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DOI:

[10.1038/s41590-018-0230-z](https://doi.org/10.1038/s41590-018-0230-z)

*Document Version*

Peer reviewed version

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*Citation for published version (APA):*

Povoleri, G. A. M., Nova-Lamperti, E., Scottà, C., Fanelli, G., Chen, Y.-C., Becker, P. D., Boardman, D., Costantini, B., Romano, M., Pavlidis, P., McGregor, R., Pantazi, E., Chauss, D., Sun, H.-W., Shih, H.-Y., Cousins, D. J., Cooper, N., Powell, N., Kemper, C., ... Afzali, B. (2018). Human retinoic acid-regulated CD161<sup>+</sup> regulatory T cells support wound repair in intestinal mucosa. *Nature Immunology*, 19(12), 1403–1414. <https://doi.org/10.1038/s41590-018-0230-z>

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## Retinoic acid-regulated CD161<sup>+</sup> Tregs support wound repair in intestinal mucosa

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## Summary

Repair of tissue damaged during inflammatory processes is key to the return of local homeostasis and restoration of epithelial integrity. Here we define CD161<sup>+</sup> regulatory T cells (Tregs) as a distinct, highly suppressive, population of Tregs mediating wound-healing. These Tregs were enriched in intestinal lamina propria, particularly in Crohn's disease. CD161<sup>+</sup> Tregs had a retinoic acid (ATRA) gene signature and CD161 expression on Tregs could be induced by ATRA, which directly regulated the CD161 gene. CD161 itself was co-stimulatory and its ligation with the T-cell receptor induced cytokines that accelerated wound-healing of intestinal epithelial cells. We identified a transcription factor network, including BACH2, ROR $\gamma$ t, FOSL2, AP-1 and RUNX1, controlling expression of the wound-healing program and found that a CD161<sup>+</sup> Treg signature in Crohn's disease mucosa associated with reduced inflammation. These findings identify CD161<sup>+</sup> Tregs as a population critical for controlling the balance between inflammation and epithelial barrier healing in the gut.

## Introduction

Regulatory T cell (Tregs) are a non-redundant, suppressive, subset of CD4<sup>+</sup> helper T (Th) cells that are critical for the prevention of autoimmunity<sup>1</sup>. They are ideal candidates for cell-based clinical immunotherapy of autoimmunity and for prevention of transplant rejection<sup>2</sup>. Tregs express the master transcription factor (TF) FOXP3, the IL-2 receptor component CD25, the inhibitory co-receptor CTLA4<sup>3,4</sup> and depend on the TF BACH2<sup>5</sup> for differentiation in the thymus (“tTregs”) and the periphery (“pTregs”).

Conventional T cells (Tconv) are characterized by their own master TFs; these include T-BET for anti-viral Th1 cells, GATA3 for anti-helminth Th2 cells and ROR $\gamma$ t for anti-bacterial and anti-fungal Th17 cells, respectively<sup>6</sup>. These TFs were initially considered to antagonize Treg development: in mice, induction of high T-bet expression in Tregs within inflamed bowel drives Tregs into a pro-inflammatory phenotype reminiscent of Th1 cells<sup>7</sup>. This view has been challenged by the investigation of mice with deletion of these factors specifically within FoxP3<sup>+</sup> cells<sup>8-11</sup>. For example, T-bet expression within FoxP3<sup>+</sup> Tregs is required for trafficking to and suppression of Th1-mediated inflammation<sup>10</sup>, and Gata3 is required for full Treg function in the gut<sup>11</sup>. These findings support a “compartmentalized” view of Tregs, suggesting that there are multiple sub-populations of Tregs defined by the expression of transcription factors associated with effector cell lineages and by their ability to suppress particular immune cell responses or to carry out other specialized functions. Indeed, it is now recognized that the TF circuitry of Tregs is complex, with significant interplay between FoxP3 and other lineage-associated TFs<sup>12,13</sup>.

