TCR- and IL-2-dependent transcriptomes of human regulatory T cells identifies CEACAM1 as an IL-2R- and BLIMP-1-dependent checkpoint

or

CEACAM1 is an IL-2R- and BLIMP-1-dependent Treg checkpoint that marks IL-2 responsive cells during low-dose IL-2 therapy for autoimmunity

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**Abstract**

Although regulatory T cells (Tregs) constitutively express high levels of the IL-2R, the contribution of IL-2R signaling in the expansion of human Tregs is poorly understood. Here we performed genome-wide transcriptome analyses to identify IL-2R-dependent processes for human Tregs. The main role of initial IL-2R signaling in Tregs is to largely enhance gene expression induced through the TCR and CD28. *CEACAM1* was identified as one of only a few genes that was primarily induced by IL-2. Tregs expressed higher amount of CEACAM1 than T effector/memory cells in vitro or in autoimmune patients undergoing low-dose IL-2 therapy. CEACAM1 expression is promoted by extensive IL-2-dependent expansion but inhibited by TCR/CD28 activation and is associated with opening of chromatin and positive regulation by BLIMP-1. Tregs express the long isoform of CEACAM1 which contains ITIM motifs that inhibit TCR signaling. Correspondingly, knockout of *CEACAM1* in human Tregs by Crispr/Cas9 in vitro led to increased TCR-dependent Treg proliferation whereas CEACAM1+ Tregs in patients undergoing low-dose IL-2 therpy showed reduced proliferation. Collectively, our findings indicate that CEACAM1 is a checkpoint in Tregs to limit TCR dependent processes that may function to regulate Treg homeostasis and is a highly IL-2R-selective biomarker for low-dose IL-2 therapy.

**Introduction**

The homeostasis and activation of regulatory T cells (Tregs) in vivo depend not only on TCR signaling, but also co-stimulatory and cytokine signaling, especially through CD28 and IL-2R (*1-5*). For mouse Tregs in vivo, TCR signaling is essential for the development of activated effector Tregs (eTregs) while CD28 promotes Treg homeostasis by providing proliferative and survival signals, in part through Bcl-XL (*6, 7*). Under steady state conditions, IL-2R signaling supports homeostasis of Tregs, largely through survival signaling, in part through Bcl-2, where resting central Tregs (cTregs) show a more acute requirement for IL-2 (*8, 9*). The development of terminally differentiated eTregs also depends on IL-2 (*8, 10*). Although Treg proliferation can occur in vivo without IL-2, administering IL-2 to mice or humans lead to Treg expansion (*11*), which is the basis for low-dose Treg-directed therapy for autoimmunity and other inflammatory disorders (*11, 12*). These IL-2-dependent proliferative response may be shaped by endogenous TCR and co-stimulatory signaling (*2, 13*).

Our understanding of the role of TCR, CD28, and IL-2R signaling for human Tregs has been limited to in vitro studies. Optimal expansion and functional activity of Tregs in vitro require crosslinking of the TCR and CD28 plus high amounts of IL-2 (*14-17*). Extensive studies with human Tregs show that crosslinking only the TCR and CD28 supports suboptimal proliferative responses (*1, 18, 19*). Paradoxically and in striking contrast to conventional T cells activated through their TCR, Tregs fail to proliferate solely to IL-2 in vitro, even though these cells express high amounts of the high affinity IL-2R (*18, 20*). Indeed, optimizing anti-CD3, anti-CD28, and IL-2 stimulation of Tregs has been critical for the production of therapeutic numbers of Tregs for adoptive therapy to promote immune tolerance (*21*). Although signaling induced by these 3 agents are critical for expansion of human Tregs in vitro, little is known about the molecular basis concerning the relative roles of TCR/CD28 vs IL-2R signaling in initiating these responses.

The current study was undertaken with two main goals. First was to molecularly define the relative outcomes of TCR/CD28 and IL-2R signaling for human Tregs by RNA-seq to better understand the contribution of IL-2R signaling to Treg activation and proliferation. The second objective was to identify genes that were highly induced by IL-2R, but not TCR/CD28, signaling and investigate their function and as potential IL-2R-dependent selective biomarkers related to low-dose IL-2 therapy. CEACAM1 (CD66a) was identified with these properties.

CEACAM1 is upregulated as a consequence of TCR and IL-2R signaling (*22, 23*). CEACAM1 is a type 1 glycoprotein with multiple isoforms and broad tissue distribution. With respect to conventional T cells, the long isoform appears to dominate, where its expression is largely restricted to activated CD4+ and CD8+ T effector (Teff) cells (*22-24*). The intracytoplasmic region of the long isoform of CEACAM1 contains 2 immunoreceptor tyrosine based inhibitory motifs (ITIMS) that limit TCR signaling to reduced proliferation, secretion of TH1 cytokines and CTL activity (*25*). The expression, regulation and function of CEACAM1 has not been explored in Tregs. We examined these points for human CEACAM1+ and CEACAM1 knockout (CEACAM1KO) Tregs in vitro, the latter after Crispr/Cas9-mediated editing of CEACAM1. We also evaluated CEACAM1 as an IL-2-dependent biomarker in autoimmune patients undergoing low-dose IL-2 therapy.

