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IRF4 instructs effector Treg differentiation and immune suppression in human cancer

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ABSTRACT

The molecular mechanisms responsible for the high immunosuppressive capacity of CD4⁺ regulatory T cells (Tregs) in tumors are poorly known. High-dimensional single cell profiling of T cells from chemotherapy-naïve individuals with non-small cell lung cancer identified the transcription factor IRF4 as specifically expressed by a subset of intratumoral CD4⁺ effector Tregs with superior suppressive activity. In contrast to the IRF4⁻ counterparts, IRF4⁺ Tregs expressed a vast array of suppressive molecules, and their presence correlated with multiple exhausted subpopulations of T cells. Integration of transcriptomic and epigenomic data revealed that IRF4, either alone or in combination with its partner BATF, directly controlled a molecular program responsible for immunosuppression in tumors. Accordingly, deletion of Irf4 exclusively in Tregs resulted in delayed tumor growth in mice while the abundance of IRF4⁺ Tregs correlated with poor prognosis in patients with multiple human cancers. Thus, a common mechanism underlies immunosuppression in the tumor microenvironment irrespectively of the tumor type.
INTRODUCTION

Despite recent clinical breakthroughs in adoptive T cell transfer approaches and of checkpoint blockade in treating hematopoietic and solid tumors, suppression of the anti-tumor immune response in the tumor microenvironment (TME) is a major obstacle to tumor regression (1). While anti-tumor immune cells, such as T and NK cells, normally infiltrate tumors and their abundance correlates with better prognosis in certain cancers, the presence of inhibitory populations, such as regulatory CD4\(^+\) T cells (Tregs) counteract tumor rejection (2). The main role of Tregs is to maintain immune homeostasis in physiology by inhibiting effector T cells via different modes of action including expression of cell-surface inhibitors, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) (3), production of inhibitory cytokines such as IL-10 (4), TGFβ (5) or IL-35 (6), depletion of IL-2 by overexpression of the high-affinity IL-2 receptor α chain (IL-2Rα or CD25), purine-mediated suppression by CD39-dependent conversion of ATP (7), or direct cytotoxicity (8), thus preventing autoimmunity and immunopathology (reviewed in Vignali et al. (9)). Conversely, Tregs are aberrantly enriched in tumors and dampen anti-tumor immune responses (10). This detrimental effect on anti-tumor immunity is demonstrated by a plethora of preclinical data where systemic Treg depletion, or inhibition of their function promotes tumor regression (11-14). Unfortunately, it often results in severe autoimmunity, allergy and immunopathology (11-14), thus new strategies are required to safely deplete Tregs specifically in the TME. The prerequisite to do so is the identification of a specific molecular program or molecules that are exclusively present in tumor-associated Tregs, thus allowing to spare circulating Tregs and maintain peripheral homeostasis.

Transcriptional profiling recently revealed that CD4\(^+\) Tregs isolated from colon, lung and breast tumors are transcriptionally different from those isolated from the adjacent
tissues and the blood (15, 16). In particular their transcriptional profile overlaps with that of effector Tregs described in mice, which show enhanced suppressive activity. This includes the increased expression of molecules such as CTLA-4, 4-1BB, CCR8, ICOS and others (17). Noteworthy, increased expression of CCR8 (15, 16), LAYN or MAGEH1 (15) by the intratumoral Tregs correlated with poor prognosis, suggesting a pivotal role of these cells in inhibiting anti-tumor immune responses and/or favoring tumor growth.

The molecular mechanisms leading to increased Treg activity in tumors remain ill-defined. Studies performed in the last decade have elucidated in part the transcriptional network at the basis of murine effector Treg differentiation in lymphoid and non-lymphoid tissues. Once activated, Tregs undergo a program of effector differentiation that mirrors T helper cell differentiation by expressing transcription factors (TFs) that regulate T helper polarization, such as T-bet, GATA-3 or Bcl-6 (17). Additional TFs might be involved in regulating effector Tregs at specific tissue sites, such as PPARγ that controls the unique transcriptional and metabolic signature of those cells residing in the visceral adipose tissue (18). Interferon regulatory factor 4 (IRF4), initially identified as the TF responsible for the generation of those Tregs specifically controlling Th2 responses (19), plays a central role in generating effector Tregs in peripheral organs (20). IRF4 is absent in quiescent T cells but is induced by T cell receptor (TCR) signaling. Mice with specific deletion of Irf4 in Tregs develop multiorgan autoimmunity due to exacerbated Th1, Th17 and Tfh responses and plasma cell infiltration (19). Notably, IRF4-deficient Tregs largely maintain a CD62L\textsuperscript{high} naïve-like phenotype and express reduced amounts of effector and suppressive molecules, such as ICOS, CTLA-4 and IL-10 (20). IRF4 cooperates with other TFs in DNA binding and regulating transcription in CD4\textsuperscript{+} T cells, namely the AP-1 family members BATF and JUN (21, 22). Notably, IRF4 was reported to be overexpressed by non-small cell lung cancer (NSCLC)-infiltrating Tregs (23), but its role in determining the
Treg transcriptional program, functionality and regulation of anti-tumor immunity remains completely unexplored.

Here, we reported the existence of two subsets of tumor-infiltrating Tregs with differential expression of IRF4, as revealed by high-dimensional single cell analysis of lung, liver and melanoma-infiltrating T cells. IRF4 defined activated effector Tregs with enhanced suppressive activity, the abundance of which correlates with poor prognosis in multiple human cancers. Consistent with a direct requirement for IRF4 for the enhanced suppressive functions, deletion of Irf4 in Tregs enhanced anti-tumor immunity in a mouse model of cancer in vivo. We further identified a core network of immunosuppressive genes directly regulated by IRF4 in interaction with its molecular partner BATF, thereby demonstrating that IRF4 instructs the suppressive activity of Tregs in human cancer.
RESULTS