In humans, heterogeneous populations of Tregs have been reported, although these have been typically defined by surface markers rather than TFs. These include CD39, HLA-DR and CD45RA, among others<sup>2</sup>. Whether these sub-populations translate into the ability to suppress specific parts of the human immune system has yet to be fully elucidated. We have previously shown that conventional methods of delineating Treg subsets are limited by the number of markers that can be concurrently used and by a biased approach to data analysis (gating of Treg subsets)<sup>14</sup>. This has led to conflicting results, with memory Tregs reported as both non-suppressive<sup>15</sup> and highly suppressive<sup>16</sup> by different groups. By contrast, an unbiased multi-dimensional analysis approach can delineate the most suppressive Treg sub-populations, identify new ones and exclude those that are less likely to be regulatory<sup>14</sup>.

Inflammatory bowel disease (IBD) represents a complex collection of disorders in which aberrant activation of the mucosal immune system, epithelial barrier dysfunction and microbial dysbiosis all contribute to chronic inflammation and unregulated local Th1 and Th17 responses<sup>17</sup>. Bowel mucosa is a key site for induction of pTregs from naïve CD4<sup>+</sup> precursors via instruction from environmental factors, such as transforming growth factor (TGF)- $\beta$ , IL-2 and all-trans retinoic acid (ATRA)<sup>18</sup>. Tregs mediate dominant tolerance in gut

mucosa, preventing or ameliorating colitis on adoptive transfer in murine models<sup>19</sup>. Conversely, *FOXP3* mutations or disruption of other molecules key to Tregs (e.g. CTLA-4, IL-10R, TGF- $\beta$ ) cause enteropathy in both humans and mice<sup>4,20,21</sup>, again demonstrating a key role for these cells in preventing gut inflammation. Lamina propria Tregs are expanded in patients with IBD, but it is unclear why they are unable to control local inflammation or what function(s) they perform in these diseases<sup>22,23</sup>. We and others have shown that Tregs can express ROR $\gamma$ t together with IL-17A, and that in humans these predicates are restricted to a subset of Tregs expressing CD161<sup>24-26</sup>. CD161 is a C-type lectin-like receptor, encoded on human chromosome 12 and expressed on the cell surface of human NK cells<sup>27</sup> and various subsets of T lymphocytes<sup>28</sup>. CD161<sup>+</sup> conventional Th cells are memory T cells that act as Th17 precursors<sup>29,30</sup>. The cognate ligand for CD161 is lectin like transcript 1 (LLT1)<sup>31</sup>, in which single nucleotide polymorphisms (SNPs) associate with human IBD on genome-wide association studies<sup>32</sup>, suggesting that the CD161-LLT1 interaction is physiologically important.

Here, we delineate the biological repertoire of CD161<sup>+</sup> Tregs, their role in the immune system and their mechanisms of action. Our data show that CD161<sup>+</sup> Tregs are a highly suppressive and distinct subset of induced Tregs that are enriched in active areas of IBD, and that expression of CD161 is induced by ATRA. Functionally, they are highly suppressive and produce soluble factors, a property co-stimulated by ligation of CD161 itself. Their regulome, assessed by ATAC-seq, points to a TF circuitry that includes the TFs BACH2, ROR $\gamma$ t and AP-1 family members, genes that mediate wound healing. Cytokines from these cells accelerate wound healing of colorectal epithelium, suggesting that CD161<sup>+</sup> Tregs may play a critical role in the patho-physiology of IBD and could theoretically be used as cellular products for the treatment of these diseases. Accordingly, we report that a CD161<sup>+</sup> Treg signature in the mucosa of Crohn's disease is associated with ameliorated inflammation.

