Genome-wide DNA-methylation landscape defines specialization of regulatory T cells in tissues

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Regulatory T cells (Treg cells) perform two distinct functions: they maintain self-tolerance, and they support organ homeostasis by differentiating into specialized tissue Treg cells. We found that epigenetic modifications defined the molecular characteristics of tissue Treg cells. Tagmentation-based whole-genome bisulfite sequencing revealed more than 11,000 regions that were methylated differentially in pairwise comparisons of tissue Treg cell populations and lymphoid T cells. Similarities in the epigenetic landscape led to the identification of a common tissue Treg cell population that was present in many organs and was characterized by gain and loss of DNA methylation that included many gene sites associated with the Treg subset of helper T cells, such as the gene encoding cytokine IL-33 receptor ST2, as well as the production of tissue-regenerative factors. Furthermore, the ST2-expressing population was dependent on the transcriptional regulator BATF and could be expanded by IL-33. Thus, tissue Treg cells integrate multiple waves of epigenetic reprogramming that define their tissue-restricted specialization.

Regulatory T cells (Treg cells) are critical for the maintenance of self-tolerance. They modulate the functions of various immune cells and thereby affect a variety of conditions, including autoimmunity, cancer, allergy and inflammation1,2. In addition, it has been increasingly clear that specialized Treg cells in tissues are important for the promotion of organ homeostasis, a function that was initially attributed only to tissue-resident macrophages3. In the fat (visceral adipose tissue), Treg cells support metabolic functions and express PPAR-γ, a master regulator of adipocyte differentiation3,5, and the α-chain of the cytokine receptor IL-33R (ST2)6. Other examples of tissue homeostasis promoted by specialized Treg cells include injured skeletal muscles and lungs after infection with influenza A virus7,8. In both cases, Treg cells present in damaged tissues produce amphiregulin (AREG), a ligand for the epidermal-growth-factor receptor that is a tissue-regenerative factor important for tissue repair7,8.

The molecular mechanisms by which tissue-resident Treg cells acquire and stabilize their ‘tissular’ program are poorly understood. Epigenetic modifications have been linked to the establishment of tissue-resident characteristics in macrophages9,10. Similar mechanisms could be important for shaping the tissue identity of Treg cells. Our analysis of the pattern of DNA methylation (the ‘methylation’) revealed 11,000 differentially methylated regions (DMRs) associated with about 4,000 genes. Shared epigenetic profiles led to the identification of a common tissue Treg cell population characterized by the epigenetic reprogramming of parts of the pattern of the Treg subset of helper T cells and the production of AREG. Our data suggest that epigenetic events shape the characteristics and functions of tissue Treg cells.

RESULTS Identification of DMRs

To investigate the tissue-specific program of Treg cells, we performed low-input transposon-based fragmentation (tagmentation)-based whole-genome bisulfite sequencing (TWGBS) to delineate the DNA methylene of Treg cells isolated from various tissues. Using mice expressing a green fluorescent protein (GFP) reporter under the transcriptional control of Foxp3, we isolated Treg cells from abdominal fat depots, skin, liver andinguinal lymph nodes (LNs), and included CD4+ conventional T cells (Tconv cells) from LNs as a control population (Fig. 1a and Supplementary Fig. 1). Three independent biological replicates per sample were assessed, and robust data were obtained for all samples with reproducible replicates, with about 7 × 106 total reads per group and an average coverage of 20-fold for each CpG dinucleotide per population (Supplementary Fig. 2a). In pairwise

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comparisons, a strict definition of a difference of at least 30% in DNA methylation was chosen, and this revealed about 11,000 unique DMRs (Fig. 1b,c and Supplementary Fig. 2b). The average length of a DMR was about 1 kilobase pairs (kb), and annotation with genomic features derived from the reference-sequence database RefSeq illustrated that the majority of DMRs were located in intragenic regions (63%), whereas promoter regions and intergenic regions contained only 9% and 28%, respectively, of the DMRs (Fig. 1c and Supplementary Fig. 2c,d). We observed a peak of DMRs located immediately downstream of transcription start sites (Fig. 1d). Principal-component analysis showed that the methylation patterns of fat T\textsubscript{reg} cells and skin T\textsubscript{reg} cells were more similar to each other and were distinct from those of LN T\textsubscript{reg} cells and T\textsubscript{conv} cells (Fig. 1b,e). These results indicated that T\textsubscript{reg} cells in tissues had a distinguishable methylation pattern.

RNA transcriptome analysis supports DNA-methylation patterns

We performed RNA-based next-generation sequencing (RNAseq) analysis of T\textsubscript{reg} cells and T\textsubscript{conv} cells from tissues. The RNA transcriptome revealed substantial differences between T\textsubscript{reg} cells from tissues and those from LNs in their gene expression: 3,072 genes and 4,698 genes were expressed differentially by fat T\textsubscript{reg} cells and skin T\textsubscript{reg} cells relative to their expression in LN T\textsubscript{reg} cells, respectively (Fig. 2a). The comparison between fat T\textsubscript{reg} cells and skin-resident T\textsubscript{reg} cells showed that 552 genes were expressed differentially by these cells (Fig. 2a). Unsupervised hierarchical clustering of the RNAseq data confirmed that finding and grouped fat T\textsubscript{reg} cells and skin T\textsubscript{reg} cells close together, whereas liver T\textsubscript{reg} cells were located closer to LN T\textsubscript{reg} cells (Fig. 2b). Hierarchical clustering of the DMR data set resulted in a very similar grouping (Fig. 2c), which suggested that the gene-expression patterns and DNA-methylation patterns were interconnected. Indeed, integrated analysis of both data sets showed a negative correlation between gene expression and DMR methylation (median, −0.303; Fig. 2d); this indicated that in many cases, hypomethylation of a DMR correlated with expression of the corresponding gene and vice versa. Such a correlation has been observed in similar published studies\(^\text{11}\).

T\textsubscript{reg} cell–specific epigenetic signature

Methylation-based analyses of candidate regions have helped distinguish T\textsubscript{reg} cells from T\textsubscript{conv} cells. The most prominent example of this is a T\textsubscript{reg} cell–specific demethylated region in Foxp3 located in the first intron, called ‘conserved non-coding sequence 2’ (CNS2)\(^\text{1,2}\). That analysis has been extended by immunoprecipitation of methylated DNA for the analysis of differences between T\textsubscript{reg} cells and T\textsubscript{conv} cells from lymphatic organs\(^\text{13}\). That study identified a T\textsubscript{reg} cell–specific CpG-hypomethylation pattern that was established in the thymus and included, in addition to Foxp3, other T\textsubscript{reg} cell signature genes\(^\text{13}\). Since our data set included T\textsubscript{reg} cells and T\textsubscript{conv} cells from LNs, we first focused our analysis on the signature established in the thymus. Pairwise comparison of LN T\textsubscript{reg} cells and LN T\textsubscript{conv} cells revealed 339 DMRs (Fig. 1c). When we plotted the mean methylation difference (LN T\textsubscript{reg} cell − LN T\textsubscript{conv} cell) of DMRs located in promoters and located intragenically against RNA-expression data of the corresponding genes, we identified distinct anti-correlation for the association of demethylation with increased gene expression and for gain of methylation with gene repression (Fig. 3a). Our data confirmed T\textsubscript{reg} cell–specific hypomethylation at sites described in the aforementioned published study\(^\text{13}\), such as Cilia4, Ikzf2, Ikzf4 and Il2ra (Fig. 3a,b), while we also identified several previously unknown hypomethylated sites linked to genes such as Ccr6, Cish and Ifi80 (Fig. 3a and Supplementary Fig. 3). Furthermore, we identified previously unappreciated hypermethylated regions in genes that were underexpressed in T\textsubscript{reg} cells, such as Ilk, Satb1, Cox10, Fam78a and Tgfbr2 (Supplementary Fig. 3).

Since TWGBS allows resolution at the level of a single CpG, we used this to study Foxp3. The T\textsubscript{reg} cell–specific demethylation of Foxp3 went far beyond the CNS2 region initially described\(^\text{13}\) and spanned the entire first intron (Fig. 3c and Supplementary Fig. 4). Starting
from exon 2, the remaining gene was methylated in Treg cells (Fig. 3c and Supplementary Fig. 4). To verify our whole-genome sequencing data with a complementary method, we selected several regions in Foxp3 and performed PCR-based amplification of bisulfite-converted DNA, followed by sequencing of those amplicons (Fig. 3d–f and Supplementary Table 1). The amplicon-sequencing data confirmed the whole-genome methylation data and, additionally, established that demethylation of the entire intron 1, as well as an upstream region 1, can occur during differentiation of Treg cells in the thymus (Fig. 3d and Supplementary Fig. 5). Furthermore, that Foxp3 pattern was present in naive Treg cells but not in cells induced by transforming growth factor-β (TGF-β) in vitro (Fig. 3e and Supplementary Fig. 6). In addition, methylation at Foxp3 was not responsible for the differences between Treg cells in their Foxp3 expression (Supplementary Fig. 7).