**Results**

**IL-2R signaling mainly functions to enhance TCR/CD28-dependent activation in Tregs**

To define the relative contributions of initial IL-2R- vs TCR/CD28-dependent signaling in human Tregs, FACS-purified CD4+ CD25hi CD127lo (typically>90% Foxp3+; **Fig. S1A**) human Tregs were cultured for 4 and 16 hr in media alone or with IL-2, anti-CD3/anti-CD28 plus anti-IL-2 to inhibit potential endogenous IL-2 from any contaminating Teff cells, or a combination of anti-CD3/anti-CD28 and IL-2. Genome-wide profiling of the Tregs from these cultures by RNA-seq revealed that in comparison to cells cultured in only media, 2616 and 6239 unique genes were up- or down-regulated 1.5-fold (FDR<0.01) in one or more culture conditions at 4 and 16 hr, respectively (**Fig. 1A**). At 4 and 16 hr, IL-2 regulated many fewer genes (455 and 1405) than stimulation through TCR/CD28 (1663 and 4698) or IL-2/CD3/CD28 (2309 and 5899). At both time points, 42.8% and 79.6% of the IL-2-dependent genes were also activated by TCR/CD28 whereas approximately 3% are solely responsive to IL-2.

Hierarchical clustering of the differentially expressed genes at 4 and 16 hr confirmed that few genes are solely responsive to IL-2, e.g. cluster 4 at 4 hr and cluster 6 at 16 hr (**Fig. 1B**), whereas in the former cluster the response by IL-2/TCR/CD28 likely reflects that induced by IL-2. Rather, the role of IL-2 is largely to enhance the up- or down-regulation of many genes induced after stimulation by TCR/CD28, where this effect is most noticeable at 16 hr. At this time point, IL-2/TCR/CD28 stimulation substantially down-regulated gene expression (clusters 1 and 2), but only those genes in cluster 1 showed some responsiveness to only IL-2. Reciprocally, cluster 3-5 were highly up-regulated by IL-2/TCR/CD28 stimulation, while responsiveness to solely IL-2 minimally increased in the genes represented in these clusters. Collectively, these data reveal that the main role of IL-2 in Tregs is to augment activation induced by TCR and CD28 signaling.

Pathway analysis was performed to determine the relative contribution of IL-2R signaling to that in fully activated Tregs. Pathways related to all upregulated IL-2-dependent genes were compared to those upregulated after combined activation through the IL-2R, TCR and CD28. At 4 hr post stimulation, IL-2 was associated only with pathways related to receptor signaling whereas this pathway and those related to RNA processing, translation, purine, pyrimidine, cholesterol metabolism and TCR signal were evident after stimulation through IL-2/TCR/CD28 (**Fig. S2**). At 16 hr post-stimulation, IL-2 supported a somewhat more robust response that on its own not only support cytokine signaling but pathways related to cholesterol biosynthesis, rRNA processing, mitochondrial translation, and TRiC folding (**Fig. 1C**, top). These were largely a subset of more complex pathways regulated after activation through the IL-2R, TCR, and CD28, with only the cholesterol-related biosynthetic pathway unique to IL-2R signaling (**Fig. 1C**, bottom). Thus, at 16 hr, fully activated Tregs express genes in pathways that are required to prepare these cells for proliferation, differentiation and expression of some immune functions.

**CEACAM1 is identified as a highly IL-2-dependent gene in Tregs**

The above analyses indicated that the majority of genes induced in Tregs by IL-2 are also upregulated by TCR and CD28 signaling. To identify genes highly dependent on IL-2, but not TCR/CD28 signaling, we first stratified all genes expressed >3-fold after stimulation with IL-2 at 4 hr in relationship to their basal level of expression when cultured only in medium. (**Fig. 2A**). *CEACAM1*, *MEOX1*, *TREML2*, and *OSM* were among those genes that were low at baseline and highly induced after IL-2 stimulation, with *CEACAM1* as the highest IL-2-inducible gene with these characteristics. The induction of these genes at 4 and 16 hr by IL-2 was then compared to the fold-increase after activation by TCR and CD28 signaling in the presence of anti-IL-2. *CEACAM1* and *MEOX1* remained as the two genes most highly induced by IL-2 with low responsiveness to TCR and CD28 signaling (**Fig. 2B, C**). *IL-2RA* and *CISH* are two genes that are well known to be IL-2-dependent, but these are also highly induced by TCR and CD28 signaling.

Several other genes with higher basal expression, e.g. *DPP4*, also were highly IL-2 dependent genes as they show equivalent fold increase when activated through the IL-2R and IL-2R/TCR/CD28, but were minimally affected by TCR/CD28 signaling (**Fig. 2B**). Other genes, such as *MYC* and *LRRC32*, are more responsive to TCR/CD28 signaling than IL-2R signaling. In contrast, *CEACAM1* and *MEOX1*, show lower expression when stimulated through IL-2/TCR/CD28 than IL-2R alone, indicating that IL-2-dependent increases in these two genes are down-regulated by TCR/CD28 signaling. Collectively, these data identify *CEACAM1* and *MEOX1* as two targets that are highly and selectively responsive to IL-2R signaling and suggest they are co-regulated by TCR and CD28 signaling.

**CEACAM1 is more highly induced in Treg than Teff cells**

Since CEACAM1 is an important regulator of Teff proliferation and function (*26, 27*) and is readily identified by flow cytometry, we further characterized the requirements for its expression in human Tregs and compared these to Teff cells. When purified CD4+ T cells were cultured with only human IL-2, CEACAM1 was induced in a greater percentage of Tregs than Teff cells (**Fig. 3A,** left). This experiment also revealed that to achieve an equilivent percentage of CEACAM1+ Treg or Teff cells, Teff cells required an approximately 50-fold higher amount of IL-2 (**Fig. 3A**, right). However, >85% of this expression was inhibited by anti-IL-2, indicating that endogenously produced IL-2 from conventional CD4+ T cells was largely controlling the expression of CEACAM1 in both cell types (**Fig. 3B**). This finding is consistent with CEACAM1 being highly dependent on IL-2R signaling.