Treg heterogeneity in the TME and its relation to IRF4. To gain insight into CD4$^+$ T cell phenotypes at the tumor site, we initially investigated T cells in a cohort of 53 NSCLC patients (Table S1) (24) with a 27-parameter polychromatic flow cytometry panel encompassing markers of memory and effector T cell differentiation, activation, metabolic activity and exhaustion as well as Treg markers (Table S2). We profiled tumors (n = 53), paired adjacent cancer-free lung tissues (n = 45), and peripheral blood samples (PB; n = 22) of treatment-naïve patients (Table S1). We next used Uniform Manifold Approximation and Projection (UMAP), a dimensionality reduction visualization approach that preserves the local and the global structure of single cell data, to simplify the visualization of marker co-expression in a 2D space (Fig. 1A, B). Overall, CD4$^+$ T cells from the three body sites displayed a different single cell profile (Fig. 1A). Besides previously described dynamics such as the loss of CD45RA$^+$, CCR7$^+$, CD27$^+$ cells (identifying naïve and early memory cells) and the accumulation of HLA-DR$^+$ activated and PD1$^+$ T cells, we also noticed the accumulation of CD25$^+$Foxp3$^+$ Tregs expressing the TF IRF4 in tumor vs. adjacent lung and blood samples (Fig. 1B-D). Manual gating of flow cytometry data indicated that $\sim$40% of CD25$^+$Foxp3$^+$ Tregs in tumors expressed IRF4, while the rest was IRF4$^-$ (Fig. 1C, D), suggesting Treg heterogeneity at the tumor site. In addition, the majority of conventional CD4$^+$ T (Tconv) cells lacked IRF4 expression (Fig. 1C, D). Flow cytometric analysis also revealed that IRF4$^+$ Tregs retain increased expression of PD1, TIGIT and TIM3 receptors, and CD71 and CD98 metabolic markers compared to both IRF4$^-$ Tregs and Tconv in tumors (Fig. 1E). These markers are generally upregulated with activation. In line with our results, reanalysis of a published single cell RNA-sequencing (scRNAseq) data (25) of CD4$^+$ tumor-infiltrating lymphocytes (TILs) from NSCLC patients identified IRF4 expression as largely confined to a subpopulation of CTLA$^+$ Tregs (subset 9-CTLA4; Fig. 1F), while it was relatively lower in other cell subsets,
including in non-activated Tregs (8-FOXP3). \( \text{CTLA4}^{\text{high}} \) Tregs, which also express \( \text{FOXP3} \), displayed an effector signature including expression of \( \text{CCR8}, \text{ICOS}, \text{TNFRSF4} \) (encoding OX-40), \( \text{TNFRSF9} \) (encoding CD137/4-1BB) and the IRF4 transcriptional partner \( \text{BATF} \) (Fig. 1G). We identified a similar subset of \( \text{IRF4} \)-expressing Tregs by scRNAseq analysis of CD45+ cells infiltrating hepatocellular carcinoma (HCC; Supplementary Fig. 1A) (26). Notably, \( \text{IRF4} \) expression correlated with multiple Treg genes but not with TFs related to other T helper cell subsets in single CD4+ T cells isolated from melanomas (Supplementary Fig. 1B) (27). Thus, a common phenotypic and gene expression architecture defines tumor infiltrating Tregs in multiple human cancers.

**IRF4 expression defines effector Tregs with enhanced suppressive potential and capable to promote tumor growth in vivo.** We further characterized IRF4+ and IRF4− Tregs by bulk RNAseq. As the intranuclear localization of TFs precluded the isolation of viable cells based on IRF4 expression levels, we used a surrogate surface staining strategy for fluorescence-activated cell sorting (FACS)-based isolation of Treg subsets. Bulk Tregs from tumors, defined as CD25++CD127−, were further separated according to CCR8 and ICOS to identify IRF4+ and IRF4− Tregs (Fig. 2A and Supplementary Fig. 2A; see Methods). As expected, both of these subsets expressed high levels of FOXP3 compared to Tconv (Supplementary Fig. 2B) and their gene expression significantly overlapped with a tumor infiltrating Treg signature as obtained from a NSCLC scRNAseq dataset (25) (Supplementary Fig. 2C), confirming the Treg identity of these subsets.

A multi-dimensional scaling plot of gene expression profiles showed that sorted intratumoral \( \text{CCR8}^{-}\text{ICOS}^{+} \) (IRF4+) and \( \text{CCR8}^{-}\text{ICOS}^{-} \) (IRF4−) clustered separately, indicating substantial differences at the transcriptional level (Supplementary Fig. 2D). Overall, we identified 2,674 differentially expressed genes (DEGs; FDR<0.05; Table S3). \( \text{CCR8}^{+}\text{ICOS}^{+} \)
Tregs expressed high amounts of transcripts encoding molecules involved in effector differentiation (e.g., TNFRSF9, TNFRSF4, TNFRSF18, IL2RA), transcriptional regulation of effector Treg (BATF), proliferation (MKI67), Treg identity (IKZF2, encoding the transcription factor Helios (28)) and suppressive function (ICOS, CTLA4). Additional transcripts included IL32, CCL22 and CX3CR1. By contrast, CCR8− ICOS− Tregs expressed high amounts of transcripts encoding molecules associated with early differentiation or quiescence including, CCR7, KLF2, LEF1, TCF7 (29), BACH2, the latter repressing effector programs to stabilize Treg-mediated immune homeostasis (30), and TXNIP, a negative regulator of AKT signaling and glycolytic metabolism (Fig. 2B and Table S3). Gene set enrichment analysis (GSEA) further revealed that CCR8 ICOS− Tregs were largely quiescent and displayed transcriptional signatures of Wnt/β-catenin and TGF-β signalling. In contrast, CCR8+ICOS+ Tregs displayed enhanced signatures of metabolic activity, including oxidative phosphorylation and glycolysis, mTORC1-dependent activity, previously shown to favor murine Treg-cell activation and prevention of autoimmunity (31), and reactive oxygen species metabolism, consistent with enhanced activation and/or mitochondrial respiration (Fig. 2C, Table S4) (32). To identify transcriptional regulators of CCR8+ICOS+ intratumoral Tregs, we performed computational analysis of TF binding motif (TFBM) enrichment at the promoters (-950, +50 bp from the transcriptional start site) of DEGs obtained from bulk RNAseq data. This analysis showed enrichment of binding motifs of TFs, including IRF4, predicted to be preferentially active in the CCR8+ICOS+ Treg subset (Fig. 2D). We additionally identified NRF1, involved in antioxidant defence, lipid metabolism and mitochondrial respiratory function (33), MYC, a master regulator of metabolic reprogramming in activated T cells (34) and the NF-κB family members REL, RELA and RELB. In particular, RelA has been previously shown to regulate effector Treg activity in nonlymphoid tissues downstream of TNFRSFs (35). Thus, our transcriptional profiling revealed that intratumoral IRF4+ Tregs are highly activated compared
to IRF4− Tregs, and supported the notion that they have enhanced suppressive potential. To confirm this hypothesis, FACS‐sorted CCR8ICOS+ (IRF4+) and CCR8ICOS− (IRF4−) intratumoral Tregs were further tested for their capacity to suppress proliferation of autologous CD4+CD25− Tconv in vitro (Fig. 2E). Both Treg subsets were effective in this regard at 1:1 Tconv:Treg ratio, while only CCR8ICOS+ maintained substantial suppressive capacity at a 2:1 ratio (Fig. 2E).