To further investigate the universality of that Treg cell–specific methylation signature, we isolated peripherally induced (pTreg cells) and thymus-derived Treg cells (iTreg cells). pTreg cells are characterized by expression of the transcription factor RORγt and do not express the transcription factor HELIOS (encoded by Ikzf2), whereas iTreg cells do not express RORγt but do express HELIOS14. We sorted both populations from the spleen and performed PCR-based amplification of bisulfite-converted DNA to analyze regions in CItla4, Ikzf2, Il2ra and Foxp3. Although CItla4, Il2ra and Foxp3 were uniformly hypomethylated in both Treg cell populations, Ikzf2 was completely methylated in pTreg cells (Fig. 3g and Supplementary Fig. 8), which identified differences in the methylation of Ikzf2 as an epigenetic mark for distinguishing pTreg cells versus iTreg cells. In summary, we found that TWGBS was a powerful method for studying Treg cell–specific differences in methylation at the level of a single CpG.

**Epigenetic landscape of tissue Treg cells**

While our pairwise comparison of LN Treg cells and LN Tconv cells identified 339 DMRs, the number of DMRs in fat Treg cells versus LN Treg cells and in skin Treg cells versus LN Treg cells was about fivefold larger (1,593 and 1,645, respectively) (Fig. 1c). Many of the DMRs were shared by fat Treg cells versus LN Treg cells and by skin Treg cells versus LN Treg cells (Fig. 4a), which indicated either a common effector-memory T cell program or specific tissue Treg cell programs. We extracted 106 genes that showed differential methylation and corresponding gene-expression changes in both comparisons (Fig. 4b).

To distinguish between a common effector-memory T cell program versus tissue Treg cell programs, we compared that signature with RNAseq data from Tconv cells isolated from the same peripheral tissue (Fig. 4b and Supplementary Fig. 9). Although we were able to identify individual genes in this list (for example, Foxp1 or Lef1) as being related to a common effector-memory T cell program, the majority of the 106 genes were distinct and part of tissue Treg cell programs (Fig. 4b and Supplementary Fig. 9). Pairwise comparisons of Treg cells and CD4+ non-Treg cells from the same tissue revealed that the signature of 106 genes was significantly biased toward tissue Treg cells, such as the gene set upregulated in tissue Treg cells relative to their expression in lymphoid Treg cells in the comparison of fat Treg cells versus fat Tconv cells (P = 9.2 × 10⁻¹⁴) or of skin Treg cells versus skin Tconv cells (P = 1.5 × 10⁻¹⁴) (Supplementary Fig. 9b).

In many cases, methylation was similar for the groups at the promoter sites but began to differ just after the transcription start site in the first intron, as observed for Foxp3 (Fig. 4c and Supplementary Fig. 10). For selected genes, we confirmed differential expression at the protein level by flow cytometry (Fig. 5a). For example, KlrG1 (which encodes the differentiation marker KLRL1) displayed hypomethylation in tissue Treg cells, and KLRL1 was expressed by more than 80% of Treg cells in the skin and fat but by only about 10% of Treg cells from the LNs (Figs. 4c and 5a). Results obtained for the hypomethylation of Tigit (which encodes the immunoreceptor TIGIT) and Il1rl1 (which encodes ST2) and the expression the
Figure 3  Methylation changes of a T\textsubscript{reg} cell–specific epigenetic signature. (a) Methylation difference for promoter and intragenic DMRs (mean values; horizontal axis) plotted against expression of the corresponding genes (vertical axis; log\textsubscript{2} values) in LN T\textsubscript{reg} cells versus LN T\textsubscript{conv} cells (along axes): red, selected demethylated and upregulated genes; blue, hypermethylated and downregulated genes; gray diagonal line, linear regression. (b) Methylation profiles of genes encoding products known to be related to T\textsubscript{reg} cell function (Foxp3, Ctla4, Ikrft2, Ikrft4, Il2ra and Il2rb) in fat, skin, liver and LN T\textsubscript{reg} cells and LN T\textsubscript{conv} cells (key), presented as beta values (average value of three replicates per group) ranging from 0 (unmethylated) to 1 (methylated); below plots, gene diagrams, showing gene body (black bars), annotated promoter region (red bar), exons (blue bars), location of individual CpG sites (‘tick marks’) and gene direction (arrow). (c) Detailed analysis of the methylation (key) of Foxp3 at individual CpG sites (one per circle) in fat, skin, liver and LN T\textsubscript{reg} cells (along axes): red, skin and liver T\textsubscript{reg} cells and LN T\textsubscript{conv} cells (left margin); above plot, exons (open and red-filled boxes; numbered from −2a to +4), promoter region (PRO), conserved non-coding regions CNS1–CNS3, and regions for amplicon-based validation via bisulfite sequencing (R1–R3); below plot, genomic position of individual CpG sites. (d–g) PCR amplicon sequencing of bisulfite-converted genomic DNA in thymic T\textsubscript{reg} cells and precursors of T\textsubscript{reg} cells (d), LN T\textsubscript{reg} cells (d), LN T\textsubscript{reg} cells, T\textsubscript{reg} cells and \textit{in vitro}-induced T\textsubscript{reg} cells (\textit{iT}_{reg}) (e), T\textsubscript{reg} cells derived from fat, skin and liver tissue (f), and p\textsubscript{T}_{reg} cells and iT\textsubscript{reg} Cells derived from the spleen and splenic T\textsubscript{conv} cells (g), showing unmethylated or methylated CpG sites (key); numbers in plots indicate total reads analyzed. Data are from three experiments (a–c) or are representative of two experiments (d–g).
Figure 4 Identification of epigenetic and transcriptional changes in tissue-resident T\textsubscript{reg} cells. (a) Methylation difference versus RNA expression (plotted as in Fig. 3a) for DMRs in fat T\textsubscript{reg} cells versus LN T\textsubscript{reg} cells (top) or skin T\textsubscript{reg} cells versus LN T\textsubscript{reg} cells (bottom). (b) DMR methylation (left; key above) and expression (middle and right; key above) of 106 candidate genes (left margin) in skin, fat and LN T\textsubscript{reg} cells (left and middle) or in T\textsubscript{conv} cells isolated from LNs, skin and fat (right); all gene-expression data were row-normalized together. (c) Methylation profiles of selected genes from b in fat, skin, liver and LN T\textsubscript{reg} cells and LN T\textsubscript{conv} cells (key, top left plot), presented (as in Fig. 3b) as average values from three individual replicates. Data are from three experiments.
proteins encoded were similar (Fig. 5a). The substantial protein expression observed for TIGIT, KLRG1 and ST2 in tissue T\textsubscript{reg} cells was not found for T\textsubscript{conv} cells isolated from the skin and fat (Fig. 5a).

In addition to being interested in their shared characteristics, we also wanted to study the differences among tissue T\textsubscript{reg} cells (Fig. 5b). The comparison of fat T\textsubscript{reg} cells and skin T\textsubscript{reg} cells revealed that Pparγ, which encodes the key transcription factor (PPAR-γ) for the differentiation of T\textsubscript{reg} cells in visceral adipose tissue, was hypomethylated in fat T\textsubscript{reg} cells and, concomitantly, this gene also had high expression in fat T\textsubscript{reg} cells (Fig. 5c). On the other hand, skin T\textsubscript{reg} cells had several specifically hypomethylated loci, including Ahr (which encodes the transcription factor AhR), Icos (which encodes the inducible costimulator ICOS), Itgac (which encodes the integrin CD103) and Gpr55 (which encodes a G-protein-coupled cannabinoid receptor), and all the corresponding genes were overexpressed in skin T\textsubscript{reg} cells (Fig. 5b,d,e and Supplementary Fig. 11). Overall, these results showed that the epigenetic landscapes shared many characteristics, but individual, tissue-specific features were also detectable.