## Results

### *CD161-expressing Tregs are a discrete Treg population with a distinct TCRV $\beta$ repertoire*

We used an unbiased multi-dimensional analysis pipeline via cytometry by time-of-flight (CyTOF) to identify and study biologically important human Treg sub-populations. Visualized stochastic neighbor embedding (viSNE) was used to create a map of CD4<sup>+</sup> T cells freshly isolated from blood and arrange cells along t-distributed stochastic neighbor embedding (t-SNE) axes based on per-cell phenotypic similarity<sup>14,33,34</sup> (**Fig. 1a-b**). Tregs, identified by high expression of CD25 and FOXP3 and low expression of CD127, clustered together and could be resolved into naïve and memory Treg populations (**Fig. 1a-b**). Likewise, Tconv, identifiable by low CD25 and FOXP3 and high CD127 expression, clustered together and distinctly from Tregs (**Fig. 1a-b**). In this unsupervised analysis, the C-type lectin CD161 was expressed by a sub-population of memory Tregs (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>FOXP3<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup>) and a group of memory Tconv (broadly CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup>FOXP3<sup>-</sup>CD45RA<sup>lo</sup>CD45RO<sup>+</sup>) (**Fig. 1a-b**). To identify distinct clusters of T cells, we performed a spanning-tree progression analysis of density-normalized events (SPADE)<sup>35</sup> based on t-SNE values. Differential expression of different markers within each identified SPADE node was used to further cluster T cells. This analysis resolved CD4<sup>+</sup> T cells into 50 sub-populations, grouped into four main populations: naïve Tregs, memory Tregs, naïve Tconv and memory Tconv, each characterized by a different expression profile (**Fig. 1c** and **Supplementary Fig. 1a**). CD161-expressing cells represented a distinct sub-population of memory Tregs and several sub-populations of memory Tconv, which were all distinct from Tregs (**Fig. 1c**). In order to further distinguish Tregs from Tconv, we performed a similar analysis of transcriptomes by single-cell RNA-seq using CD4<sup>+</sup>CD25<sup>+</sup> cells as input (**Supplementary Figs. 1b-e**). This pipeline resolved cells into 9 clusters, of which two were Tregs (clusters 0 and 3), five were Tconv (clusters 1, 2, 4, 5 and 6) and two were probably Tregs, although too small in number to sub-categorize (clusters 7 and 8) (**Supplementary Figs. 1b-e**). The two Treg clusters (clusters 0 and 3) were clearly separate from the Tconv clusters and one of them (cluster 3) expressed KLRB1, the gene encoding CD161 (**Supplementary Figs. 1b-e**). Consistent with our CyTOF data, expression of KLRB1 in the CD161<sup>+</sup> Treg population was lower than that in the CD161<sup>+</sup> Tconv sub-population. A heatmap of Treg markers (IL2RA, IL7R, FOXP3), KLRB1 and naïve/memory markers (CD62L and CCR7) confirmed clustering of the CD161<sup>+</sup> Tregs (cluster 3) independently from Tconv cells (**Supplementary Fig. S1e**) and was similar to heatmaps constructed from protein expression derived by CyTOF (**Supplementary Fig. 1f**). A similar unsupervised pipeline applied to CD4<sup>+</sup> T cells flow-stained with just 5 markers, CD4, CD25, CD127, CD45RA and CD161 produced similar viSNE and SPADE plots to mass cytometry (**Supplementary Fig.**

**1g-h)** and could be used to FACS-sort Treg sub-populations from peripheral blood for further analysis (**Supplementary Figs. 1i-j**). Henceforth, these Tregs are referred to as “CD161<sup>+</sup> Tregs” (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup>CD161<sup>+</sup>; denoted in purple), “naïve Tregs” (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>+</sup>CD161<sup>-</sup>; denoted in orange) and CD161<sup>-</sup> memory Tregs, abbreviated to “memory Tregs” (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup>CD161<sup>-</sup>; denoted in yellow).