To further examine the requirements for CEACAM1 expression, FACS-purified Tregs (**Fig. S1A**) and TEM cells (**Fig. S1B**) were initially cultured with IL-2 or anti-CD3/CD28 and IL-2 for 5-6 days without subculturing. Tregs again showed greater expression of CEACAM1 than Teff when cultured only with IL-2 (**Fig. 3C**). In each of two experiments for Tregs, this amount of CEACAM1 decreased when stimulated with IL-2 plus anti-CD3/CD28 whereas this effect was variable for TEM cells. To more carefully quantify the CEACAM1 expression over time, purified Treg and TEM cells were activated with anti-CD3/CD28 plus IL-2 and on days 3 and 6 were sub-cultured with only IL-2. Decreased CEACAM1 mRNA was noted on days 1 and 3 (**Fig. 3D**) and minimal CEACAM1 surface protein was detected by flow cytometry (**Fig. 3E, Fig. S3**) on both cell types at day 3. Indeed, this amount of surface CEACAM1 for Treg and TEM cells was lower than after culturing these cells with only IL-2 for 2 days (**Fig. 3A**). This finding and the mRNA results further support the notion that initial TCR/CD28 signaling limit IL-2R-dependent induction of CEACAM1. However, after subculture with IL-2, expression of CEACAM1 was readily detected, and these amounts were greater for Tregs (**Fig. 3D, 3E, S3**). In contrast to CEACAM1, CD25 mRNA and surface protein were rapidly upregulated, over this time course. Collectively, these data demonstrate that CEACAM1 is an IL-2-dependent activation molecule that is more prominently induced in Tregs when compared to activated TEM cells, where initial TCR/CD28 signaling appears to readily initially down-regulate CEACAM1 in these cell types.

**CEACAM1 is an TCR checkpoint in Tregs**

The long isoform of CEACAM 1 limits TCR signaling in activated conventional T cells through ITIM motifs associated with its cytoplasmic tail (*25, 28, 29*). The long CEACAM1 isoform was also readily detected in Treg and TEM cells after anti-CD3/CD28/IL-2 activated cells were further sub-cultured with only IL-2 (**Fig. 4A**).

To directly assess the role of CEACAM1 in Tregs, purified Tregs were activated with anti-CD3/CD28/IL-2 (**Fig. S4A**), and 3 days later, guide RNAs to exons 2 and 4 of *CEACAM1* and CRISPR/Cas9 (**Fig. S4B**) were used to abrogate expression of CEACAM1. High editing efficiency (>98% on exon2 and >69% on exon 4, respectively) were noted at the DNA level by the T7 endonuclease I mismatch assay (**Fig. S4C**) and protein level by Western blotting (**Fig. S4D**). Flow cytometry revealed reduced CEACAM1 expression at day 3 and essentially undetectable levels at day 7 post CRISPR editing (**Fig. 4B**).

The absence of CEACAM1 on Tregs did not affect their suppressive activity in vitro (**Fig. 4C**). However, in each of four independent experiments, when IL-2 expanded Tregs were restimulated with only anti-CD3 or anti-CD3/CD28, CEACAM1KO Tregs exhibited increased proliferation (**Fig. 4D**, top, one representative experiments; the other individual experiments are in **Fig. S5**). On average these proliferative responses increased 2.2-2.3-fold (**Fig. 4D**, bottom). As expected, the greatest proliferation occurred when Tregs were stimulated with anti-CD3/CD28 and IL-2, and these responses were typically similar for wild-type and *CEACAM1*KO Tregs.

The contribution of CEACAM1 to IL-2R and TCR signaling was also determined. *CEACAM1*KO Tregs showed a slight decrease in expression of CD25, but not CD122 and CD132 (**Fig. 5A**). Correspondingly, CEACAM1 showed a slight lowering of responsive to IL-2 as assessed by IL-2-dependent activation of pSTAT5 (**Fig. 5B**) as on average the IC50 of WT vs. CEACAM1KO Tregs increased from, 0.56 to 1.1 U/ml, respectively. This small difference did not affect Treg proliferation because the cells were cultured with a high amount (500 U/ml) of IL-2. In contract, anti-CD3 activation of CEACAM1KO Tregs led to a greater activation of pS6, downstream of AKT and mTORC1, when compared to wild-type Tregs (**Fig. 5C**). Collectively, these data indicate the CEACAM1 functions to limit TCR signaling, which in an IL-2 poor setting will inhibit Treg proliferation, but not function.

**CEACAM1 expression in Tregs depends upon PRDM1 (BLIMP-1)**

To better understand the mechanism by which CEACAM1 expression is regulated, the effect of TCR/CD28 and IL-2 on *CEACAM1* chromatin accessibility was assessed by ATAC-seq. Five regions of chromatin accessibility was detected in and around *CEACAM1* (**Fig. 5A**). Region 1 comprises 2 adjacent peaks and is just upstream the *CEACAM1* start site, which likely defines the promoter. Only this region was found to be open in baseline unstimulated Tregs. The four other regions were detected as open at days 3 and 6. Region 2 is within an intron while region 3-5 are downstream the last exon of *CEACAM1*. The opening of these 4 regions on day 3 after anti-CD3/CD28/IL-2 activation indicates that these changes in chromatin accessibility precedes a large increase in CEACAM1 mRNA (**Fig. 3D**) and surface protein (**Fig. 3E**).