IRF4 has been shown to promote effector Treg differentiation in peripheral tissues and to limit autoimmunity (20), but its importance in suppressing anti‐tumor immune responses is unknown. To test the functional role of IRF4 in tumor‐infiltrating Tregs, we used mice that allow tamoxifen‐inducible deletion of IRF4 specifically in Tregs (Irf4fl/flFoxp3EGFP‐cre‐ERT2) and controls (Foxp3EGFP‐cre‐ERT2) (Fig. 2F). Induced deletion of Irf4 in FoxP3+ cells in MC38 tumor bearing mice resulted in a significant delay in tumor growth (Fig. 2F), indicating that IRF4+ Tregs suppress antitumor immunity. To further test the Treg intrinsic role of IRF4, we reconstituted lethally irradiated mice with a mix of congenically marked bone marrow from wildtype (WT) mice and mice with a T cell‐specific deletion of IRF4 (Irf4fl/flCd4Cre). We also generated mixed control chimeras containing wildtype and Cd4Cre control bone marrow. Flow cytometric analysis of tumor‐infiltrating Tregs in these chimeric mice showed severely impaired representation of IRF4‐deficient Tregs at the tumor site but not in the spleen and lack of ICOS expression, a direct target of IRF4. In contrast, control chimeras showed similar contribution of Tregs at both sites and robust ICOS expression (Supplementary Fig. 2E, F).

IRF4 and its partner BATF control a molecular program of effector Treg differentiation and suppression in tumors. Next, we formally tested the role of IRF4 and its transcriptional partner BATF in determining the features of tumor‐infiltrating Tregs by integrating our data from human Tregs with gene expression profiles and epigenomic data.
from mouse Tregs (Table S5). As mouse vs. human gene regulation is difficult to be inferred due to species differences in non-coding regions, we first defined a conserved tumor-infiltrating Treg signature by integrating DEGs of CCR8^+ICOS^+ vs CCR8^−ICOS^− Tregs from Fig. 2B and Table S3, and DEGs of tumor-infiltrating Tregs vs. spleen Tregs from a murine model (36), as outlined in Fig. 3A. In total, we identified 382 transcripts that were specifically up or downregulated in mouse and human tumor-infiltrating Tregs (Fig. 3A, Table S6). Next, we defined the transcriptional program that is dependent on IRF4 or BATF expression by performing RNAseq and analysis of gene expression profiles of splenic Tregs from wild-type vs Irf4^−/− mice. We also reanalyzed a published data set comparing wild-type vs Batf^−/− Tregs (37). Overall, this identified 1,241 and 232 genes that were controlled by IRF4 and BATF, respectively (Fig. 3B and Table S5). GSEA revealed that many genes involved in lymphocyte activation, proliferation and differentiation were under the joint control of IRF4 and BATF, those involved in apoptosis were controlled by BATF alone and those related to immunosuppression, i.e. IL-37 and IL-10-dependent signaling pathways, were under the control of IRF4 alone (Supplementary Fig. 3B). A relevant proportion (94 genes; 24.6%) of the conserved tumor Treg signature between humans and mice was controlled by IRF4 and/or BATF (Fig. 3B; IRF4 and BATF DEGs vs tumor-infiltrating Treg signature: P=1.1e^{-14} and P=3.3e^{-18}, respectively; hypergeometric test; not shown). We further made use of our chromatin immunoprecipitation deep sequencing (ChIPseq) data to assess IRF4 occupancy at the loci of interest (Table S7) and thus define a direct role of this TF in controlling gene expression in tumor-infiltrating Tregs. Supplementary Fig. 3B shows the distribution of regions in the genome that are bound directly by IRF4 according to their distance from transcriptional start sites (TSS) (see also Table S7). Despite a small fraction mapped in promoter regions (0-1 Kb), the majority of IRF4-bound sites mapped at 10-100 Kb distance from the TSS, suggesting regulation of gene expression at enhancer regions. Tumor-
infiltrating Treg genes dependent on IRF4 included several Tnfr family members involved in effector Treg differentiation such as Tnfrsf1b, Tnfrsf8, Tnfrsf18, Tnfrsf9 (the latter also dependent on Batf expression) (35), chemokine receptors such as Ccr8, Cxcr3 and Ccr5, likely involved in the localization of Tregs to tumors or to the lung and expressed by murine effector Tregs (38), and Zbtb32, Mki67, Map2k3, Kif23,Rrm2, and Aurkb, previously linked to cell cycle in other cell types and likely contributing to Treg proliferation (Fig. 2C). Instead, Icos, Ikzf2 (encoding Helios), and Il1rl1 (also known as ST2 or IL-33 receptor), were dependent on both Irf4 and Batf. Thus, Irf4 and Batf directly and indirectly control a program of effector Treg differentiation and immunosuppression in cancer.

scRNAseq-guided high-dimensional flow cytometry profiling reveals that CCR8\(^+\)ICOS\(^+\) (IRF4\(^+\)) effector Tregs associate with multiple exhaustion traits of T cells. To further characterize the T cell phenotypic landscape associated with Treg subsets in NSCLC cancer patients in an unbiased manner, we designed a second high-dimensional flow cytometry panel guided by results obtained with scRNAseq (25) and bulk RNAseq (Figure 2 and Table S2). We preferred markers identified by fluorochrome-conjugated antibodies providing a high signal-to-noise ratio (e.g., CCR8 and ICOS instead of IRF4), so to enhance the identification of subsets by the clustering algorithm, as recently suggested (39). We profiled millions of single cells from tumors (n = 45), paired adjacent cancer-free lung tissues (n = 23), and peripheral blood samples (n = 23) of treatment-naïve patients (Table S1). A schematic representation of the analysis pipeline is shown in Fig. 4A. By applying PhenoGraph, a computational algorithm capable to unbiasedly cluster single cells according to the relative expression of these molecules in the multidimensional space (40), we identified 14 different CD4\(^+\) and 15 different CD8\(^+\) T cell clusters and defined their abundance as percentage of total CD4\(^+\) or CD8\(^+\) T cells in each sample type (Fig. 4B). Also for this panel of markers, UMAP of single cell
distributions and principal component analysis (PCA) of cluster abundance in the different samples clearly distinguished T cells from the peripheral blood, lung tissues and tumors (Supplementary Fig. 4A, B), indicating that different sites have highly distinct T cell profiles. We next calculated the integrated median fluorescence intensity (iMFI) values of each marker in each PhenoGraph cluster so to obtain information on cluster identity (Fig. 4B; see Methods) (39). Metaclustering of PhenoGraph clusters (rows) and markers (columns) grouped subpopulations with similar immunophenotypes. Similar to previous findings (24), we documented the loss of subsets of naïve (CD4\(^+\): C2; CD8\(^+\): C12) and cytotoxic (CD4\(^+\): C7; CD8\(^+\): C1, C9, C2) T cells and the accumulation of exhausted T cells (CD4\(^+\): C13; CD8\(^+\): C5, C15) in tumors compared to the blood or the adjacent lung tissue (Fig. 4B). Moreover, in line with data shown in Fig. 1, we revealed the increased presence of CCR8\(^+\)ICOS\(^+\) (IRF4\(^+\)) activated Tregs (CD4\(^+\): C6) in tumors (Fig. 4B). Next, we investigated the association of these cells with specific T cell subsets in the TME by performing a Pearson correlation analysis of the abundance of CD4\(^+\) and CD8\(^+\) T cell clusters as identified by PhenoGraph in 45 patients (Fig. 4C). Notably, intratumoral CCR8\(^+\)ICOS\(^+\) Tregs correlated with CD4\(^+\) (C13) and CD8\(^+\) (C5 and C15) T cells with features of exhaustion (i.e., expressing PD1, TIM3 and TIGIT in different combinations and intensities). At the same time, CCR8\(^+\)ICOS\(^+\) Tregs negatively correlated with cytotoxic CD8\(^+\) T cells (CTL; C2, co-expressing GZMB and GNLY and thus armed for rapid effector functions) and with clusters of T cells bearing a central memory T (T\(_{CM}\)) phenotype (CD4\(^+\): C11 and CD8\(^+\): C14). In summary, high-dimensional single cell profiling identifies a T cell signature with increased frequencies of CCR8\(^+\)ICOS\(^+\) effector Tregs associated with T cell exhaustion.