**Epigenetic reprogramming of T\textsubscript{h}2 cell–associated loci**

To further delineate common principles of tissue T\textsubscript{reg} cells, we performed a gene-ontology analysis of the 106 genes with differential methylation and expression patterns (Fig. 4b). Most prominent in this analysis were gene-ontology terms describing T cell differentiation (Fig. 6a). In particular, four genes encoding transcription factors (Gata3, Irf4, Rora and Batf) accounted for those T cell–differentiation gene-ontology terms, and all four genes were hypomethylated and overexpressed in fat and skin T\textsubscript{reg} cells (Fig. 6b and Supplementary Fig. 12). GATA-3 and IRF4 are key transcription factors that determine the T\textsubscript{h}2 cell fate ‘decision’ of CD4+ T cells\textsuperscript{15}. To investigate whether fat and skin T\textsubscript{reg} cells show a generalized type 2 profile, we used a selected gene list of T\textsubscript{h}2 cell–biased probes\textsuperscript{16} and plotted their expression in skin T\textsubscript{reg} cells versus LN T\textsubscript{reg} cells and in fat T\textsubscript{reg} cells versus LN T\textsubscript{reg} cells. In both comparisons, genes overexpressed in T\textsubscript{h}2 cells as well as those repressed in T\textsubscript{h}2 cells were significantly biased toward tissue T\textsubscript{reg} cells (Fig. 6c), indicative of type 2 polarization. For example, Il1rl1 and Il10, which are both associated with type 2 conditions\textsuperscript{15,17}, were hypomethylated in tissue T\textsubscript{reg} cells (Fig. 6d and Supplementary Fig. 13). Il10 displayed two hypomethylation regions, one about 9 kb upstream of the promoter and a second in an intragenic region (Fig. 6d and Supplementary Fig. 13). That upstream region was in a previously described DNase I–hypersensitive site of the Il10 locus in T\textsubscript{h}2 cells\textsuperscript{18}, Il1rl1 was hypomethylated in the first intron, and about 90% of the fat T\textsubscript{reg} cells and 60% of skin T\textsubscript{reg} cells expressed its product, ST2, compared with fewer than 10% of their spleen counterparts (Fig. 6d and Supplementary Fig. 13). If tissue T\textsubscript{reg} cells from fat and skin were type 2 biased, it would be possible to recapitulate parts of their phenotype by treating lymphoid T\textsubscript{reg} cells with IL-4. Indeed, treatment of lymphoid T\textsubscript{reg} cells with IL-4 substantially induced the expression of Gata3, Irf4, Il1rl1 and Il10 but repressed the expression of Msha40b, a T\textsubscript{h}1 cell–associated gene that was repressed in tissue T\textsubscript{reg} cells, in a dose-dependent manner (Fig. 6e and Supplementary Fig. 14).

Demethylation at CG dinucleotide sites can allow transcription factors to modulate gene transcription\textsuperscript{19}. We were interested in transcription-factor-binding motifs for which hypomethylated DMRs in fat and skin T\textsubscript{reg} cells showed enrichment. To investigate this, we clustered DMRs across T\textsubscript{reg} cells isolated from various tissues and identified cluster 2 as showing enrichment for hypomethylated DMRs in fat and skin T\textsubscript{reg} cells (Fig. 6f). Among the transcription-factor-binding motifs in cluster 2, 12 showed substantial enrichment relative to their abundance in the DMRs in clusters 1, 3 and 4 (Fig. 6f). Analysis of the expression of genes encoding the correspondingly transcription factors for which cluster 2 showed binding-motif enrichment, 11 of the 12 were also overexpressed at the level of the gene in fat and skin T\textsubscript{reg} cells, including those encoding RORα (Rora) and BATF (Batf), as well as those encoding various members of the JUN and Fos families (Fig. 6f). The BATF–JUN complex promotes binding (to DNA) of IRF4, and the IRF4–JUN–BATF heterotrimERIC complex has been shown to be critical for IRF4-mediated transcription in T cells\textsuperscript{20}; therefore, the complex might reinforce IRF4-mediated type 2 polarization.

**Characterization of tissue ST2+ T\textsubscript{reg} cells**

We called the T\textsubscript{h}2 cell–biased subset of T\textsubscript{reg} cells identified here that expressed ST2 and dominated the T\textsubscript{reg} cell population in fat and skin tissue ‘tissue’ T\textsubscript{reg} ST2 cells (‘tisT\textsubscript{reg}ST2’ cells). These were characterized by epigenetic and expression differences of 106 genes, including Gata3, Irf4, Batf, Rora, Maf, Il1rl1, Il10, CD200r1, Tigit and Klrk1 (Fig. 4b). Fat and skin T\textsubscript{reg} cells showed demethylation and overexpression of the gene encoding c-Maf (Maf) (Supplementary Fig. 15a), originally described as a T\textsubscript{h}12 cell–associated transcription factor able to bind to the Il10 promoter and induce Il10 transcription\textsuperscript{16}. In addition, one of the fundamental characteristics of tisT\textsubscript{reg}ST2 cells was expression of AREG (Supplementary Fig. 15b), a T\textsubscript{h}2 cell–associated ligand for the epidermal-growth-factor receptor\textsuperscript{21}. The Areg locus harbored two hypomethylated regions upstream of the promoter in fat and skin T\textsubscript{reg} cells (Supplementary Fig. 15b). Since expression of ST2, KLRG1, TIGIT and GATA-3 characterized tisT\textsubscript{reg}ST2 cells in fat and skin, we used these markers to screen various organs for the presence of this cell type. While fat and skin had the highest fraction of tisT\textsubscript{reg}ST2 cells within the Foxp3+ T\textsubscript{reg} cell compartment (about 80–90% and 50–60%, respectively), other peripheral organs, such as the lungs, bone marrow and liver, had 10–20% tisT\textsubscript{reg}ST2 cells among Foxp3+ T\textsubscript{reg} cells, and lymphoid organs had the lowest fraction, with less than 5% (Fig. 7a,b). We were unable to detect a ST2+KLRG1+ cell population in the T\textsubscript{conv} cell compartment in skin, liver, blood, bone marrow or lungs and found only a minor population (<5%) of such cells in fat tissue (Supplementary Fig. 16a). While tisT\textsubscript{reg}ST2 cells from the various organs had, for example, similar high expression of GATA-3, the corresponding T\textsubscript{conv} cell population did not resemble tisT\textsubscript{reg}ST2 cells (Fig. 7a and Supplementary Figs. 9b and 16c).

To further study the influence of signaling via and activation of the T cell antigen receptor (TCR) on the tisT\textsubscript{reg}ST2 cell population within the T\textsubscript{reg} cell compartment in tissues, we separated T\textsubscript{reg} cells on the basis of their expression of CD44, an effector–memory marker. The tisT\textsubscript{reg}ST2 cell population was present almost exclusively in the CD44hi effector–memory compartment (Supplementary Fig. 17a), in accordance with published literature reporting that tissue-resident T\textsubscript{reg} cells have an effector–memory phenotype\textsuperscript{22}. Since high expression of CD44 in tisT\textsubscript{reg}ST2 cells suggested a previous activation event via TCR signaling, we analyzed this population in mice expressing GFP as a reporter for Nr4a1 (which encodes the nuclear hormone receptor Nur77), in which TCR signal strength is measured by reporter activity\textsuperscript{23}. Therefore, we subdivided the T\textsubscript{reg} cell pool into GFP\textsuperscript{lo}, GFP\textsuperscript{int} and GFP\textsuperscript{hi} fractions to determine whether the presence of tisT\textsubscript{reg}ST2 cells depended on ongoing TCR signaling. Although the frequency of tisT\textsubscript{reg}ST2 cells was much lower in lymphatic organs than in fat tissue (1–2% versus 90%, respectively), the ‘per-organ’ fraction of tisT\textsubscript{reg}ST2 cells was not influenced by whether current TCR-signaling was on or off (Supplementary Fig. 17b).