*PRDM1* is an IL-2- and STAT5-dependent gene whose expression is associated with effector Tregs (eTregs) (*30-33*), including Klrg1 that marks Tregs that have extensively expanded in response to IL-2 (*34*). Since high expression of CEACAM1 mRNA and protein was detected after considerable IL-2-dependent expansion (**Fig 3D, E**), we assessed whether CEACAM1 depends on BLIMP-1. First, the open chromosome regions were examined for *STAT5* and *PRDM1* motifs. Regions 1, and 3-5 contained *STAT* motifs and regions 4 and 5 contained *PRDM1* motifs (**Fig. 6A**), where the most significant change is chromatin opening associates with STAT and PRDM1 motifs in peak 5 (**Fig. 6B**). Thus, CEACAM1 has to potential be a direct target of STAT5 and BLIMP-1.

To determine if BLIMP-1 was involved in the regulation of *CEACAM1*, Crisper/Cas9 was used to abrogate *PRDM1* in Tregs in a manner identical to that used for gene targeting of CEACAM1 (**Fig. S4A**, and **Fig. S6A**). By targeting exons 2-5, the regulatory PR/SET domain and the DNA-binding Zinc-finger domain would be deleted. RT-PCR of BLIMP-1 showed >95% targeting of *PRDM1* (**Fig. 6C**). Flow cytometry revealed that surface expression of CEACAM1 was reduced on average by 1.5-fold in the absence of BLIMP-1 (Fig. 6D). Thus, high expression of CEACAM1 depends on chromatin remodeling and Blimp-1.

**CEACAM1 as an IL-2-dependent biomarker for low-dose IL-2 therapy**

To assess the relevance of CEACAM1 in vivo, we examined the expression of CEACAM1 in 27 patients undergoing low-dose IL-2 therapy with 8 different autoimmune diseases, i.e., systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, Crohn’s disease (CD), sclerosing cholangitis (SC), ankylosing spondylitis, Sjogren’s Syndrome, and systemic sclerosis (SSc). These patients initially received 1 x106 IU of human IL-2 s.c each day for 5 days and then every 15 days for 6 months, except SLE, where IL-2 was administered weekly. When compared to baseline (Day 1), CEACAM1 was highly induced in Tregs when examined 3 days after the 5-day induction course of low-dose IL-2 (Day 8) (**Fig. 7A, B**). This expression was variable (range 28.8-64.7%) and transient, as CEACAM1 returned to baseline levels when measured at 3 months, 2 weeks after the last maintenance injection, or two months after low-dose IL-2 therapy ceased (Month 8) (**Fig. 7B**). CEACAM1 was induced on averge to 10-fold lower levels, (range 0.9-7.0%) on CD4+ CD45RO+ TEM cells, consistent with selectivity of low-dose IL-2 toward Tregs.

The percentage of CEACAM1 (**Fig. 7C**, left) and the MFI of CD25 (**Fig. 7C**, middle), a TCR and IL-2 induced protein, was determined for Tregs relative to baseline for each patient. However, the overall upregulation of CEACAM1 and CD25 on Tregs showed at best a weak correlation (**Fig. 7C,** right). This result likely reflects somewhat distinctive factors that affect *IL2RA* gene expression to low-dose IL-2 in vivo, where CEACAM1 is more dependent on IL-2R than CD25 (**Fig. 2**). In contrast, consistent with the selectivity of low-dose IL-2 for Tregs and analogous to CEACAM1 (**Fig. 7B**), CD25 amounts were minimally affected by low-dose IL-2 for CD4+ TEM cells (**Fig. S7**).

Our in vitro studies showed that CEACAM1 limits the proliferative response of Tregs to TCR/CD28 stimulation (**Fig. 4D**). On day 8, CEACAM1+ Tregs exhibited a lower proliferative response than CEACAM1neg Tregs to low-dose IL-2 based on KI67 expression (**Fig. 7D**). This finding raises the possibility that TCR/CD28 signaling contributes to the expansion of Tregs to low-dose IL-2. The CEACAM1+ Tregs, however, expressed greater amounts of CD26, Bcl-2, and Foxp3, but not TIGIT, where the former 3 are known to be regulated by IL-2 (**Fig. 7E**). Collectively, these data indicate that CEACAM1 is an IL-2-dependent biomarker related to an acute response of Tregs to IL-2 and further supports the notion that CEACAM1 is a Treg checkpoint.

**Discussion**

Although expansion of human Tregs is highly dependent on the interplay between TCR, CD28 and IL-2R signaling (*17, 35*), the relative contribution of these pathways is not well 4understood. Since signal transduction through the IL-2R and TCR/CD28 are distinctive, one potential outcome was that IL-2R and TCR/CD28 signaling would largely induce unique genes and pathways that cooperate to drive Treg proliferation. Instead, we found that IL-2R signaling activated a much smaller set of genes than TCR/CD28 and that the role of IL-2R signaling was to mostly enhance the up- or down-regulation of genes controlled by TCR/CD28 signaling. Indeed cholesterol-related biosynthesis was the only pathway that depends primarily on IL-2R signaling 16 hr post-activation, a time when substantial gene activation is detected as Tregs are preparing for cell division. This finding is consistent with past results where the selective loss of IL-2R signaling in peripheral mouse Tregs resulted in down-regulation of genes related to cholesterol biosynthesis (*8*). Thus, IL-2R signaling appears to distinctively control the production of cellular cholesterol to support Treg proliferation and homeostasis.