T cell CD161 expression is associated with a restricted range of T cell receptors (TCRs): TCR V $\alpha$ 7.2 in mucosal-associated invariant T (MAIT) cells<sup>28</sup> and invariant TCR V $\alpha$ 24-J $\alpha$ 18 in iNKT cells<sup>36</sup>. Neither TCR was significantly enriched in CD161<sup>+</sup> Tregs (**Supplementary Fig. 1k**). To determine the clonality of CD161<sup>+</sup> Tregs and their relationship to other Tregs and Tconv, we *TCRVB* sequenced the three populations of Tregs as well as Tconv (defined as CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup>) and CD161<sup>+</sup> Tconv (defined as CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup>CD45RO<sup>+</sup>CD161<sup>+</sup>) as controls. Among the TCRBV families, the three highest contributors to the total TCR repertoire (BV05, BV06 and BV07) and one of the lower contributors (BV19) were chosen to represent the variability of the cellular repertoire by spectratyping. CD161<sup>+</sup> Tregs were polyclonal, had normally distributed CDR3 length in the different TCRBV families (**Fig. 1d**) and a similar contribution to the overall TCR repertoire compared with other T cell subsets (**Supplementary Fig. 1l**). We then asked whether the total TCR repertoire of CD161<sup>+</sup> Tregs overlapped with either the Treg or Tconv populations. We found limited clonality shared between the different subsets; in particular, CD161<sup>+</sup> Tregs shared only 4.5% and 4.8% of their TCR sequences with Memory Tregs and CD161<sup>+</sup> Tconv cells, respectively (**Supplementary Fig. 1m**). We next calculated the Morisita-Horn similarity index to measure the TCR composition overlap between the different T cell populations<sup>37</sup>. This index ranges between 0 (minimal similarity) and 1 (maximal similarity). Analysis showed limited TCR repertoire overlap between any of the populations other than between CD161<sup>+</sup> Tconv and Tconv (a value of 0.413) (**Fig. 1e**). Hierarchical clustering of the samples, based on the Morisita-Horn similarity index, showed two subdivisions, separating the two Tconv populations from all three Treg populations; moreover, CD161<sup>+</sup> Tregs clustered with memory Tregs within the regulatory T cell main branch (**Fig. 1f**). These data indicate that CD161<sup>+</sup> Tregs have a distinct TCR repertoire from other Tregs and do not represent a clonal expansion from a Tconv population.

#### *CD161<sup>+</sup> Tregs share features with both CD161<sup>+</sup> Tconv and classical Tregs*

To identify similarities and differences between the 3 sub-populations, we first compared their transcriptomes using microarray and Gene Set Enrichment Analysis (GSEA) (**Supplementary Fig. 2a-d**). Despite CD161<sup>+</sup> Tregs being a sub-population of memory Tregs, there were 549 genes differentially expressed when comparing CD161<sup>+</sup> to memory Tregs (**Fig. 2a, Supplementary Fig. 2b-d and Supplementary Table 1**). CD161<sup>+</sup> Tregs were

enriched in genes expressed by other CD161<sup>+</sup> cells<sup>28</sup> and also expressed the core transcriptional profile of Tregs<sup>38</sup> (**Fig. 2b-c** and **Supplementary Fig. 2e-f**). This data suggested that, in addition to core Treg genes they express a range of other transcripts related to CD161 induction or signalling.

All three Treg populations had similar FOXP3 protein expression (**Fig. 2d**). Epigenetic modifications, notably the degree of methylation of the Treg-specific demethylation region (TSDR) within the *FOXP3* gene locus, can give an indication of the stability of FOXP3 expression, with tTregs having a demethylated locus and more stable FOXP3 expression, compared with iTregs that retain higher region methylation<sup>39</sup>. We next measured the methylation status of the *FOXP3* TSDR and two other key Treg-associated genes, *IL2RA* and *CTLA4*, in the three Treg populations and, for comparison, Tconv (**Supplementary Fig. 2g**). Methylation at *IL2RA* and *CTLA4* loci were similar between the three Treg populations but distinct from the more highly methylated Tconv (**Fig. 2e** and **Supplementary Fig. 2h**). At the *FOXP3* TSDR we noted CD161<sup>+</sup> Tregs to have region methylation intermediate (55%) between naïve (25%) or memory (28%) Tregs and Tconv (88%) (**Fig. 2e** and **Supplementary Fig. 2h**). In summary, CD161<sup>+</sup> Tregs express the Treg master TF FOXP3, same surface markers as Tregs, and express the Treg gene transcription profile. Their methylation pattern at key gene loci are similar to other Tregs, albeit with intermediate TSDR methylation.