Next we sought to investigate whether tisT\textsubscript{reg}ST2 cells were part of the induced T\textsubscript{reg} cell population in tissues. Published articles have
Figure 5 Confirmation of the common tissue Treg cell signature and identification of tissue-specific patterns. (a) Flow-cytometry analysis of TIGIT, KLRG1, ST2, BCL-2, TCF7 and LEF1 in fat, skin and LN Treg cells (CD19+MHCIICD3CD8-CD4+CD25+Foxp3-) and the corresponding Tconv cells (CD19-MHCIICD3CD8-CD4+CD25-Foxp3-), presented as concatenated files representative of four replicates (top group); below, quantification of results above. Numbers adjacent to outlined areas (top group) indicate percent cells in each throughout. ***P < 0.001 (one-way analysis of variance (ANOVA) with Bonferroni post-test). (b) Methylation difference versus gene expression in Treg cells versus skin Treg cells (plotted as in Fig. 3a). (c) Methylation profile of Pparg (left) and its corresponding expression (right); presented as reads per kilobase per million mapped reads (RPKM) in LN, skin and fat Treg cells (key (left) or horizontal axis (right)); one individual replicate per line (n = 3 replicates). ***P < 0.001 (Wald test with Benjamini and Hochberg correction). (d) Methylation profiles of Itgae and Gpr55 (left) and expression of Gpr55 (bottom) in skin, fat and LN Treg cells (presented as in c), and quantification of CD103 data in e (top right). **P < 0.001 (one-way ANOVA with Bonferroni post-test. (e) Flow-cytometry analysis of CD103 on LN, fat and skin Treg cells. Each symbol (a bottom) and d (top right) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). Data are representative of two experiments with n = 4 mice (a) or are from three experiments (b,c; mean ± s.d. in c), two experiments with n = 9 mice (d; mean ± s.d.) or two experiments (e).
Figure 6  Fat and skin T_{reg} cells are polarized like T_{H}2 cells. (a) Gene-ontology term analysis of the 106 genes in Figure 4b, showing gene-ontology terms (horizontal axis) with frequency enrichment in the sample relative to background frequency; top right, genes encoding the six factors responsible for the GO-term call. (b) Methylation profile of Gata3, Irf4, Rora and Baff (left) and expression of Irf4, Rora and Baff (right middle and bottom) (presented as in Fig. 3b), and frequency of GATA-3± cells (top right), in fat, skin, liver and LN T_{reg} cells and LN T_{conv} cells (key (top left plot) and horizontal axes (right)), presented as average methylation values derived from three individual replicates. ***P < 0.001 (Wald test with Benjamini and Hochberg correction). (c) Expression of T_{H}2 cell-associated signature genes upregulated (red; left) or downregulated (blue; right) in skin T_{reg} cells versus LN T_{reg} cells (top) or fat T_{reg} cells versus LN T_{reg} cells (bottom); plotted against P values (Wald test with Benjamini and Hochberg correction); numbers in corners indicate total genes in each plot area. (d) Methylation profile of Il1r1 and Il10 (far left and right middle) and expression of Il10 (far right) (presented as in Fig. 3b), and frequency of ST2± cells (middle left; based on Fig. 5a), in cells as in b. ***P < 0.001 (one-way ANOVA with Bonferroni post-test (Il1r1) or Wald test (Il10)). (e) qPCR analysis of genes in T_{reg} cells or T_{conv} cells (key) cultured in the presence (+) or absence (−) of IL-4 (25 ng/ml); results are presented relative to those of the control gene Hprt. *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired two-tailed Student’s t-test). (f) Methylation (key) of DMRs (one per row) in tissue T_{reg} cells (above plot; one replicate (n = 3) per column (above columns), grouping DMRs into four specific methylation-expression clusters (right margin) (top); motif-enrichment scores for various transcription factors (genes, left margin; one per row) for the four clusters identified at top (above columns) (middle); and expression (key) of transcription-factor-encoding genes with binding-motif enrichment in cluster 2 in tissue T_{reg} cells (as at top) (bottom). Each symbol represents an individual mouse (d (middle left)) or replicate (e); small horizontal lines indicate the mean (± s.d.). Data are from three experiments (a–c,f; mean ± s.d. in b), two experiments with n = 4 mice (d; mean ± s.d.), or four experiments (e).
Figure 7 Identification of tisTregST2 cells. (a) Flow cytometry (top row) of Treg cells from the inguinal LNs, fat, skin, liver and spleen (Spl), identifying tisTregST2 cells (CD8−CD19+MHCI−CD3+CD4+CD25+Foxp3+ST2+KLRG1−GATA-3+) (R1; top right) and control ST2−KLRG1+ Treg Cells (R2; bottom left, number above); below, GATA-3 staining of Treg cells among R1 cells (red line) or R2 cells (blue line). Top row, concatenated files of four or more biological replicates. (b) Frequency of tisTregST2 cells among Treg Cells in various tissues (horizontal axis). PP, Peyer’s patches; BLN, brachial LNs; ILN, inguinal LNs; CLN, cervical LNs; MLN, mesenteric LNs. (c) Frequency distribution of tisTregST2 cells (% in tissue Treg cells (top row) and spleen Treg cells (bottom row)) stimulated with PMA and ionomycin and then gated (far left) as tisTregST2 cells (CD45+TCRB+CD4+CD8−Foxp3+KLRG1−ST2+; R2 (red)) or control KLRG1−ST2− Treg Cells (R1 (blue)); far right, quantification of results obtained as at left (additional controls, Supplementary Fig. 19). *P < 0.05 and ***P < 0.001 (one-way ANOVA and Bonferroni post-test). (e) qPCR analysis of the expression (key) of various genes (left margin) in tisTregST2 cells and ST2−KLRG1− Treg Cells isolated from the spleen (Spl) by flow cytometry; bracketing (left margin), hierarchical clustering. (f) Methylation (key) of CG dinucleotides in DMRs at various locations (left margin, top plot) of Gata3, Klg1 and Left1 (above plots) in tissue Treg cells (above top plot). (g) Expression of Gpr55 and Pparg, based on the gene-expression data set derived from e; results are presented relative to those of Hprt. (h) Single-cell RNAseq analysis of genes encoding tisTregST2 cells–associated markers (Helios, Gata3, Klg1 and Tgfb) and skin-associated markers (Igkae, Ahr and Gpr55) in tisTregST2 cells (n = 101) derived from the spleen (top), and t-SNE analysis of single tisTregST2 cells with a skin Treg cell signature (dot size indicates Ahr expression (key)) (bottom). Each symbol (c, d far right), g represents an individual mouse; small horizontal lines indicate the mean (± s.d.). Data are representative of two experiments (a, b, e, g, h); three experiments with a total of n = 10 mice (c) or four experiments (e) or are from one experiment representative of two experiments with n = 4 mice in each (d) or one experiment with 127 cells (h).
reported that the colon has two distinct Treg cell populations: pTreg cells and iTreg cells. Unlike HELIOS+RORγ+ iTreg cells, pTreg cells are induced by commensal bacteria in the colon. The tisTregST2 cells were present only among the iTreg cell population of the colon, where they represented about 40% of all iTreg cells (Fig. 7c and Supplementary Fig. 18a). The RORγ+ pTreg compartments in the colon and spleen were completely devoid of tisTregST2 cells (Fig. 7c and Supplementary Fig. 18a). Notably, tisTregST2 cells located in the colon had higher expression of GATA-3 than that of pTreg cells or 'non-tisTregST2' iTreg cells in the same tissue (Supplementary Fig. 18b). In summary, we were able to identify tisTregST2 cells in almost every peripheral tissue.

tisTregST2 cells in the spleen

We investigated whether the small population of tisTregST2 cells found in lymphatic organs had a pattern of gene expression and methylation resembling that of the tisTregST2 cell found in peripheral tissues. One of the important characteristics that we found for tisTregST2 cells in skin and fat was the production of AREG and IL-10 (Fig. 6d and Supplementary Fig. 15b). Therefore, we probed the function of fat and spleen tisTregST2 cells and stimulated the cells with the phorbol ester PMA and ionomycin to mimic TCR stimulation in combination with protein transport and metalloprotease inhibitors. About 80% of KLRG1+ST2+ tisTregST2 cells from spleen produced AREG and showed higher levels of IL-10 than that of spleen KLRG1+ST2− Treg cells; thus, they demonstrated an effector profile very similar to that of tisTregST2 cells isolated from fat tissue (Fig. 7d and Supplementary Fig. 19a,b). CD8+ T cells and Tconv cells did not produce AREG or IL-10 under the same conditions (Supplementary Fig. 19a). To further confirm the similarities, we sorted the tisTregST2 cell population from spleen and analyzed the characteristic tisTregST2 cell profile, including additional genes that showed epigenetic changes in fat and skin Treg cell populations, such as Lmna (which encodes lamin A) and Osbp3 (which encodes an oxyosterol-binding-like protein) (Fig. 7e). All genes analyzed, including Gata3, Rora, and Ifi4, were expressed differentially in the tisTregST2 cell fraction from the spleen relative to their expression in the global splenic Treg cell pool, which matched the profile of tisTregST2 cells (Fig. 7e). In particular, the effector-molecule-encoding genes Il10 and Areg were considerably overexpressed, with difference of 65-fold and 16-fold, respectively, in their expression in the tisTregST2 cell fraction from the spleen relative to their expression in the global splenic Treg cell pool (Fig. 7e and data not shown), which confirmed our protein-expression data (Fig. 7d). To investigate whether the methylation changes characteristic of tisTregST2 cells were also present in their counterparts in the spleen, we sequenced PCR-based amplicons of bisulfite-converted DNA on the basis of the DMRs identified in our whole-genome approach (Fig. 4). Genes specifically demethylated in fat and skin tisTregST2 cells, such as Gata3 or Klrq1, were also hypomethylated in splenic tisTregST2 cells but not in splenic KLRG1+ST2− Treg cells (Fig. 7f).

To further investigate the tisTregST2 cells found in lymphoid organs, we first assessed the expression of individual markers. Almost all Treg cells in the skin had high expression of CD103, whereas less than 5% of fat-resident tisTregST2 cells expressed this marker (Supplementary Fig. 19c). Analysis of tisTregST2 cells from the spleen showed that about 40% expressed CD103 (Supplementary Fig. 19c), indicative of heterogeneity of the tisTregST2 cell population in this organ. The induction of PPAR-γ expression in the fat tisTregST2 cell population represented an additional functional tissue-based adaptation (Fig. 5c and Supplementary Fig. 20) comparable to the demethylation and expression of Gpr55 in skin Treg cells (Fig. 5d and Supplementary Fig. 20). When assessing expression of the marker-encoding genes Pparg and Gpr55, which indicate tissue-restricted adaption of the tisTregST2 cell population in fat and skin, we found both were substantially overexpressed (>30-fold) in the spleen tisTregST2 cell population relative to their expression in the KLRG1+ST2− Treg cell population (Fig. 7g).