Paradoxically, of the 50 genes that were highly (≥3-fold) induced by IL-2R signaling, most were negatively regulated by TCR/CD28 signaling. This was particularly striking for several targets such as *MEOX1* and *CEACAM1* where the amount of their mRNA induced solely by IL-2 was reduced by approximately 16-fold when Tregs were co-cultured with anti-CD3/CD28 plus IL-2. Thus, although a large majority of the genes induced by IL-2 appear to synergize TCR/CD28 signaling, several others are repressed by TCR/CD28 signaling, suggesting coordinated control by a regulatory circuit.

To explore the potential relevance of this down-regulatory pathway, we focused on CEACAM1 because it is the most highly induced gene by IL-2 that is also substantially limited by TCR/CD28 signaling. Relative to the wealth of information about CEACAM1 in Teff cell (*26*), the function of CEACAM1 in Tregs has been minimally investigated. Our findings indicate that CEACAM1 is more highly expressed in Tregs than Teff cells and that this expression is primarily driven by IL-2 because anti-CD3/CD28 plus IL-2-dependent induction of CEACAM1 was very effectively blocked by anti-IL-2. Although short-term (4-16 hr) culture with IL-2 was sufficient to reveal CEACAM1 as a direct IL-2 responsive gene, its expression of mRNA and surface protein continued to substantially increase after subculture of the anti-CD3/CD28/IL-2 primed T cells only with IL-2. The mechanism for this late IL-2-driven CEACAM1 upregulation is likely related in part by chromatin remodeling leading to increased opening of *CEACAM1* that precedes enhance mRNA and protein levels. BLIMP-1 was also implicated in contributing to CEACAM1 expression after Crispr/Cas9 ablation of PRDM1. This effect may be direct through interaction with *PRDM1* sites found in newly opened chromatin or indirect through an effect on another transcriptional regulator that in turn enhance BLIMP1 expression.

Since CEACAM1 is upregulated by IL-2, but not TCR/CD28 signaling, it likely represents a much more specific marker for a T cell response to IL-2 than the more commonly used CD25, which is well known to be activated by through TCR and IL-2R signaling (*36*) and confirmed in our study. Indeed, linear regression analysis of CEACAM1 vs. CD25 surface protein after the induction course of low-dose IL-2 showed at best a weak relationship, suggesting the upregulation of these genes during low-dose IL-2 may reflect distinctive processes. CEACAM1 expression on T cells in autoimmune patients undergoing low-dose IL-2 therapy paralleled that found in vitro. CEACAM1 was much more highly expressed on Tregs when compared to TEM cells after the five-day induction treatment with low-dose IL-2. This finding is consistent with the selectivity of low-dose IL-2 for Tregs. The upregulation of CEACAM1 on Tregs showed a high amount of variance, suggesting that this finding might reflect patient-specific properties that support CEACAM1, one of these being heterogeneity in responding to IL-2. As such, following CEACAM1 during low-dose IL-2 may be a useful biomarker to ascertain the capacity of an individual to response to IL-2. Future low-dose IL-2 clinical trials should assess expression of CEACAM1 along with CD25 in relationship to a therapeutic response. If an association is found, one might then have a means to determine whether a patient would benefit from this therapy.

In a manner analogous to Teff cells, our study revealed that CEACAM1 acts as a TCR checkpoint for Treg proliferation. Several lines of evidence are consistent with a role for CEACAM1 in limiting TCR signaling. First, like Teff cells, the activated Tregs exclusively expressed the long CEACAM1 isoform that recruits SHP-1 to two ITIMs that have been shown to inhibit TCR signaling (*25*). Second, after Crispr/Cas9-mediate gene editing of CEACAM1, the cultured CEACAM1KO Tregs showed a higher proliferative response to restimulation by TCR/CD28 signaling. Third, CEACAM1KO Tregs showed lower downstream TCR signaling, i.e., activation of pS6, while IL-2R signaling was minimally affected. Lastly, when autoimmune patients were treated with a five-day initial course of low-dose IL-2, the CEACAM1+ Tregs showed a lower proliferative response based on Ki67 expression.

Overall, our findings have uncovered a new regulatory loop between regulation of CEACAM1 expression and responsiveness of human Tregs to TCR/CD28 and IL-2R signaling that likely impacts their homeostasis. Initially, TCR/CD28 activation of Tregs is favored in part by an inhibitory mechanism that limits expression of the CEACAM1 TCR checkpoint. Under conditions of persistent IL-2R signaling, chromatin opening of *CEACAM1* occursthat contributes to substantial upregulation of CEACAM1 that down-regulates TCR signaling in the IL-2 expanded Tregs.

The physiological relevance of this regulatory loop remains to be determined. One possibility is this represents a mechanism to promote a sequential but regulated response of Tregs, first to autoantigen followed by IL-2-dependent amplification of these autoantigen-stimulated Tregs. This type of a response likely occurs locally when autoantigen-reactive T cells provide IL-2 to expand relevant Tregs that in turn suppress the autoreactive T cell (*37*). Once suppression has been achieved, the source of IL-2 is removed and autoantigen activation and persistence of large number of such Tregs are no longer required. As such the IL-2-dependent upregulation of CEACAM1 may reflect a key homeostatic checkpoint. Another not mutually exclusive possibility is that upregulation of CEACAM1 in an environment of excess IL-2 represents a mechanism to limit TCR activation of bystander Tregs. Such a mechanism would also contribute to Treg homeostasis and likely comes into play during low-dose IL-2 therapy as CEACAM1 was upregulated in many Tregs after a five-day induction course of low-dose IL-2.