CCR8\(^+\)ICOS\(^+\) (IRF4\(^+\)) effector Tregs define a signature of disease progression in NSCLC. We finally tested whether a phenotypic landscape of T cells involving IRF4\(^+\) effector
Tregs could be found to be different in cancer patients with different prognoses. First, a subset of our cohort of patients (n=25) was subdivided in two groups according to the median distribution of the maximum standardized value of fluorodeoxyglucose uptake (SUVmax), a positron emission tomography (PET) indicator of tumor glycolysis and aggressiveness. PCA revealed a bimodal separation of the SUVmax$^{\text{low}}$ and SUVmax$^{\text{high}}$ groups of patients according to the relative frequencies of CD4$^+$ and CD8$^+$ PhenoGraph clusters (Fig. 5A, left panel), indicating that they display a different T cell profile as a whole. Analysis of PCA loadings identified clusters contributing the most to such distribution (Fig. 5A, right panel). Specifically, SUVmax$^{\text{high}}$ patients harbored increased frequencies of CCR8$^+$ICOS$^+$ Tregs (C6), subsets of CD8$^+$ (C5 and C15) and CD4$^+$ T cells (C5) with traits of exhaustion, or memory T (Tm) cells expressing GZMK, EOMES (contributing to T cell dysfunction in humans (41)) and PD1 (CD8$^+$: C3, C7), while SUVmax$^{\text{low}}$ patients harbored increased frequencies of CCR5$^+$ clusters (CD8$^+$: C4, C6 and CD4$^+$: C3, C4) as well as terminal effector, cytotoxic T cells (CD8$^+$ C1 and CD4$^+$ C7) (Fig. 5A). We observed similar trends when considering a parameter of disease progression, i.e. the international TNM classification, according to which CCR8$^+$ICOS$^+$ Tregs (CD4$^+$ C6) as well as T cells featuring exhaustion/activation markers (CD8$^+$: C5 and C15) were more abundant in patients with advanced pathological stage (pStage) II and III (Fig. 5B).

Infiltration of CD8$^+$ T cells is a predictor of good prognosis in multiple types of cancer (42). We thus investigated disease free survival (DFS) of our patients according to the Treg/CD8$^+$ T cell ratio (as determined by their frequency among CD3$^+$) at the tumors site, and found that a higher ratio is significantly associated with early tumor relapse (Fig. 5C). As predicted by their superior suppressive activity, this was due to the contribution of CCR8$^+$ICOS$^+$, but not of CCR8$^-$ ICOS$^-$ Tregs (Fig. 5C). We next validated our findings in larger cohorts of individuals such as those from the cancer genome atlas (TCGA). In these, the degree of infiltration of specific subpopulations was determined by enrichment of
transcriptional signatures (see methods). Similar to results from our NSCLC cohort, we found that a higher CCR8^+ICOS^+ Treg/CD8^+ T cell signature in bulk RNAseq data from primary biopsies was associated both with worse DFS and overall survival (OS) in lung adenocarcinoma (Fig. 5D) (43), hepatocellular carcinoma (Supplementary Fig. 5A) (44) and melanoma (Supplementary Fig. 5B), thereby underlying a common biology of Treg-mediated suppression of anti-tumor immunity in multiple human cancers.
DISCUSSION

Human tumors are often infiltrated by large numbers of CD4+ Tregs that display a highly activated phenotype and enhanced suppressive capacity compared to those present in the peripheral and the adjacent tumor-free tissues (15, 16). We have now shown that the transcriptional program associated with such activation and suppression in the TME is driven by the TF IRF4 in combination with its molecular partner BATF. Signals downstream of the TCR are likely involved in IRF4 induction (45), possibly suggesting that human Tregs suppress anti-tumor immunity in an antigen-specific way.

IRF4, previously linked to effector Treg differentiation in murine tissues in physiology (20), is now shown to control the formation of ICOS+ effector Tregs also in the TME that in turn favor tumor growth in a mouse model of cancer. IRF4 controls gene expression of tumor-infiltrating Tregs both directly, by binding gene promoters or distal regulatory regions, and indirectly, by inducing the expression of additional transcriptional regulators such as IKZF2 (Helios), required for Treg stability (28). IRF4 alone binds DNA poorly; however, binding is increased when IRF4 is part of a macromolecular complex involving AP-1 family members BATF, JUN, JUNB or JUND, recognizing DNA motifs known as AP-1/IRF composite elements (AICEs) (21, 46). Although the regions bound by these TFs are largely overlapping, IRF4 and BATF also bind unique regions (21), possibly explaining why their deficiency has differential, specific effects on gene expression. We did not formally test whether BATF is responsible for the expression of specific genes by direct binding; however, additional computational investigations identified AICE motifs in the proximity of genes that are linked to effector Treg differentiation and function, including Ccr8, Icos, Ctla4, Cxcr3, Il12rb1, Tnfrsf1b and Tnfrsf8 (not shown). In relation to this, JunB has recently been shown to control murine effector Treg differentiation in lung and colon (47) while additional TFs, such as STAT3, may physically interact with c-Jun (48). STAT3 mRNA is upregulated and the STAT3 binding motif
is enriched in IRF4⁺ vs. IRF4⁻ Tregs (data not shown), overall suggesting that a complex range of molecular interactions is cooperating to shape the effector differentiation and enhanced suppressive activity of intratumoral Tregs.