To extend that analysis, we performed single-cell RNaseq of tisTregST2 cells isolated from spleen. As expected, all single cells expressed Helios, Gata3 and Klrq1 (Fig. 7h). In contrast to that, genes that were biased toward the skin-resident tisTregST2 cell population, such as Iigae, Ahr and Gpr55, were expressed in only a fraction of individual cells (Fig. 7h), which indicated the presence of subgroups of tisTregST2 cells in the spleen that probably represented individual tissue characteristics. This suggested that fat- and skin-resident Treg cells included a recirculating fraction present in the lymphatic tisTregST2 cell pool.

tisTregST2 cells are distinct

To investigate whether tisTregST2 cells were a distinct differentiation state of Treg cells, we first analyzed the presence of these cells over time. Our data showed that the fraction of tisTregST2 cells among Treg cells in various tissues in mice was stable over a time period of 5−25 weeks of age (Fig. 8a and Supplementary Fig. 21a,b), indicative of homeostasis of the tisTregST2 cell compartment within tissues. To understand the origin of tisTregST2 cells, we investigated whether a Treg cell population derived from lymphoid organs and depleted of tisTregST2 cells could be the precursor of the tisTregST2 cells found in tissues. To generate space in the Treg cell compartment, we depleted host mice of Treg cells by injecting diphtheria toxin into Foxp3−DTR mice, which express the diphtheria toxin receptor under the control elements of Foxp3. We injected congenically marked KLRG1+ST2− Treg cells into the host mice and analyzed their skin and fat tissue for the presence of tisTregST2 cells after 10 d. These experiments showed that lymphoid-organ Treg cells had the ability to seed the peripheral tissues and differentiate into tisTregST2 cells (Fig. 8b).

To study the stability of already differentiated tisTregST2 cells, we cultured fat-derived tisTregST2 cells for 6 d in vitro under well-defined conditions with beads coated with antibody to the TCR complex component CD3 (anti-CD3) and antibody to the costimulatory receptor CD28 (anti-CD28), plus IL-2. The cultured tisTregST2 cells showed a very stable expression pattern of characteristic markers such as Il1rt1, Pparg, Osbp3, Batf, Tigit, Gata3 and Klrq1 comparable to that of tisTregST2 cells freshly isolated from fat (Fig. 8c). In parallel, cultured non-tisTregST2 Treg cells did not upregulate those characteristic genes (Fig. 8c), which indicated that the expression of these genes was not merely a function of Treg cell activation and that the tisTregST2 program was not a temporary state but was a stable program.

To identify a transcriptional regulator essential for tisTregST2 cells, we focused on Batf. As reported above, our data showed that Batf was overexpressed in tisTregST2 cells and its locus was heavily hypomethylated in tissue Treg cells from fat and skin, and we identified enrichment for BATF DNA-binding motifs in regions of genes that were specifically hypomethylated in tissue Treg cells (Fig. 6f). Indeed, BATF-deficient mice showed a substantially reduced number of tisTregST2 in all organs analyzed, including skin, fat, lung, bone marrow, LN and spleen, relative to the number of such cells in BATF-sufficient mice, while the number of other Treg cells in the same tissues was not reduced (Fig. 8d). These data identified BATF as a transcriptional regulator essential for tisTregST2 cells.

As tisTregST2 cells were characterized by the expression of ST2, we sought to determine if IL-33 could act as a growth factor to amplify
Figure 8 Characterization of the tisTregST2 cell population. (a) Flow cytometry (left) of cells from the skin of mice at 5 or 25 weeks of age (concatenated files representative of five replicates), identifying tisTregST2 cells (gated as in Fig. 7), and frequency (right) of tisTregST2 cells in the fat, skin, lymph nodes and spleen (key) of mice 5, 10, 15, 20 and 25 weeks of age (horizontal axis) (additional plots, Supplementary Fig. 21). (b) Flow cytometry of cells from the spleen, fat and skin (left margin) of untreated (UT) or diphtheria-toxin-treated (DT) CD45.2+Foxp3-DTR host mice 10 d after injection of CD45.2+KLRG1−ST2−Treg cells isolated from congenically marked CD45.2+ donor mice, identifying tisTregST2 cells (as in Fig. 7a). (c) qPCR analysis of the expression (key) of various genes (above plots) in tisTregST2 cells (from the fat) and KLRG1−ST2−Treg cells (from the spleen) analyzed immediately after isolation (ex vivo) or cultivated and activated for 6 d in vitro with microbeads coated with anti-CD3 and anti-CD28 plus IL-2 (in vitro); bracketing (left margin), hierarchical clustering; R1–R4 (right margin), independent experiments. (d) Flow cytometry (left) of tissue Treg cells (above plots) from Baff−/− and Baff+/− mice (left margin), identifying tisTregST2 cells (as in Fig. 7a) (concatenated files representative of six to ten replicates), and quantification of results obtained as at left (far right). (e) Flow cytometry of tissue Treg cells (above plots) from wild-type mice treated with PBS or IL-33 (left margin), identifying tisTregST2 cells (as in Fig. 7a) (concatenated files representative of four replicates; additional plots, Supplementary Fig. 21), and quantification of results obtained as at left (far right). Numbers above plotted points (bottom right) indicate total tisTregST2 cells after IL-33 treatment relative to that before. Each symbol (d.e. right) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). NS, not significant (P > 0.05); *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired two-tailed Student’s t-test). Data are representative of one experiment with n = 5 mice (a), one experiment with one replicate representative of four replicates (b), four experiments (c), one experiment with n = 6–10 replicates (d) or one experiment with n = 4 replicates (e).
the tisTregST2 cell pool in-vivo. The administration of recombinant IL-33 substantially expanded the tisTregST2 cell population in all organs assessed: the number of tisTregST2 cells increased by 10-fold in fat, 5-fold in skin, 13-fold in liver and 60-fold in lung tissue (Fig. 8e). This expansion did not change the identity of the cells, as GATA-3 was still overexpressed in cells after expansion compared with its expression in ‘non-tisTregST2’ Treg cells isolated from the same organs (Supplementary Fig. 21c). Collectively, these data showed that tisTregST2 cells were a distinct state. They required the transcription factor BATF and underwent population expansion via IL-33 in situ.

**DISCUSSION**

The present study has provided evidence that tissue Treg cells undergo extensive epigenetic reprogramming. Changes in the methylome can be used to determine the underlying functional programs. The similarities in the epigenetic landscape of fat-resident Treg cells and that of skin-resident Treg cells allowed us to identify a common tissue Treg cell population characterized by the expression of KLRG1 and ST2, a T1/2 cell–like expression program and the production of tissue-regenerative factors.

Classically, Treg cells have been viewed as regulators of other immune cells. With the characterization of fat Treg cells, that view has been extended to a second critical function: support of organ homeostasis. In visceral adipose tissue, about 80–90% of Treg cells had the tisTregST2 cell phenotype described here. In the skin, that proportion was somewhat lower, at about 50–60% of Treg cells, and in the lungs and liver, that proportion was between 10% and 20%. These findings explain why fat-resident Treg cells and skin-resident Treg cells shared a closer relationship to each other than to liver Treg cells in the DNA-methylome analysis. It was notable that this T1/2 cell–biased tisTregST2 cell subset was present in almost every organ. ST2, as well as the transcription factors BATF and IRF4, has been shown to be required for the differentiation of Treg cells in fat6. On the basis of our data, we concluded that BATF and IL-33 are important not just for Treg cells in the fat but also for tisTregST2 cells in all tissues, which extends the perspective from an adipose-centered view to a global one.

Our data also showed that tissue Treg cells integrated epigenetic changes from multiple differentiation steps. The first specific epigenetic reprogramming occurred during thymic differentiation and stabilized the universal Treg cell identity11. The second line of epigenetic modifications solidified the functional ‘tisTregST2 cell’ specialization via selective hypomethylation of a signature that included more than 100 genes. Thus, the tisTregST2 cell population acquired a unique reprogramming landscape. On top of that tisTregST2 cell–specialization program, we found tissue-specific epigenetic reprogramming. In the Treg cell population from the fat, we identified differences in the methylation of Pparg (among other genes) relative to such methylation in skin and LN Treg cells. In addition, Treg cells from the skin had several notable epigenetic differences, among which Ahr and Gpr55 might be of specific relevance. AhR signaling is important for immune cells and their function at barrier organs such as the skin24, Gpr55, as a cannabinoid receptor, is associated with algesia linked to inflammatory and neuropathic pain and could enable nociception by skin Treg cells in an organ with a strong pain perception25,26.

Where does such epigenetic reprogramming take place? Are most organs independently able to induce the common tisTregST2 cell program, and, in addition, add organ-specific ‘flavors’? Alternatively, one organ or very few organs might induce the tisTregST2 cell reprogramming and, via circulation, cells might reach the individual tissues in which they further specialize. Among the last, fat tissue could be such a candidate, as the vast majority of Treg cells in this tissue were reprogrammed to be tisTregST2 cells. However, parabiosis experiments have detected only a low degree of chimerism in the fat tissue Treg cell compartment, indicative of little exchange of tissue Treg cells via the circulation27. It is also possible that tisTregST2 cells found in the spleen or LNs represent a backup population that can be quickly recruited to support the homeostasis of ‘distressed’ organs. In addition, it is very unlikely that local conversion from Tconv cells to Treg cells was responsible, since our data showed that tisTregST2 cells were present only in the tTreg cell fraction, not the pTreg cell fraction, and were demethylated at the IκBζ2 locus.