**MATERIALS AND METHODS**

**Study subjects**

Peripheral blood samples from healthy adult donors were purchased from the Continental Blood Bank, Miami, FL. Frozen peripheral blood samples were analyzed from 27 autoimmune patients undergoing low-dose IL-2 clinical trial (Trial registration number, [NCT01988506](https://ard.bmj.com/lookup/external-ref?link_type=CLINTRIALGOV&access_num=NCT01988506&atom=%2Fannrheumdis%2F78%2F2%2F209.atom)). The study was approved by the the Pitié-Salpêtrière Hospital Ethical Committee (EudraCT: 2013-001232-22). All patients signed written informed consent. Patients were selected based on common and disease-specific exclusion and inclusion criteria. The main inclusion criteria were a documented diagnosis of at least one of the selected diseases of mild to moderate activity and being on stable standard therapy for ≥2 months at the time of inclusion. The main exclusion criteria were having another severe or progressive autoimmune/inflammatory disease, hematological disorders, vital organ failure, cancer, and active HIV, Hepatitis B Virus (HBV) or Epstein-Barr Virus (EBV) infections. All patients received 1 million international Units (MIU)/day of IL-2 from day 1 to day 5 (the induction period), and then every 2 weeks from day 15 to day 180 (the maintenance period). A follow-up visit was made 2 months after the end of the IL-2 treatment (day 240).

**Antibodies and flow cytometry**

The following monoclonal anti-human antibodies (with the clone names in parenthesis) were obtained from BD Biosciences (San Jose, CA), or Biolegend (San Diego, CA): FITC/PerCP-Cy5.5/Pacific Blue-αCD4 (RPA-T4), APC-αCD127 (A019D5), PerCP-Cy5.5-αCD127 (HIL-7R-M21), APC-Cy7/Alexa Fluor 700-αCD45RA (HI100), BV711-αCD3 (OKT3), PE/BV421-αCD25 (M-A251), Alexa Fluor 488-αCD25 (BC96), BV421-αCD122 (Mik-β3), BV650-αCD132 (TUGh4), FITC-αCD66 (B1.1), BV421-αCTLA4 (BNI3), FITC-αCD39 (A1), BV605-αCD73 (AD2), PE-Cy7-αCD26 (BA5b), BV421-αTIGIT (A15153G), PE/Alexa Fluor 647/Pacific Blue-αFoxp3(259D), Alexa Fluor 700-αKi67 (B56), PE-αBcl2 (100), FITC-αphosphorylated STAT5 (pSTAT5)(pY694)(47/Stat5(pY694)), and PE-αpS6 (pS235/pS236)(N7-548). PE-αCEACAM1/CD66a (283340) was purchased from R&D Systems (Minneapolis, MN). IL-2 monoclonal antibody (AB12-3G4, functional grade) and Fixable Viability Dye eFluor 455UV were purchased from ThermoFisher Scientific (Vilnius, LT). Cell-surface staining was performed with antibodies in FACS buffer (HBSS, 0.2% BSA, 0.1% sodium azide) for 15 min at 4° C. Intracellular staining for Foxp3/Ki67/Bcl2 was performed after fixing and permeabilizing using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. For pSTAT5 and pS6 staining, cells were fixed with paraformaldehyde and permeabilized using ice-cold 100% methanol (see below). FACS analysis was performed using BD LSRFortessa or CytoFLEX LX (Beckman Coulter) flow cytometer, where typically 100,000 events were collected for PBMCs and 10,000-25,000 events were collected for in vitro expanded Treg or Teff cells. Data were analyzed using BD FACSDiva 8.0.1 or FlowJo v10.7.1 software, where viable cells were gated based on forward versus side light scatter profiles and doublets were excluded based on forward light scatter area versus scatter width.

**Cell purification and culture**

Heparinized leukocyte unit was diluted in PBS (1:1 final ratio), layered on Ficoll-Paque Plus (GE Healthcare, Little Chalfont, U.K.) and centrifuged at 400g for 30 min at room temperature, without braking. PBMCs from the interphase cells were collected and washed once with PBS. Human CD4+ T cells were enriched from PBMCs by negative selection with the MACS CD4+ T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). The CD4+ T cells were stained by FITC-αCD4, BV421-αCD25, APC-αCD127, and Alexa Flour700-αCD45A, and were sorted into Tregs (CD4+ CD25hi CD127lo) and TEM (CD4+ CD25med CD127hi CD45RA-) by using a BD FACS Aria-II sorter. Purity of sorted Tregs and TEM was further evaluated by Foxp3 expression after counter staining with PE-αFoxp3.

Sorted Treg or TEM cells (5-8 x 105 /well/ml) were cultured in 24 well plate on Day 0 with initial TCR/CD28 stimulation (anti-CD3/CD28 Dynabeads, ThermoFisher Scientific) and human IL-2 (500 unit/mL, Novartis) in OpTmizer CTSTM T-cell expansion medium (designated as SFM) (Life Technologies, Grand Islands, NY). The ratio of Dynabeads to cells are 4:1. Cells were split to a density of 1-3x105 /mL in fresh SFM plus hIL-2 (500 unit/mL) when the cell density exceeded 1 x 106 /mL. For Crispr/Cas9 knockout experiments, Tregs were collected on Day 3 for electroporation with guide RNAs and Cas9. After electroporation, cells were sub-cultured with hIL-2 (500 unit/mL) in SFM (see below).