#### All-trans retinoic acid (ATRA) directly regulates CD161 expression

We noted almost complete absence of CD161<sup>+</sup> Tregs in human cord blood<sup>24</sup> and thymus (our unpublished observations), suggesting that CD161<sup>+</sup> Tregs are induced to develop or to specialize in the periphery. We have previously observed that long-term culture of Tregs with ATRA supports the persistence of cells expressing CD161<sup>40</sup>. We found significant enrichment of ATRA-regulated genes in CD161<sup>+</sup> Tregs compared to memory Tregs (**Fig. 3a**) by GSEA, including two gut homing markers, CCR9 and ITGA4, which are classically regulated by ATRA (**Fig. 3a** and **Supplementary Fig. 3a**); CCR9 was confirmed at the protein level by flow cytometry (**Supplementary Fig. 3b**). Activation of freshly isolated Tregs with ATRA induced expression of both CCR9 and CD161 (**Fig. 3b**). Since Tregs interact with DCs in the gut, we determined if mature DCs generate ATRA via a functional assay for the key enzyme aldehyde dehydrogenase (ALDH). LPS-matured DCs expressed substantial amounts of this enzyme (**Fig. 3c**) together with lectin like transcript 1 (LLT1), the natural ligand for CD161 (data not shown)<sup>31</sup>. In the absence of DCs, Tregs lost expression of CD161; whereas co-culture of freshly isolated Tregs with DCs induced CD161. The addition of BMS493, a pan-retinoic acid receptor (RAR) inverse agonist blocked CD161 expression in a dose-dependent manner (**Fig. 3d** and **Supplementary Fig. 3c**). To see whether ATRA can



directly induce CD161, we scanned the *KLRB1*, which encodes CD161, and *CCR9* gene loci (as control), for retinoic acid receptor alpha (RARA) DNA binding motifs (**Supplementary Fig. 3d**). One potential RARA binding site in *KLRB1* and multiple sites in *CCR9* were identified (**Supplementary Fig. 3e**). By ChIP-qPCR for RARA we found that ATRA enhanced RARA binding at both loci in T cells, evident as significant increment in percentage of input for both *KLRB1* and *CCR9* target sequences after culture with ATRA, compared to untreated cells (**Fig. 3e**). Collectively, these data indicate that ATRA can induce CD161 expression on Tregs.

#### *CD161<sup>+</sup> Tregs are a highly suppressive Treg population*

To study the function of CD161<sup>+</sup> Tregs, we tested first their ability to suppress Tconv proliferation *ex vivo* by measuring the ratio of Tregs to Tconv required to suppress proliferation by 50% (IC<sub>50</sub>) for each population<sup>41</sup> (**Fig. 4a-b** and **Supplementary Fig. 4a-b**). We found that CD161<sup>+</sup> Tregs had a lower mean IC<sub>50</sub> compared to memory Tregs while naïve Tregs did not reach 50% suppression *ex vivo* (and therefore IC<sub>50</sub> could not be calculated) (**Fig. 4b** and **Supplementary Fig. 4b**). CD161<sup>+</sup> Tregs remained stably regulatory even after *in vitro* expansion (**Supplementary Fig. 4c**). Separation of Tregs from target cells by a transwell abrogated the majority of the suppressive function of CD161<sup>+</sup> Tregs (**Supplementary Fig. 4d**), consistent with other Treg populations<sup>42</sup>. The presence of anti-CD161, anti-PDL1, anti-TGFβRII or anti-IL-10R antibodies caused no significant impairment of CD161<sup>+</sup> Treg suppression, indicating that suppressive function is likely not due to a single factor (**Supplementary Fig. 4e**). Furthermore, suppression assays carried out under Th1 and Th17 skewing conditions did not impair the regulatory function of CD161<sup>+</sup> Tregs (**Supplementary Fig. 2f**). CD161 is found on NK cells<sup>27</sup> and cytotoxicity is a suppressive mechanism of some Tregs<sup>43</sup>. However, we found neither perforin nor granzyme (A or B) in any of the Treg populations (**Supplementary Fig. 4g**) and no evidence of Tconv cytolysis after co-culture with CD161<sup>+</sup> Tregs (data not shown).