The tisTregST2 cells did not express the T1/2 cell–associated cytokines IL-4 and IL-13 but did express IL-10 and AREG. Areg expression could presumably be induced by ST2 signaling and has been shown to be important for tissue repair in the lungs8. The tisTregST2 cell population might therefore represent the prototype of tissue-repair-prone Treg cells that mediate tissue homeostasis by using the tissue-regenerative factor AREG. This could have clinical implications for the adoptive transfer of Treg cells for the treatment of autoimmune and graft-versus-host diseases20. Deliberate type 2 conditioning by IL-4 and IL-33 during the in vitro population expansion of blood-derived Treg cells might amplify their therapeutic potential, especially in the context of supporting tissue-repair functions. Indeed, ST2-dependent protective Treg cell functions have been demonstrated in the colon and adipose tissue6,29.

As demethylation of Foxp3 is used as a diagnostic marker for the detection of Treg cells12,30, peripheral reprogramming events can be used to study the functional capacity of Treg cells. We identified thousands of DMRs at single-CpG resolution, which characterized the universal Treg cell identity and peripheral reprogramming. Such differences should allow the design of diagnostic probes for ampli-con-based sequencing to follow the origin and cell fate of Treg cells in various pathological conditions. Indeed, the difference in the methylation of IκBζ2 in tTreg cells relative to that in RORγt+ pTreg cells described here is such an example. Analyzing the epigenetic landscape is more than a complementary approach with which to describe Treg cells; it will help to define Treg cell identities and the permanent underlying molecular programs.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

M.D., C.D.I., A.B., A.H.-W., Q.W., F.F., C.H., B.B. and M.F. analyzed data; M.D., D.W., C.P. and M.F. designed the study; M.D., D.W., P.A.L. and M.F. designed experiments; M.D., D.W., U.T., A.-C.H., D.K., J.-P.M. and K.B. performed the experiments; and M.D. and M.F. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
Identification of regulatory networks in HSCs and

The putative cannabinoid receptor GPR55 plays a role in γ

The transcriptional regulators IRF4, BATF and IL-33

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**ONLINE METHODS**

**Mice.** Wild-type C57BL/6 mice, congenic B6.129PtpcrPepcR/Botcri (CD45.1^+^) mice, and congenic B6.PL-Thy^1^Cy (CD90.1^+^) mice and NraG1-FRP mice (C57BL/6-T(−Mor−NraG1-EGFP) cr) were obtained from Jackson Laboratory (Stock Number: 013757). Jackson stock number: 016167^3^ was studied from Charles River Breeding Laboratories (Wilmington, MA, USA) or the Jackson Laboratory (Bar Harbor, ME, USA). B6.N(129-Gp)−Foxp3^tm3Ayr^ mice (Foxp3:1RES-DTR/GFP) were bred to CD45.1^+^ or CD90.1^+^ mice in the animal facility of the German Cancer Research Center (DKFZ), B6.N(129-Cp)−Foxp3^tm3Ayr^ and B6.N(129-Cp)−Foxp3^tm3(YFP/cre)Ayr^ mice, Jackson (Foxp3:1RES-YFP/Cre^3^) were used to sort YFP^+^ or GFP^+^ Treg cells. BAF3− deficient mice (129B-1tm^1^Kimm/Fjr) were obtained from Jackson Laboratories (Stock Number 013757).

All animals used in this study were male and between 15 and 30 weeks old, unless otherwise indicated. Animals were housed under specific pathogen-free conditions at the DKFZ animal care facility, and the governmental committee for animal experimentation (Regierungspräsidium Karlsruhe, Germany) approved all experiments involving animals.

**Tissue digestion for sorting of cells by flow cytometry.** For cell isolation, we used Foxp3^{ERT^2} or Foxp3^{IRES^3} reporter mice (Foxp3:1RES-DTR/GFP or Foxp3:1RES-YFP/Cre). T cells were extracted from gonadal visceral adipose tissue (called ‘fat’ here), skin, liver and inguinal LNs. For LNs, single-cell suspensions were established and red blood cells were lysed. Epidymal visceral abdominal tissue was first mechanically dissected, followed by digestion with a buffer containing collagenase II (1 mg/ml), BSA (20 mg/ml) and DNAse (20 µg/ml) for 45 min at 37 °C in a slowly shaking water bath. Afterwards, the cell suspension was incubated briefly with 0.5 Mol EDTA-H₂O₂, pelleted and further filtered. To isolate cells from skin tissue, back skin area was depilated by shaving and hair removal cream, followed by mechanical dissection and digestion using a buffer containing collagenase IV (4 mg/ml), FCS (2% vol/vol), and DNAse (10 µg/ml) for 45 min at 37 °C in a slowly shaking water bath, followed by filtration steps. Liver tissue cells were isolated from perfused mice, mechanically dissected and treated with digestion buffer containing collagenase II (1 mg/ml), BSA (5 mg/ml), and DNAse (20 µg/ml) for 45 min at 37 °C in a slowly shaking water bath, followed by a Percoll gradient centrifugation step and filtrations. Cells from lungs were isolated from perfused mice following a digestion step similar to that used for fat cells (collagenase II (1 mg/ml), BSA (20 mg/ml) and DNAse (20 µg/ml)).

After purification, cells were filtered through a 70-µm filter mesh and were either stained for flow-cytometry-based isolation of target cells or pre-enriched with anti-CD25 magnetic beads, followed by column-based isolation. Cells were stained with antibodies as indicated below (next subsection). Live-dead cell exclusion was performed with a fixable live/dead stain.

**Sorting by flow cytometry and purification of DNA and RNA.** RNA sorting for lineage cell precursors were sorted as CD4−CD8−Foxp3(GFP)+ cells from spleen and lymph nodes. Genomic DNA was isolated using a gDNA MicroPrep Kit (Zymo Research), and concentrations were measured with a Qubit fluorometer. RNA was isolated with the RNEasy mini kit (Qiagen) and concentration was determined with a 2100 Bioanalyzer instrument (Agilent Technologies).

**TWGBS.** We applied TWGBS for very low input of DNA sequencing according to a published procedure^3^ with some modifications. Double-stranded pre-adaptors consisted of oligo Tn5SmErer([phos]CGTCTTCCTTATACACTCT) and either methylated oligo Tn5mC-Apt1 (TGCtGGcACGTCAGTGTGA TAAAGAGACG) or methylated oligo Tn5mC-Apt2 (TGTcGTGCGcCAGGAGATGTGTTATAAGAGACG); lower case ‘c’ base indicates 5-methyl cytosine. Pre-adapters were combined at a ratio of 1:1 to generate a 10 µM load adapter mixture. The transposome was assembled by mixing 12 µl load adapter and 10 µl Ez-Tn5 transposase (Epichere). About 10 ng genomic DNA and 5 pg unmethylated λ-DNA were used for tagmentation with 1 µl transposase. Tagmented DNA was purified with AmpPure beads (Beckman Coulter) and was repaired with Bst DNA polymerase (NEB) and SmC-dNTP mix (Zymo). After a further bead purification, the DNA was bisulfite-converted with the EZ DNA methylation kit (Zymo) according to the manufacturer's instructions. From each converted DNA sample, four differently barcoded sequencing libraries were generated by PCR (95 °C, 3 min; 12 cycles of 95 °C, 20 s; 62 °C, 15 s; 72 °C, 40 s) using Kapa 2G Robust HotStart ReadyMix (Kapa Biosystems), SYBRGreen reagent (Life Technologies), primer Tn5mCP1 (A ATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC) and barcoded Tn5mC reverse primers (CAACGAGAAGCCGCATACAGAT- eight bases of barcode)-GTCTCCTGGGTcCAGGTC) and library pools were 100bp paired-end sequenced using Illumina HiSeq 2000.

**Ultra-low RNAseq.** cDNA was generated and amplified using 0.8 ng of total RNA and SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (Clontech Laboratories) according to the manufacturer's protocol. Then, sequencing libraries were prepared using the NEXT ChIP-Seq Library Prep Master Mix Set for Illumina (New England BioLabs) according to the manufacturer’s instructions with the following modifications: the adaptor-ligated double-stranded cDNA (10µl) was amplified using NEBNext Multiplex Oligos for Illumina (New England BioLabs, 25 µm primers), NEBNext High-Fidelity 2x PCR Master Mix (New England Biolabs) and 15 cycles of PCR. Final libraries were validated using Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit fluorometer (Invitrogen), normalized and pooled in equimolar ratios. 50bp single-read sequencing was performed on the Illumina HiSeq 2000 v4 according to the manufacturer’s protocol.