**RNA-seq**

Sample preparation: FACS-sorted human Tregs and CD4+TEM (3 x 105/well in 300 µl of SFM) were cultured in 24 well plate with media alone, recombinant human IL-2 (100 U/mL, Novartis), anti-CD3/CD28 beads (15 µl of anti-CD3/CD28, Dynabeads, ThermoFisher Scientific) and anti-IL-2 (10 µg/ml) as indicated in the Figure legends. 4 and 16 hr later, cells were pelleted and resuspended in TRIzol (Thermo Fisher Scientific) and RNA isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. Quality control analysis of RNA samples using the Bioanalyzer 2100 platform (Agilent Technologies) and libraries generation using the KAPA’s RNA Hyperprep with RiboErase HMR protocol were carried out by the Oncogenomics Core at the University of Miami. Libraries were sequenced with 25 Million single-end 100 base reads/sample (per lane) using the Illumina HiSeq SE100 at the sequencing core of the Center for Genome Technology, Hussman Institute for Human Genomics, University of Miami.

Sample analysis: Reads from the RNA-seq were mapped to the (**reference genome**, **version**) using (**software**) (**version**; (**Reference**)). Raw counts were generated based on (**software, version, and reference**). Differentially expressed genes (DEG) were identified using (**software, version, and reference**) and determined by a threshold of false discovery rate (FDR) ≤ 0.01.

**ATAC-seq**

Sample preparation: FACs-sorted human Tregs and TEM were treated as those for RNA-seq. ATAC-seq was performed as previously described (*38*). 50,000 unfixed nuclei derived from Treg, or TEM cells were tagged using the Tn5 transposase (Nextera DNA sample prep kit; Illumina) for 30 min at 37° C. Libraries were generated using the Ad1\_noMX and Ad2.1-24 barcoded primers (*39*) and amplified for 10-12 cycles. Resulting libraries fragments were purified using a DNA Clean & Concentrator-5 kit (Zymo Research). Size-selection was performed using the Agencourt AMPure XP PCR purification system (Beckman Coulter) to remove large fragments > 1 kb and primers to maintain high library complexity. Quality control analysis of libraries using the Agilent Bioanalyzer High-Sensitivity DNA kit and sequences with a 75 bp pair-end run to a minimum depth of 40 million reads per sample using the Illumina NextSeq 500 High Output Kit (150 cycle; 400 M flow cell) were performed at the Oncogenomics Core of the University of Miami.

Sample analysis:

**Cas9 RNP assembly and electroporation**

Cas9 RNPs were prepared immediately before experiments as follows: chemically synthesized tracrRNA and crRNAs (Integrated DNA Technologies, Newark, NJ) targeting scramble control (5’-GGTTCTTGACTACCGTAATT-3’), exon 2 of CEACAM1 (5’-GATGGCAACCGTCAAATTGT-3’), exon 4 of CEACAM1 (5’- CACGCCAATAACTCAGTCAC-3’), exon 2 of PRDM1 (5’-CATTGTGAACGACCACCCCT-3’), and exon 5 of PRDM1 (5’-CGGATGGGGTAAACGACCCG-3’) were reconstituted with Nuclease-Free IDTE Buffer (Integrated DNA Technologies) to generate 160 μM RNA stocks. crRNA and tracrRNA were mixed at 1:1 ratio, heated at 95 ℃ for 5 min, and then cooled to room temperature on the benchtop to generate 80 μM sgRNA. 40 μM purified *S. pyogenes* Cas9-NLS (Macrolab, University of California, Berkeley) was slowly added to the 80uM sgRNA at 1:3 ratio and incubated at 37 ℃ for 15 mins to generate Cas9 RNPs.

Electroporation was performed using the Amaxa P3 Primary Cell Kit and 4D-Nucleofecter (Lonza). FACs-purified human Tregs were initiated with anti-CD3/CD28 dynabeads and IL2 (500 unit/mL) for 3 days. Tregs (5 x 105)with dynabeads were re-suspended in 10 μL P3 buffer and mixed with 10 μL Cas9 RNP. Cells were nucleofected using program EH-115. 80 μL pre-warmed SFM was added to wells and cells were allowed to recover 15 mins at 37 ℃. Cells were then seeded at 5 x 105 cells/mL/well in SFM with IL-2 in 24 well plate.

**T7 Endonuclease I (T7EI) assay**

After gene editing cells were lysed in QuickExtract DNA Extraction Solution (ThermoFisher Scientific) and genomic DNA was used for PCR amplification of target sites with primers as follows: site on exon 2 of CEACAM1: forward: 5’-CCTCACTTCTAACCTTCTGGTTC -3’ reverse: 5’-GGTATACATGGAACTGTCCAG -3’; site on exon 4 of CEACAM1: forward: 5’-ATGGCCCGGACACCCCCAC -3’ reverse: 5’-CAGTGACTATGATCGTCTTGAC -3’. The hybridization of PCR products was performed on a thermocycler with the following setting: 95 ℃, 10 min, 95-85 ℃ at -2 ℃/s, 85 ℃ for 1 min, 85-25 ℃ at -0.3 ℃/s, 25 ℃ for 1 min, and hold at 4 ℃. T7 endonuclease I (NEB) was added to digest the reannealed DNA. The mixture was incubated at 37 ℃ for 3 hr and was resolved on 2% agarose gel after adding 6 x gel loading dye. The image was developed using Odyssey Fc Dual Mode Imaging System (Li-COR, Lincoln, NE).

**[3H]-thymidine proliferations assay**

Cells (1 x 105 cell/well) were cultured as indicted in the Figure legends in 96-well plate in 200 µL SFM as needed for 3 days. 1 μ Ci [3H]-thymidine was added to each well 4 hr before collecting the cells. The radioactivity emitted by the cells in the individual wells was measured using a beta scintillation counter. Data of each sample are reported as the means of triplicate values that consistently varied by <10% from each other.