The mouse CD161 ortholog is not expressed on T cells (**Supplementary Table 2**). To confirm suppressive ability of CD161<sup>+</sup> Tregs *in vivo*, we used a humanised mouse model of severe xeno-graft versus host disease (GvHD) in NOD/scid/*Il2rγ*<sup>-/-</sup> mice by injecting CD25-depleted human PBMC with or without *in vitro* expanded memory or CD161<sup>+</sup> Tregs. Mice receiving either memory or CD161<sup>+</sup> Tregs were protected from xeno-GvHD, surviving significantly longer than mice injected with PBMC alone. This was associated with a significant reduction in clinical disease scores (**Fig. 4c**). Thus, CD161<sup>+</sup> Tregs had a comparable suppressive activity to traditional Tregs *in vivo*. These data indicate that CD161<sup>+</sup> Tregs are highly regulatory both *in vitro* and *in vivo*.

### CD161 ligation stimulates cytokine production in CD161<sup>+</sup> Tregs

To determine the role of CD161 expression by Tregs, we first investigated the specific effects of TCR triggering by examining transcriptomes of Tregs stimulated with  $\alpha$ -CD3 +  $\alpha$ -CD28 for 4 hours. Approximately 1500 transcripts showed changes from baseline in CD161<sup>+</sup> Tregs (**Fig. 5a and Supplementary Fig. 5a-b**). While a core set of transcripts, including *KLRB1*, *RORC* and *CCR9* differed between CD161<sup>+</sup> and memory Tregs both before and after these cells were activated, there was surprisingly little overlap (**Fig. 5b-c**). Of note, a number of cytokine genes, including *IL10*, *IL17A*, *IL17F* and *IL21* (**Fig. 5c**), were enriched in CD161<sup>+</sup> Tregs (**Supplementary Fig. 5b**). We confirmed preferential accumulation of IL-10, IL-17A, IL-22 and IL-4, but not IFN- $\gamma$ , in three-day supernatants of  $\alpha$ CD3/CD28-activated CD161<sup>+</sup> Tregs (**Fig. 5d**).

CD161 in Tconv is a marker of Th17 cells. Furthermore, CD161<sup>+</sup> Tregs expressed RORC, the key TF of Th17 cells, along with IL-17 itself. We next determined whether IL-17 production was compatible with suppressive function in CD161<sup>+</sup> Tregs, by isolating IL-17<sup>+</sup>CD161<sup>+</sup> and IL-17<sup>-</sup>CD161<sup>+</sup> Tregs using an IL-17 capture assay (**Supplementary Fig. 5c-d**) and testing their respective suppressive functions. IL-17<sup>+</sup>CD161<sup>+</sup> Tregs remained highly suppressive despite producing IL-17 (**Fig. 5e**).