**Read-pair preprocessing of TWGBS data.** To determine whether read pairs originated from the original strand in the genome (C converted to T in read 1, and G converted to A in read 2, during bisulfite treatment) or the reverse complementary (G converted to A in read 1, and C converted to T in read 2), we performed the following preprocessing before the alignment: since the C residues in CpG sites are mainly methylated and thus are unconverted, they were excluded from the analysis; i.e., CpG, TpG, GpC and GpT dinucleotides were masked. For each read in every read pair, we then calculated the base ratios T/C and A/G, denoted as R1_{TC}, R1_{AG} and R2_{TC}, R2_{AG}. We then compared these ratios between the first and second read in a read pair to determine if they were assigned in (R1-R2) or (R2-R1) order or could not be assigned to any order. The following rules were applied: for read pairs for which the condition R1_{TC} > R2_{TC} and R1_{AG} < R2_{AG} held true, we assumed the read pair came from the original strand and assigned them the (R1-R2) read order; for read pairs for which the conditions R1_{TC} < R2_{TC} and R1_{AG} > R2_{AG} were met, we assumed the read pair came from the reverse complementary strand and read order (R2-R1) was assigned; and read pairs that did not meet one of the conditions above were ambiguous and thus were eliminated from the analysis.
Mapping of whole-genome bisulfite sequencing data and methylation calling. The TWGBS data were processed as described\(^4\). The mm10 reference genome (GRCh38.73) was transformed in silico for both the top strand (C to T) and bottom strand (G to A) using MethylCtools\(^5\). Before alignment, adaptor sequences were trimmed using SeqPrep (https://github.com/jstjohn/SeqPrep). The first read in each read pair was then C-to-T converted and the second read in the pair was G-to-A converted. The converted reads were aligned to a combined reference of the transformed top strands (C to T) and bottom strands (G to A) using BWA (bwa-0.6.2-tpx)\(^6\) with default parameters but with disabling of the quality threshold for read trimming (-q) of 20 and the Smith-Waterman for the unmapped mate (-s). After alignment, reads were converted back to the original states, and reads were mapped to the antisense strand of the respective reference were removed. Duplicate reads were marked, and the complexity was determined using Picard MarkDuplicates (http://broadinstitute.github.io/picard/). Reads with alignment scores of less than 1 were filtered before subsequent analysis. Total genome coverage was calculated using the total number of bases aligned from uniquely mapped reads over the total number of mappable bases in the genome. At each cytosine position, reads that maintain the cytosine status were considered methylated, and reads in which cytosine was converted to thymine were considered unmethylated. Only bases with Phred-scaled quality score of ≥20 were considered. In addition, the five base pairs at the two ends of the reads were excluded from methylation calling according to M-bias plot quality control. For the TWGBS libraries, the first nine base pairs of the second read and the final nine base pairs before the adaptor of the first read were excluded from methylation calling.

**Calling of DMRs.** The raw counts of methylated and unmethylated reads for each CpG site from different libraries were merged for each replicate. BSmooth\(^7\) was used (default parameters, version 1.2.0) to call DMRs for all possible ten pairwise tissue–tissue comparisons. Each comparison contained three replicates in each group (three-versus-three comparison). To account for possible false-positive DMR results reported by BSmooth, due to smoothing in uninformative and low-coverage regions, we applied additional filtering procedures. On autosomes, we selected for DMRs that had a mean CpG coverage per DMR greater or equal to five in all six replicates in a particular comparison, based on raw read counts. We applied a paired Wilcoxon test to each DMR on the basis of the beta values of the six replicates. To correct for multiple testing, the resulting \( P \) values underwent Benjamini-Hochberg correction, and only DMRs with a \( P \) value lower or equal to 0.05 were selected. Due to the fact that all mice in this study were male, we expected lower coverage in uninformative and low-coverage regions, we applied additional filters for possible false-positive DMR results reported by BSmooth, due to smootht. For the TWGBS libraries, the first nine base pairs of the second read and the final nine base pairs before the adaptor of the first read were excluded from methylation calling.

**Principal-component analysis and hierarchical clustering on DMRs.** The methylation values of called DMRs from all ten possible tissue–tissue comparisons were joined into a single matrix. Duplicate DMRs (exact same start and end bp position) were eliminated from the matrix. Principal-component analysis of all replicates using R (3.1.2) and pcomp was performed. A complete-linkage clustering was performed, which allowed clustering of rows (DMRs) and columns (replicates) using R (3.1.2) and heatmap.

**Identification of promoter, intragenic, intergenic regions and transcription start site.** Unique DMRs from all ten comparisons have been annotated (bedtools-2.24.0 closest\(^8\)) using parameters -d to report distance and -t `first` to handle ties. We used ReSeq September 2013 version and defined promoters as 2,000 bp upstream for genes located on the plus strand and 500 downstream for those located on minus strand. The transcription start site (TSS) was defined as the first base pair downstream for the plus strand and the first base pair upstream for the minus strand. Whole-genome data were visualized in a circo plot as described in\(^9\).

**Calculation of average distance of DMRs from the TSS.** To calculate the distance of DMRs from the TSS, we first normalized all DMRs to the same DNA strand, subtracted the mean DMR genomic localization from the TSS coordinates, grouped the results into 500-bp clusters from position –10000 to position +10000, and quantified DMRs in each cluster. DMRs more than 10,000 bp from the respective TSS were disregarded.

**Pearson correlation of gene expression and methylation.** For this analysis, we included DMRs that were intragenic or within 5kb upstream or downstream of the nearest gene according to RefSeq annotation. Methylation values and RPKM values were associated with each other using the ReSeq gene identifiers. For each gene we applied a correlation test using Pearson correlation with a cutoff of -0.7 or less, or 0.7 or more, which allowed negative correlation as well as positive correlation.

**Hierarchical clustering of DMRs and RNA data.** From the DMRs with an absolute Pearson correlation coefficient of 0.7 or greater, we created a new matrix of methylation values including all replicates. We applied complete-linkage hierarchical clustering to generate a heatmap using R (3.1.2) and pheatmap. We further applied R (v3.1.2) and mclust(v.5.1)\(^{10}\) to estimate the number of clusters using parameters: mclust(..., G = 1:20). The resulting number of clusters was four, and we cut the resulting tree for the DMRs from the complete-linkage using cutree (…, k = 4) into four different clusters.

**Motif analysis.** For each of the four clusters identified, we merged overlapping DMRs (bedtools-2.24.0 merge) to avoid possible bias by over-representation and extracted the corresponding genomic DNA sequences (bedtools-2.24.0fasta). All genomic regions in the four clusters were assigned scores with the JASPAR motif library\(^{48}\) using the total binding affinity (TBA) score. In brief, the TBA score was computed for each genomic region by summing for each position the maximum PWM score between the plus strand and minus strand\(^41\). Then, for each PWM, the regions were ranked according to their TBA score in decreasing order. For each PWM, we determined the recovery curve for the regions in a specific cluster, and the area under the curve (AUC) was computed over the first 300 regions. AUC values were converted into a z-score by computation of the mean and s.d. of the AUC over 1,000 randomizations of the ranks. For each set of regions inside a cluster, the z-scores for the PWMs are presented as a heat map. Only motifs with an absolute z-score above 3 in one of the clusters were presented hew.

**Mapping of RNAseq data, statistical evaluation and plotting.** For all samples, low-quality bases were removed with Fastq _quality_filter from the FASTX Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html), with 90% of the read needing a quality phred score of >20. Homertools 4.7 (ref. 42) was used for poly(A)-tail trimming, and reads with a length of <17 were removed. PicardTools 1.78 (https://broadinstitute.github.io/picard/) was used to compute the quality metrics with CollectRNASeqMetrics. With STAR 2.3 (ref. 43), the filtered reads were mapped against mouse genome 38 using default parameters. Count data were generated using HTSeq\(^{44}\) for the genes. For the comparison with DESeq2 (ref. 45), the input tables containing the replicates for groups to compare were created by a custom perl script. For DESeq\(^2\), DESeq\(^2\)Data SetFromMatrix was applied, followed by estimateSizeFactors, esti-mateDifferences and nbinomWald testing. The resulting tables were annotated with gene information (gene symbol and gene type) derived from the genome.vM8.gtf file. For the RPMK table of non-coding RNAs, a custom perl script separated non-coding RNA genes from protein-coding genes and calculated their RPMK values from the HTSeq count values. Mapping of filtered reads against protein-coding transcripts using a custom pipeline generated the RPMK table of the protein coding genes. These genes (status ‘KNOWN’) were extracted from the Mouse EnsEMBL (Ref. 80) database. Mapping was carried out with bowtie2 version 2.2.4 (ref. 46) against union mouse genes; every gene is represented by a union of all its transcripts (exons). The count values (RPMK and raw counts) were calculated by running CoverageBed from Bedtools v.2.17.0 (ref. 38) of the mapped reads together with a specific mouse annotation file for protein coding genes (based on Enssembl 80) in gtf format and parsing of the output with custom perl scripts. MA plots were generated as described\(^{47}\). For hierarchical clustering of RNA data a complete-linkage clustering was performed using R (3.1.2) and heatmap.