Our study reveals an additional important aspect, namely that effector differentiation is not a feature of all intratumoral Tregs. Rather, a subset of these cells with increased expression of IRF4 is preferentially expanded in lung, liver and melanoma tumors compared to the adjacent, tumor-free tissues and peripheral blood, as revealed by scRNAseq. These IRF4⁺ Tregs express high amounts of molecules associated with enhanced immunosuppression, have increased metabolic demand and are phenotypically and transcriptionally distinct from the more quiescent IRF4⁻ Tregs. While in humans ~40% of Tregs express IRF4 as detected by flow cytometry, deletion of *Irf4* in mice results in the near complete loss of Tregs in tumors, possibly implicating that low levels of IRF4 are also present in CCR8 ICOS⁻ Tregs. An alternative hypothesis is that slow tumor development as seen in humans results in a balanced infiltration of less and more activated Tregs. In line with this, ~40% of Tregs infiltrating lung adenocarcinoma forming in the K-ras<sup>G12D</sup> autochthonous model (which better recapitulates the physiology of lung adenocarcinoma) express the IL-33 receptor ST2 (encoded by *Il1rl1*) (49), a direct genomic target of IRF4 (Figure 3C). Similarly, a recent human study found that ~50% of breast cancer-infiltrating Tregs express CCR8 (50), thereby corroborating our results of Treg heterogeneity in the TME. Despite not reaching the capability of scRNAseq in terms of number of parameters being measured, the high-dimensional flow cytometry employed here investigates T cell phenotypes as a whole in a large cohort of patients compared to those recently investigated by scRNAseq (25) and is thus capable to further define the relationships between Treg subsets and other T cell populations within the TME. In this way, we revealed that IRF4⁺ Tregs positively correlate with multiple subsets of exhausted and activated CD4⁺ and CD8⁺ T cells, and their abundance is associated both with relapse and poor overall survival
in multiple human cancers. Therefore, deep immunophenotyping with improved and high-throughput single cell technologies can pinpoint those subsets associated with slower tumor growth and favorable anti-tumor responses with enhanced precision. We anticipate that the simple addition of an effector Treg marker (e.g., IRF4, ICOS or CCR8 or a TNFR superfamily member) to FoxP3 staining, as done by immunohistochemistry in immunoscore approaches (51), will improve the definition of patients with improved prognosis.

The identification of CD4⁺ Treg heterogeneity in the TME and, as a consequence, of a molecular program mastering the differentiation of those Tregs with enhanced suppressive capacity offers novel opportunities to reverse immunosuppression while favoring anti-tumor immune responses. Depletion of Tregs has been tested in a number of preclinical approaches to promote anti-tumor responses (11-14), however novel strategies interfering selectively with the activated, effector Treg state are emerging as a promising tool to boost effective anti-tumor immunity without resulting in overt autoimmunity due to the loss of peripheral tolerance (14, 52). We expect that targeting those signals leading to IRF4 activation, or downstream, IRF4-dependent Treg activation would result in a similar scenario. This approach may be widely applicable, as our data show that IRF4-driven effector Treg differentiation is common to at least three human tumor types, i.e., lung cancer, hepatocellular carcinoma and melanoma. Further definition of the molecular network orchestrating the suppressive capacity of intratumoral Tregs and, most importantly, the identification of specific players that are not active in anti-tumor infiltrating lymphocytes is anticipated to benefit cancer immunotherapy strategies.
MATERIALS AND METHODS

Study design. The characteristics of the patients and of the samples used in this study as well as the procedures of cell isolation were previously described (24). Details on the patients’ characteristics included in this manuscript are further indicated in Table S1. Information on the pathological stage (pStage), determined by an institutional pathologist (some patients were re-staged as III following examination of the tumor), was available for all patients, while results of the preoperative FDG PET-CT scan was available for 25 patients. Details on obtainment of PET scans were previously described (24).

Polychromatic flow cytometry and cell sorting. Antibodies used in the study are listed in Table S2. Flow cytometry procedures of high-dimensional single cell panel development were previously described (24, 39). Additional panels used for further characterizing the IRF4+ CD4+ cell subset are listed in Table S2. All data were acquired on a FACS Symphony A5 flow cytometer (BD Biosciences) equipped with 5 lasers (UV, 350 nm; violet, 405 nm; blue, 488; yellow/green, 561 nm; red, 640 nm; all tuned at 100 mW, except UV tuned at 60 mW) and capable to detect 30 parameters. Flow cytometry data were compensated in FlowJo by using single stained controls (BD Compbeads incubated with fluorescently-conjugated antibodies), as described (53). CCR8+ICOS+ and CCR8–ICOS–, pregated as CD4+ Aqua– CD25+CD127–, were isolated from tumor samples with a FACSAria cell sorter (BD Biosciences). CCR8 and ICOS proved to be the best combination over other markers to isolate Treg subsets with differential IRF4 expression (data not shown).

Suppression of T cell proliferation by Tregs. Live (Aqua–) CD4+ CD25– Tconv were isolated from patients’ blood samples with FACSAria cell sorter and stained with CellTrace CFSE kit (final concentration: 2µM; ThermoFisher Scientific) according to the manufacturer’s
protocol. Subsequently, cells were plated in R10 U-bottom 96-well plates (10,000 cells/well) and stimulated with human Treg Suppression Inspector beads (Miltenyi Biotec Ltd., UK) for 5 days at 37°C. Tconv cultured alone, in the absence of bead stimulation, were used as non-proliferating, negative control. CCR8^+ICOS^+ and CCR8^+ICOS^- Tregs were FACS-sorted from tumors and were added to autologous Tconv cell cultures at different ratios (Tconv:Treg ratio= 1:1, 2:1 and 4:1). CFSE dilution was evaluated at day 5.

**High-dimensional flow cytometry data analysis.** Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 9, and analyzed by standard gating to remove aggregates and dead cells, and identify CD3^+CD4^+ or CD8^+ T cells. 3,000 CD4^+ and CD8^+ T cells per sample were subsequently imported in FlowJo version 10, biexponentially transformed and exported for further analysis in Python (version 3.7.3) by a custom-made script that makes use of PhenoGraph (scikit-learn package) with fixed seed “123456” (https://github.com/luglilab/Cytophenograph). Blood, adjacent lung tissue and tumor samples were labelled with a unique computational barcode for further identification and converted in comma separated (CSV) files and concatenated using in a single matrix by using the merge function of pandas package. K value, indicating the number of nearest neighbors identified in the first iteration of the algorithm, was set at 40 and 60 for CD4^+ and CD8^+ T cells clustering, respectively. Clusters representing less than 1% of total CD4^+ or CD8^+ T cells were removed in subsequent analysis. The data were then re-organized and saved as new CSV files, one for each cluster, that were further analyzed in FlowJo to determine the frequency of positive cells for each marker and the corresponding median fluorescent intensity (MFI). These values were multiplied to derive the integrated MFI (iMFI, rescaled to values from 0 to 100; **Fig. 4B**) (24, 39). The heatmap, showing the iMFI of each marker per cluster, and the subsequent metaclustering was performed using the gplots R package. Hierarchical metaclustering of all
samples, based on the frequency of PhenoGraph clusters (Fig. 4B), was performed in R according to the Ward minimum variance method. UMAP was obtained by UMAP Python package and visualized in FlowJo 10.

