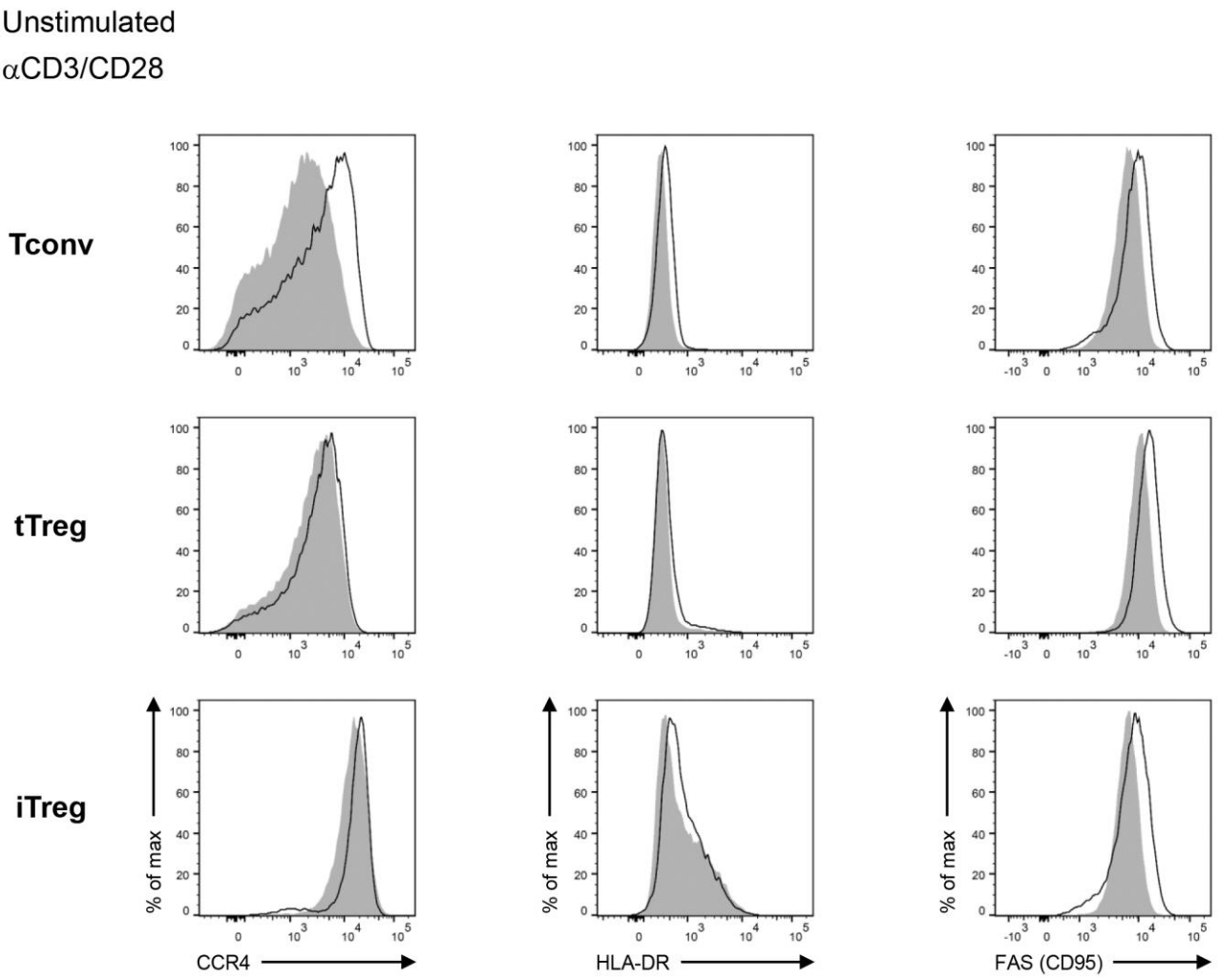
Supplementary Figure 4. Analysis of the proteomic responses of Tconv, tTreg and iTreg cells to CD3/CD28-mediated restimulation. **(A)** GO (biological processes) enrichment analyses of unique differentially expressed proteins in restimulated Tconv, tTreg or iTreg cells ($p < 0.05$). **(B)** Linear regression analysis of the proteomic response of iTreg cells (x-axis) compared to the responses of either Tconv (red, y-axis) or tTreg (blue, y-axis) cells, revealing different regression lines ($p < 0.0001$).

Supplementary Figure 5



Supplementary Figure 5. Representative flow cytometry histograms showing protein expression of total NFATC2 (*n* = 4), surface ITGA4 (CD49d) (*n* = 5) and surface ICOS (*n* = 5) on Tconv, tTreg and iTreg cells that were restimulated via CD3/CD28 or not.

Supplementary Figure 6



Supplementary Figure 6. Representative flow cytometry histograms showing protein expression of surface CCR4 (*n* = 4), surface HLA-DR (*n* = 4) and surface FAS (CD95) (*n* = 4) on Tconv, tTreg and iTreg cells that were restimulated via CD3/CD28 or not.

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1. All 4224 detected proteins. **(Tab 1)** List of all 4224 detected proteins that were analyzed in this study. **(Tab 2)** List of all 4224 detected proteins that were analyzed in this study (without missing value imputation).

Supplementary Table 2. List of 1290 proteins that were differentially expressed between unstimulated Tconv, tTreg and iTreg cells (Figure 3B, $p < 0.05$) (all samples are shown).

Supplementary Table 3. Detected proteins of the effector Treg cell signature. **(Tab 1)** List of detected proteins of the effector Treg cell signature, using a different filter for valid values as described in the Methods. Values in yellow and blue indicate the upregulated and downregulated part of the effector Treg cell signature, respectively. **(Tab 2)** List of detected proteins of the effector Treg cell signature, using a different filter for valid values as described in the Methods, without missing value imputation. Values in yellow and blue indicate the upregulated and downregulated part of the effector Treg cell signature, respectively.