**Unsupervised clustering, computation of heatmaps, and methylation plotter.** Unsupervised hierarchical clustering was performed with the Gene
Isolation of RNA and reverse-transcription followed by qPCR. Sorted cell populations were lysed and RNA was isolated using the RNeasy Mini Kit (Qiagen). Synthesis of cDNA was performed with SuperScript Reverse Transcriptase II and oligo(dT) primers (both Life Technologies) according to the manufacturer’s instructions. Real-time PCR was performed using a ViiA7 instrument (Applied Biosystems) and Taqman master mix (Applied Biosystems). Gene-expression values were normalized to those of the control gene (Hprt).

Purification and bisulfite conversion of genomic DNA. Sorted cell populations were resuspended in PBS and genomic DNA was purified according to the manufacturer’s guidelines using the DNEasy Blood and Tissue kit (Qiagen). For isolation of pTreg cells and iTreg cells, cells were fixed and stained with the Foxp3 Fix/perm buffer set (eBiosciences) according to the manufacturer’s instruction. Cells were then sorted into lysis buffer included in the QIAamp DNA micro kit (Qiagen). Reverse cross-linking of gDNA was performed for 1 h at 56 °C and 1 h at 90 °C, followed by isolation of genomic DNA. DNA purity and concentration were measured with a NanoDrop or Qubit photometer. Bisulfite conversion was performed using the EpiTect Bisulfite Conversion Kit (Qiagen), and converted DNA was used immediately after purification or divided into aliquots and stored at −20 °C.

Computation and testing of bisulfite-DNA primers. Genomic DNA was bisulfite converted in silico using Bisulfite Primer Seeker software (http://www.zymoresearch.com/tools/bisulfite-primer-seeker). Primer sequences were calculated on the basis of the manufacturer’s recommendations. Primer pairs were tested on bisulfite-converted genomic DNA to determine optimal annealing temperature range and cycle number for each specific reaction. Once parameters were optimized, adaptor sequences for 454 sequencing and barcodes to distinguish individual samples were attached to each primer pair sequence and synthesized. Alternatively, Illumina adaptor sequences were attached to primer pairs for Illumina sequencing. Based on optimal annealing temperature and PCR cycle number, primers were used to generate PCR amplicons from bisulfite-converted DNA for each cell type tested. An overview of primers used for our sequencing experiments is provided in Supplementary Table 2. Once genomic DNA had been bisulfite-converted, PCR with bisulfite-specific primers was performed. PCR amplicons were separated from primer dimers on 1–2% agarose gels and were visualized using ethidium bromide. Specific bands were excised under UV light exposure, and DNA amplicons were purified using a Quick Gel Extraction Kit (Life Technologies). Equimolar amounts of amplicons were combined and processed on a GS Junior Sequencer (Roche). Sequence reads were aligned to the bisulfite-converted mouse genome, and methylation levels were visualized in heat maps. Alternatively, Illumina adaptor sequences were processed on an Illumina MiSeq V3 machine with Paired-End 300bp or Paired-End 250bp settings. Raw data were aligned to the mouse genome, and Cpg methylation was calculated. Detailed genomic positions of amplicon data are either labeled in the corresponding graph or are presented here: R3 (Fig. 3c; CG#1: 7,583,950; CG#2: 7,583,986; CG#3: 7,584,002; CG#4: 7,584,036; CG#5: 7,584,063; CG#6: 7,584,050), Il2ra (Fig. 3g; CG#1: 69,670,284; CG#2: 69,670,291; CG#3: 69,670,370; CG#4: 69,670,377; CG#5: 69,670,385), Il2r (Fig. 3f; CG#1: 11,645,653; CG#2: 11,645,705; CG#3: 11,645,718; CG#4: 11,645,738), Cited (Fig. 3e; CG#1: 60,912,472; CG#2: 60,912,521; CG#3: 60,912,536; CG#4: 60,912,573), Gata3 (Fig. 7d; CG#1: 9,868,708; CG#2: 9,868,720; CG#3: 9,868,768; CG#4: 9,868,798; CG#5: 9,868,820; CG#6: 9,868,844; CG#7: 9,868,855; CG#8: 9,868,858; CG#9: 9,868,883; CG#10: 9,868,948; CG#12: 9,868,958), Lef1 (Fig. 7e; CG#1: 131,116,109; CG#2: 131,116,113; CG#3: 131,116,152; CG#4: 131,116,173; CG#5: 131,116,187; CG#6: 131,116,191; CG#7: 131,116,196; CG#8: 131,116,257; CG#9: 131,116,288; CG#10: 131,116,302; CG#11: 131,116,327; CG#12: 131,116,356).
Stability of tisTregST2 cells in vitro. KLRF1+ST2+ tisTregST2 cells from fat were isolated by flow cytometry. In addition, KLRF1−ST2− Treg cells were isolated from the spleen by flow cytometry. Samples were frozen for the 0 h time-point analysis, or tisTregST2 and KLRF1−ST2− cells were incubated with microbeads coated with anti-CD3 and anti-CD28 (Dynabeads, Thermo Fisher Scientific) and 5000 U/ml IL-2 for 6 d at 37 °C. Then, cells were lysed and RNA was isolated. Gene expression was determined by qPCR with Taqman probes.

Transfer of congenically-labeled KLRF1−ST2− Treg cells into host mice depleted of Treg cells. Treg cells were isolated from CD45.2+ Foxp3-YFP, Cre mice by CD25 head-based pre-enrichment and a reduced staining protocol (CD8−CD19−CD25−Foxp3−YFP−KLRF1−ST2−). Cells were then injected intravenously into CD45.1+ recipient mice. Control mice received injection of PBS. In addition, all recipient mice were treated by intraperitoneal injection of diphtheria toxin to eliminate all host-resident Treg cells. Injection of diphtheria toxin was repeated after 24 h. Mice were analyzed 10 d after injection of Treg cells or PBS. Four recipient mice received Treg cells from five donor mice. Analysis of untreated control mice ensured proper gating.

Population expansion of tisTregST2 cells with IL-33 in vitro. Foxp3+GFP mice were given injection of either 5 μg recombinant mouse IL-33 or PBS into the peritoneum on day 1 and day 3 (BioLegend). On day 6, mice were killed, and the frequency of tisTregST2 cells in tissues was evaluated by flow cytometry.

Bioinformatics and statistical analysis. Massive parallel sequencing data underwent statistical testing as described above, and those statistical values were used in Figures 2a (MA plots), 5c (Pparg), 5d (Gpr55), 6b (Ifnγ, Rora, Batf) and 6d (Il10) and Supplementary Figures 12, 13a, 13b, 15a, 15b and 20, with n = 3 for all comparisons. Data based on flow cytometry or real-time PCR were tested with the unpaired two-tailed Student’s t-test in Figures 6e (n = 4), 7c (n = 10), 7d,g (n = 4), 8d (n = 6–10) and 8e (n = 4) and Supplementary Figures 18a (n = 10), 19a (n = 4), 19b (n = 4), 21b (n = 5) and 21c (n = 4). The one-way ANOVA with Bonferroni post-test was used in Figures 5a (n = 4–19), 5d (CD103, n = 9), 6b (GATA-3, n = 4–19) and 6d (ST2, n = 4), and Supplementary Figures 13c (n = 4–19), 14b (n = 6–7), 16c (n = 4–19), 17b (n = 4) and 19c (n = 8). Dunnett’s post-test to compare all columns versus control column was used in Supplementary Figure 14a (n = 4). All graphed results represent the mean of at least three biological replicates ± s.d. **P < 0.01, ***P < 0.001, ****P < 0.0001 and *P < 0.05. To identify the T1i2 cell bias of tissue Treg cells, we used a data set derived from m vitro–differentiated T1i1, T1i2, T1i17 and iTreg cells26. Genes specifically up- or down-regulated in T1i2 cell–polarized cells were identified in the following comparisons: T1i2 cells versus T1i1 cells, T1i2 cells versus T1i17 cells, and T1i2 cells versus naive T cells. T1i2 cell–specific genes had to have differential expression in all three comparisons (over twofold). We plotted both T1i2 cell–specific gene lists (upregulated and downregulated) on our gene expression data set (Fat Treg cells versus LNTreg Cells, and skin Treg cells versus LNTreg cells). The significance of bias was evaluated by χ2 testing.

A Life Sciences Reporting Summary is available for this paper.

Data availability. Fastq files from TWGBS, RNAseq and single-cell RNAseq that support the findings of this study have been deposited in European Nucleotide Archive (ENA) with the accession code PRJEB14591.

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Corrigendum: Genome-wide DNA-methylation landscape defines specialization of regulatory T cells in tissues

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In the version of this article initially published, the methods section lacked information on the provenance of the BATF-deficient mice used in the study. The first paragraph of Online Methods should end as follows: “BATF-deficient mice (129S-Batf<sup>tm1.1Kmm</sup>/J) were obtained from Jackson Laboratories (Stock Number 013757).” The error has been corrected in the HTML and PDF versions of the article.