**scRNAseq analysis**

**Melanoma dataset.** Normalized scRNA-seq counts were retrieved from Gene Expression Omnibus (dataset [GSE72056](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72056)). Analysis was restricted to the cells labelled as “T cells,” as previously defined by Tirosh et al. (27). T cells were divided into CD4+ and CD8+ based on the normalized expression levels (E) of CD4 (E > 4), and CD8 (average of CD8A and CD8B, E > 4). Among all samples composing the dataset, 466 were identified as CD4+. To asss which genes have the expression profile most correlated with the one of IRF4, a Pearson correlation coefficient, indicated as score in the Supplementary Fig. 1B, and a corresponding P value were computed between each gene expression profile in the matrix (23,686) and IRF4. In order to obtain a CD8 signature used in Supplementary Fig. 5B, differentially expressed genes in the pairwise comparison between CD8+ and CD4+ T cell subsets were determined by the “FindAllMarkers” function coded in the Seurat R package (version 2.3.4) with default parameters (54). In this way, we obtained 225 specific genes for the CD8+ subpopulation respect to the CD4+ T cells.

**Lung and Liver datasets.** We took advantage of the webserver for exploration of NSCLC ([http://lung.cancer-pku.cn](http://lung.cancer-pku.cn)) and of hepatocellular carcinoma ([http://hcc.cancer-pku.cn/](http://hcc.cancer-pku.cn/)) single T cell RNA-seq data to assess the expression levels of markers of interest within the CD4+ intratumoral subpopulations. Lung and liver tumor infiltrating CD8+ signatures ([Fig. 5D](#) and Supplementary Fig. 5A) were obtained by combining all the DEGs from the CD8+ tissue-specific clusters and by further excluding the circulating clusters labelled as CD8-C1-LEF1 (26, 27).
Mice. *Irf4*-/- mice (55) were originally provided by Tak Mak. *Irf4*-/- mice were crossed to Foxp3<sup>RFP</sup> mice. Foxp3<sup>eGFP-Cre-ERT2</sup> (JAX #016961) (56), were crossed to *Irf4* floxed mice (JAX stock #009380) (57) to allow for specific deletion of *Irf4* in Foxp3<sup>+</sup> cells following Tamoxifen treatment.

Mouse procedures. Irradiation: mice were irradiated 2x5.5 Gy and reconstituted by i.v injection of 200 µL of bone marrow isolated from femur and tibia of donor mice. Following irradiation mice were treated with Neomycin in drinking water for 4 weeks and allowed to recover for at least 8 weeks. Tumor: MC38 cells (provided by Stephen Nutt, WEHI, Melbourne) were grown in DMEM with 10% FCS and 1% PenStrep (Gibco) in 37°C, 5% CO<sub>2</sub> incubator and passaged every 2-3 days. For tumor inoculation, 1x10<sup>6</sup> cells were resuspended in 100ul PBS and injected sub cutaneously into the right flank of the mice. Tumor growth was assessed with a digital calliper every 2-3 days. Tamoxifen (2 mg/mouse) was administered in 200 uL Sunflower oil i.p. for five consecutive days. Upon the experimental endpoint, mice were euthanized with CO<sub>2</sub> and cervical dislocation and tumors were excised with forceps and scissors. Tumors were mechanically dissociated and digested in 1mg/ml Collagenase IV (Gibco) in RPMI medium for 45min at 37°C, with constant agitation.

Treg isolation from mice. Single cell suspensions from spleens were enriched for CD4<sup>+</sup> T cells by depleting CD8<sup>+</sup> T cells and B cells using anti-CD8 and B220 antibodies. CD4<sup>+</sup> T cell enriched cell suspension was stained with CD4 (RM 4-5) and TCR<sub>β</sub> (H57-597) antibodies. CD4<sup>+</sup> TCR<sub>β</sub><sup>+</sup> Foxp3<sup>RFP</sup><sup>+</sup> cells were sorted from Foxp3<sup>RFP</sup> (WT) and *Irf4*-/- Foxp3<sup>RFP</sup> mice.
**Mouse RNA isolation and sequencing.** RNA purification was performed following the manufacturer’s protocol using the RNAeasy Plus Mini Kit (Qiagen). RNA from WT and *Irf4<sup>−/−</sup>* Tregs were sequenced using Illumina platform (75bp paired end reads).

**RNA-sequencing and bioinformatic analysis.** RNA isolation from the FACS-purified CCR8<sup>+</sup>ICOS<sup>+</sup> and CCR8<sup>−</sup>ICOS<sup>−</sup> Treg cells was performed following the manufacturer’s protocol using the Quick-RNA Microprep kit (Zymo research, CA, USA). RNA quality control was performed with the Agilent 2200 Tape Station system and only RNAs having a RIN>8 were used for library preparation. Libraries for mRNA-sequencing were prepared starting from 1.5 ng tot RNA for each sample by using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech-Takara). All samples were sequenced on an Illumina NextSeq 500 at an average of 32.9 million 75-bp single-end reads. After quality control, raw reads were aligned to the human genome (GRCh38.p12) using the STAR aligner with default parameters (version 2.7.0) (58). Gene-based read counts were then obtained using HTSeq count (59) module (version 0.11) and GENCODE v29.gtf annotation (60). The read counts were imported into R statistical software and differential gene expression analysis was performed using the edgeR package (version 3.22) (61). For pair-wise comparisons, raw read counts were normalized using the TMM method (trimmed mean of log-ratio values) (62) and genes that failed to achieve a counts per million (CPM) mapped reads value greater than 1 in at least two libraries were not considered. P-values were adjusted using the Benjamini-Hochberg method. Genes were considered differentially expressed when FDR< 0.05 and had an expression change of >1 log<sub>2</sub> fold change. The heatmap representing the log<sub>2</sub> of CPM was obtained using pheatmap R package (version 1.0.12) with the distance method "correlation" for both rows and columns.
Effector Treg differentiation in cancer

**Gene Signature Identification (RNAseq).** Gene Set Enrichment Analysis (GSEA) was performed using GSEA (version 3.0) software (Broad Institute, MIT) and gene list ranked based on log\(_2\) fold changes. The gene set enrichment analysis was conducted in pre-ranked mode with scoring scheme “classic” and 1,000 permutations. The maximum gene set size was fixed at 5,000 genes, and the minimum size fixed at 10 genes. The gene signature was retrieved from the the H collection (h.all.v6.2.symbols.gmt) of the Molecular Signatures Database (MSigDB v6.2). The GSEA in **Supplementary Fig. 3B** was performed with custom gene sets relevant to immunological signatures ([https://github.com/luglilab/ProjectScripts_Treg_Irf4/blob/master/Figure5/Mouse_ImmunePath_February_01_2019_symbol.gmt](https://github.com/luglilab/ProjectScripts_Treg_Irf4/blob/master/Figure5/Mouse_ImmunePath_February_01_2019_symbol.gmt)). Dotplot was generated with a custom Rscript available here: ([https://github.com/luglilab/ProjectScripts_Treg_Irf4/tree/master/Figure5/DotplotSuppFig5.r](https://github.com/luglilab/ProjectScripts_Treg_Irf4/tree/master/Figure5/DotplotSuppFig5.r)).