**In-vitro suppression assay**

Human PBMCs (1 x 106) were suspended in 1 ml SFM and labeled with 0.5 μM Celltrace Violet (ThermoFisher Scientific) according to the manufacturer’s instructions. For *in vitro* suppression assays, labeled PBMCs (1 x 105 cell/well) were incubated with titrated amounts of scramble or CEACAM1KO Tregs (in serially decreasing ratios) in SFM in 48-well plates. Cells were stimulated with anti-CD3 (1 μg/ml) (OKT3, Biolegend) and anti-CD28 (2 μg/mL) (CD28.2, Biolegend), and incubated at 37 ℃ under 5% CO2. After 3 days, proliferation of CD8+ cells (used as responder cells) was assessed by flow cytometry as the dilution of Celltrace Violet. Percentage of inhibition was calculated by [1- (proliferation (responder and suppressor))/proliferation (responder only)] x 100.

**pSTAT5 and pS6 phospho-flow assay**

After electroporation with Cas9 RNPs, Tregs with CEACAM1 knockout and scramble Tregs were sub-cultured in SFM with 500 unit/mL hIL-2 for 7 days. After removal of dynabeads, cells were washed with PBS 3 times and cultured in SFM (1-5 x 105/ml) for 4 hr. After this “rest culture”, IL-2 was added for 15 min at 37 ℃. pSTAT5 staining was performed as previously described (*40*). In brief, after IL-2 treatment, cells were fixed by 1.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at 37 ℃ and permeabilized by ice-cold methanol for 30 min on ice. Cells were washed twice in wash buffer (PBS containing 0.5% BSA and 0.02% sodium azide) and stained with antibodies for 60 min at room temperature in the dark. For pS6 analysis, cells were rested overnight and stimulated for 6 hr at 37 ℃. pS6 staining was performed the same as pSTAT5.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

Total RNA was purified by RNesy Mini Kit (Qiagen). Complementary DNA (cDNA) samples were synthesized with the High-Capacity cDNA Reverse Transcription Kit using oligo(dT) primers (ThermoFisher Scientific). Primers used for determining CEACAM1 isoforms are forward: 5' GCTCTACCACAAGAAAATGG, reverse: 5' CATTGGAGTGGTCCTGAG. Primers for target site ranging from exon2 to exon 5 of PRDM1 are forward: 5’-GCACTGTGAGGTTTCAGGGA -3’, reverse: 5’-AGTGATGTACGTGGGTCTCTCG-3’. PCR was done under the conditions: 95 ℃ 5min (1 cycle), 95 ℃ 30s, 59 ℃ 1min, 72 ℃ 1min, (40 cycles), 72 ℃ 10 min (1 cycle), and hold at 4 ℃.

**Western blotting**

Cell extracts were prepared using cell extraction buffer (ThermoFisher Scientific, Rockford, IL) with protease inhibitors, and 1 mM PMSF (All from Sigma-Aldrich). Immunoblotting was performed on 10% SDS-PAGE gels after 200 μg total protein was loaded per lane. Target protein was probed using anti-CEACAM1 (283324) (R&D Systems) and anti-β-actin (Biolegend) antibodies. Proteins were visualized after incubation of the blots with ECL chemiluminescence agent (ThermoFisher) and analyzed using Odyssey Fc Dual Mode Imaging System (Li-COR, Lincoln, NE).

**ELISA**

Scramble and CEACAM1KO Tregs (1.5 x 105 cells/ml) were treated with plate bound anti-CD3 (2 µg/mL) anti-CD28 (2 µg/mL) and IL2 (100 unit/mL) for 3 days and supernatant was collected. The amount of IL-10 in supernatants was determined by IL-10 ELISA kit (Invitrogen, Austria), following the manufacturer’s protocol. Absorbance was measured at 450nm and 550nm. The concentration of IL-10 (pg/ml) was determined by using the standard curve plotted with kit’s standards. The sensitivity of IL10 ELISA kit was less than 3 pg/ml.

**Statistical analyses**

Graphical representations of the data and statistical analyses were performed using GraphPad Prism 8 software. Data are shown as means ± SD. Paired or unpaired two-sided t-test, one-sample two-sided t-test, and two-way ANOVA with multiple comparisons were used to calculate statistical significance among groups, as listed in each Figure Legend. For a one-sample t test, each data point for the experiment under consideration was generated from a process that involved a comparison to a control condition, where each control group value was normalized to 1. A p-value of less than 0.05 was considered significant.

**Acknowledgements**

### We thank Jay Enten, Patricia Guevara, Shannon Saigh and Natasha Ward from the Flow Cytometry Core and Marissa Brooks, Yoslayma Cardentey and Jenny Kemper from the Oncogenomics Core of the Sylvester Comprehensive Cancer Center (supported by NIH P30CA240139). This research was supported by funding to T.R.M. from the NIH (R01 AI131648)and Bristol Myers Squibb.

# Authors’ Contributions

Conception and design: T.R.M., A.Y., Y.D., and D.K. Acquisition of data: Y.D., A.Y., M.V., and A.M. Analysis and interpretation of data: Y.D., A.Y. L.N. Z.G., N.T., M.R., A.V. and T.R.M. Manuscript Writing: T.R.M., Y.D. and A.Y. All authors edited and approved the manuscript.

# Competing interests

The authors declare no financial conflicts or competing interest.

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