**Motif enrichment analysis.** The PScan software tool (version 1.5) was used to perform the in silico computational analysis of over-represented transcription factor binding sites (TFBS) within the 5′-promoter regions of differentially expressed genes (63). PScan was ran on [-950, +50] bp upstream regions onto the Homo Sapiens JASPAR 2018 NR database (64). Results were summarized with a scatter plot where p-values were plotted against Z-score on vertical axis by using Python Matplotlib package (version 3.0.3).

**Bioinformatic analysis of microarray data.** Microarray probe fluorescence signals downloaded from the Gene Expression Omnibus (GSE89656 and GSE61077 (samples GSM1496276, GSM1496277, GSM1496274, GSM1496275) were converted to expression values using robust multiarray average procedure RMA (65) of Bioconductor Affy package. Fluorescence intensities were background-adjusted and normalized using quantile
normalization, and expression values were calculated using median polish summarization and custom chip definition files for a total of 18,075 custom probe sets for Mouse Genome 430 2.0 Array based on Entrez genes (Mouse4302_Mm_ENTREZG version 21.0.0) and 12,426 custom probe sets for Mouse Genome 430A 2.0 Array based on Entrez genes (Mouse430A2_Mm_ENTREZG version 21.0.0). To identify genes that are differentially expressed, we compared the expression profiles of Batf−/− Treg and WT Treg cells, using limma algorithm coded in the same R package (66). All data analyses were performed in R version 3.4.4 using Bioconductor libraries and R statistical packages.

**Bioinformatic analysis of ChIP-sequencing data.** Raw data were downloaded from NCBI Gene Expression Omnibus (GEO; accession number GSE98264). Reads were aligned to mouse genome GRCm38.p6/mm10 using Bowtie2 (version 2.1.0) (67) in local alignment mode. After alignment to the reference genome, mitochondrial and ambiguously mapped reads were discarded with Samtools (68), further used for sorting and indexing mapping files. Bigwig files for IGV (69) genome coverage visualization were generated with the multiBamSummary module from the deepTools suite (version 3.2.0) (70). To call peaks, we used MACS2 (version 2.1.2) (71) with these parameters: callpeak gsize mm nomodel extsize 147 and Qvalue 1e-3. Peaks within 30 kb upstream and 10kb downstream of the TSS or within intragenic regions were annotated with the closest TSS using ChIPSeeker (version 1.18.0) (72) and GENCODE M20.gtf gene annotation.

**Survival analysis** Transcriptomic and clinical data of human lung adenocarcinoma, hepatocellular carcinoma and melanoma from the TCGA database (provisional cohorts) were obtained from the cBioPortal platform. Signatures of CCR8+ICOS+ Tregs (Fig. 2B) and CD8+ T cells (see paragraph “Gene signature identification”) were used to calculate patient-specific enrichment scores from specimens of lung adenocarcinoma (LUAD), hepatocellular
carcinoma (LIHC) and melanoma (SKCM) datasets (“GSVA” R package)(73). Survival curves were calculated between groups of patients subdivided according to the percentile rank (set at 0.8) of the resulting scores. The R packages “survival” ([https://cran.rproject.org/web/packages/survival/index.html](https://cran.rproject.org/web/packages/survival/index.html)) and “survminer” ([https://cran.rproject.org/web/packages/survminer/index.html](https://cran.rproject.org/web/packages/survminer/index.html)) were used to assess statistics and obtain survival curves.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 7, unless specified otherwise. Data were first tested for normal distribution with D’Agostino-Pearson, Shapiro-Wilk, or Kolmogorov-Smirnov normality tests and then analyzed with Wilcoxon rank test (paired non-parametric data) or Mann-Whitney (unpaired non-parametric data) when comparing 2 groups; Friedman test or two-way ANOVA with Bonferroni post-hoc test for multiple comparisons when comparing 3 groups (specific tests are specified in the figure legends).

Correlation of gene expression from scRNAseq data was determined by calculating the Pearson correlation coefficient. P values of less than or equal to 0.05 were considered significant. Principal Component Analysis (PCA) was performed by the prcomp function of the R stats package. Hypergeometric tests between *Irf4* or *Batf* DEGs or scRNAseq signature (25) and the murine tumor-infiltrating Treg signature (Fig. 3B) was run with GeneOverlap R package.

**Study approvals.** All human experiments were approved by the Humanitas Clinical and Research Center Internal Review Board (IRB; approval #1501). All patients provided written informed consent. Mice were maintained and used in accordance with the guidelines of the Walter and Eliza Hall Institute and approved by the institutional IACUC.
Data Availability. The gene expression data generated in this study are available under the GEO accession number GSE128822.

Supplementary Materials

Supplementary Fig. 1 The IRF4$^+$ Treg signature is conserved among different cancer types

Supplementary Fig. 2 Bulk RNAseq of CCR8$^+$ICOS$^+$ and CCR8$^-$ICOS$^-$ tumor-infiltrating Treg subsets

Supplementary Fig. 3 Batf and Irf4-dependent gene regulation

Supplementary Fig. 4 Transcriptomics-guided FACS panel design identifies tumor-specific T cell subpopulations

Supplementary Fig. 5 CCR8$^+$ICOS$^+$ effector Treg infiltration relative to CD8$^+$ defines a signature of disease progression in different cancer types

Table S1. Characteristics of the patients analyzed in this study

Table S2. List of antibodies for flow cytometry used in this study

Table S3. Human tumor-infiltrating Tregs RNAseq DEGs: CCR8$^+$ICOS$^+$ vs CCR8$^-$ICOS$^-$

Table S4. Hallmark GSEA results from DEGs between CCR8$^+$ICOS$^+$ vs CCR8$^-$ICOS$^-$ Tregs

Table S5. List of the publicly available datasets re-analyzed in this study

Table S6. Tumor infiltrating Treg signature

Table S7. Annotated peaks, IRF4 ChIP sequencing (MM10)
AUTHOR CONTRIBUTIONS

G.A., Jo.Br., S.P., E.M.C.M., E.P.T., C.P., A.V., Jo.Bl. performed experiments, F.S.C. provided expertise with cell sorting, V.Z., C.P., A.S., provided technical support, M.A., P.N., G.V. performed surgery, A.L., Eg.Lo. and G.V. provided clinical information and helped with clinical interpretation of the data, R.R., M.K. and M.P. provided expertise in data analysis, G.A., Jo.Br., S.P., A.K. and En.Lu. conceived the study, En.Lu. supervised the study, G.A. and En.Lu. wrote the paper. All authors edited the paper. G.A., Jo.Br. and S.P. share first authorship. G.A. and Jo.Br. provided equal contribution in conceiving the overall study and in performing the vast majority of the experiments, while S.P. conceived and performed all the bioinformatic analyses. Given the importance of bioinformatics in this manuscript, these authors deserve equal contribution. The order of the first authors reflects the leadership exerted in the study.

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Bibliography


Figure 1. IRF4 identifies effector Tregs enriched in human tumors. A. UMAP analysis of concatenated CD4+ T cells (1,500 cells/sample) from tumor (n=53), adjacent lung tissue (n=45) and blood (n=22) samples from NSCLC patients. B. UMAP of relative markers expression by concatenated CD4+ T cells from the same samples in A. C. Manual gating analysis of CD4+CD25+FOXP3+ Tregs expressing IRF4+ by flow cytometry. Numbers are % of positive cells. D. Summary plot representing the IRF4 expression in CD4+ Treg and conventional T (Tconv; defined as CD25low FoxP3–) cells from the same patients as in A; Kruskal-Wallis test; **** = P<0.0001; *** = P<0.0005). E. Representative distribution by flow cytometry (top) and summary of percent expression of selected markers (bottom) in tumor-infiltrating IRF4+ and IRF4– Tregs, and Tconv cells (non parametric Friedman test, **** = P<0.0001; *** = P< 0.0005; * = P<0.01). F. Boxplot showing the log2 (TPM+1) expression of IRF4 transcript across 9 CD4+ T cells clusters as identified by single-cell RNAseq (Guo et al., Nat. Med. 2018). Each dot is a single cell (Wilcoxon test; ****=<0.0001, *=0.01). G. t-SNE plots illustrating the expression of selected genes in single CD4+ T cells from lung tumor lesions. Cell clusters, depicted on the left, were identified as in F.
Figure 2. Transcriptional and functional profiling defines the effector and enhanced suppressive nature of IRF4+ Tregs. A. Left: representative ICOS and CCR8 expression in tumour infiltrating CD25+CD127+ Treg subsets defined by IRF4 expression. Right: percent IRF4 expression in tumour-infiltrating Tregs gated as ICOS-CCR8+ or ICOS-CCR8+. B. Heatmap of differentially expressed genes (DEGs) in the FACS-sorted CCR8+ICOS+ versus ICOS-CCR8+ tumour-infiltrating Tregs, as obtained by RNAseq (FDR< 0.05). Selected DEGs are indicated. For some genes, protein names are indicated between brackets. C. “Hallmark” gene sets (MsigDB, as obtained by gene set enrichment analysis ‘GSEA’) significantly enriched in cells sorted as in B. D. Transcription factor binding motif (TFBM) enrichment analysis by pScan of RNAseq data obtained as in B. Colored dots indicate significant hits. E. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4+ CD25+ T cells (Tconv) dilution from a representative blood sample. Tconv were cocultured with Suppression Inspector MACSIBead™ beads and different ratios of intratumoral Treg subsets for 5 days. Data are representative of 5 independent experiments. F. Tumor volumes in FoxP3EGFPcreERT2 (control) or Irf4EGFPcreERT2 mice following the administration of tamoxifen. Left: tumor curves in single mice; right: mean±SEM of the same cohort. **=P<0.01, paired Student t-test.
Figure 3. IRF4 and its partner BATF directly and indirectly control a molecular program of effector Treg differentiation and suppression. A. Identification of a shared gene expression signature between tumor-infiltrating human CCR8⁺ ICOS⁺ vs CCR8⁻ICOS⁻ Treg and murine Tregs (obtained from Magnuson, PNAS, 2018). B. Venn diagram of the number of genes of the tumor-specific Treg signature obtained as in A that are differentially expressed in splenic Tregs from Batf⁻/- and Irf4⁻/- mice. Genes controlled only by Batf (n=10) were of limited interest and thus not further investigated. C. List of tumor-infiltrating Treg genes that are dependent on the expression of Irf4 or Irf4 and Batf. All genes are induced, except for Plac8 that is repressed (indicated in light blue). Those genes directly controlled by IRF4 binding to the genome as obtained from ChIPseq analysis of murine Tregs are highlighted. Genomic binding of IRF4 to the DNA for selected genes is depicted.
Figure 4. Abundance of CCR8*ICOS* intratumoral Tregs is associated with multiple features of T cell exhaustion. A. Experimental workflow. B. Heatmaps of the relative expression, depicted as integrated MFI (iMFI: MFI x % antigen expression) of markers (columns) in discrete CD4+ (left panel) and CD8+ (right panel) PhenoGraph clusters (rows). Treg: Memory; Treg: Central Memory; Treg: Naive, Exh: Exhausted, Act: Activated; CTL: Cytotoxic T Lymphocyte. Data are further meta-clustered to group subpopulations with similar immune-phenotypes. The median frequency of each PhenoGraph cluster in the different compartments is depicted by using Balloon plots. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 tumor vs. blood or vs. normal tissue samples; two-way ANOVA with Bonferroni post-hoc test. C. Correlogram showing Pearson correlation between frequencies of CD4+ (T4) and CD8+ (T8) PhenoGraph clusters in tumor samples from 45 early stage (I-III) non-small-cell lung cancer (NSCLC) patients. Non significant correlations (p-value > 0.05) are left blank.
Figure 5. CCR8+ICOS+ Tregs define a signature of disease progression in NSCLC. A. Left: Principal Component Analysis (PCA) plot showing the distribution of patients (n=48) according to the frequency of CD4+ and CD8+ PhenoGraph clusters in each patient (Treg: Memory; Tcm: Central Memory). Patients were classified according to pathological stage (pStage) I, II or III of the International TNM classification. Right: PCA loading plot of PhenoGraph clusters most contributing to the PCA output on the left. B. Left: PCA plot showing the distribution of patients (n=26) according to the frequency of CD4+ and CD8+ PhenoGraph clusters in each patient. The cohort was subdivided in two groups according to the median distribution of maximum Standardized Uptake Value (SUVmax). Right: PCA loading plot as in A. C. Kaplan-Meier progression free survival (PFS) curves according to the intratumoral frequencies of Tregs subsets over CD8+ T cells in each patient (n=38). The cohort was subdivided in two groups according to the percentile rank (set at 0.8). The p value was calculated by Gehan-Breslow-Wilcoxon test. D. Kaplan–Meier disease free survival (DFS; left) and overall survival (OS; right) curves in the TCGA LUAD lung cancer cohort (n=516). Patients were grouped by percentile rank (set at 0.8) according to the enrichment of the CCR8+ICOS+ bulk Treg signature (as obtained in Figure 2B) as relative to the CD8+ T cell signature. + indicates censored observations. P values were calculated by multivariate Cox regression. Dotted lines indicate the time at which 50% of the cohort was still free of the event.