CD161 does not have a classical ITAM/ITIM domain but its ligation can have both activating and inhibitory functions, depending on cell type<sup>28,44,45</sup>. To assess the function of CD161 in Tregs, we stimulated cells with  $\alpha$ CD3,  $\alpha$ CD28 magnetic beads additionally coated with  $\alpha$ CD161 or an IgG2 isotype. After three days of culture CD161 cross-linking significantly enhanced cytokine production from CD161<sup>+</sup> Tregs (**Fig. 5f**), indicating that CD161 acts as a co-stimulatory molecule in CD161<sup>+</sup> Tregs. Thus, CD161<sup>+</sup> Tregs are a cytokine producing population of Tregs, CD161 co-ligation is co-stimulatory to this process and IL-17 production is compatible with preserved suppressive function.

### Genome-wide chromatin landscapes define regulatory circuitry in CD161<sup>+</sup> Tregs

We next assessed the regulatory elements (REs) of naïve, memory and CD161<sup>+</sup> Tregs directly *ex vivo*, hypothesizing that these dictate differences in biological function. We examined global chromatin landscapes using assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)<sup>46</sup>. Open chromatin regions (OCRs) surrounding the signature Treg-associated genes, notably *FOXP3*, *CTLA4* and *IL2RA* were not significantly different between the three populations (**Supplementary Fig. 6a**). We focused on ~1300 OCRs that have consistent patterns among all three biological replicates but differ among the three Treg populations. These regions were in three clusters: those specific to naïve Tregs (25% of total), specific to CD161<sup>+</sup> Tregs (39% of the total) and those shared between CD161<sup>+</sup> and memory Tregs (37% of the total) (**Fig. 6a-b and Supplementary Table 3**). Notable

examples of genes with OCRs specific to CD161<sup>+</sup> Tregs include key cytokines, such as *IL17A*, transcription factors, such as *PRDMI* and *C-MAF*, and chemokine receptors, including *CCR9* (**Supplementary Table 3**). The genomic distributions of OCRs were similar in the three clusters, with the majority in intronic and intergenic regions (**Supplementary Fig. 6b**).

To determine potential TFs targeting distinct Treg regulomes, we searched for enrichment of known TF motifs above background within OCR clusters using HOMER (**Fig. 6c** and **Supplementary Fig. 6c**). CD161<sup>+</sup>-specific OCRs were enriched for motifs of ROR $\gamma$ t, RUNX, AP-1 family (e.g. BATF, FOSL2), and Cap'n'collar (CNC) family members that include BACH2 (**Fig. 6c** and **Supplementary Fig. 6c**). To test if these TFs could explain the transcriptional differences between CD161<sup>+</sup> and memory Tregs, we performed GSEA for genes regulated by BACH2, ROR $\gamma$ t, BATF, FOSL2 and RUNX1. CD161<sup>+</sup> Tregs were significantly enriched for BACH2, ROR $\gamma$ t, FOSL2 and RUNX1 regulated genes, compared to memory Tregs (**Fig. 6d**). Approximately 40% of the 549 genes differentially expressed between CD161<sup>+</sup> and memory Tregs could be attributed to regulation by *Bach2*, *Runx1*, *Rorc*, *Fosl2* and *Batf* in mice using knockout models<sup>5,47,48</sup>, with BACH2 responsible for the majority of the transcriptional differences (**Fig. 6e**). Expression of BACH2 itself was significantly reduced and RORC significantly elevated in freshly isolated CD161<sup>+</sup> Tregs compared to the other Treg populations (**Supplementary Fig. 6d**), suggesting that alteration in the expression of these TFs could explain some of the transcriptional differences we have seen in/of their targets. Peripheral blood T cells from a patient heterozygous for a BACH2<sup>L24P</sup> mutation rendering her haploinsufficient for this transcription factor<sup>49</sup> showed over-representation of the CD161<sup>+</sup> Treg subset relative to controls (**Fig. 6f**), confirming that low BACH2 expression is important for development and/or persistence of these cells. An integrated network was constructed based on these effects to illustrate the transcriptional circuitry (**Supplementary Fig. 6e**). Genes controlled solely by BACH2 regulated cell division, whereas genes co-regulated by BACH2 and the other TFs in this model were especially involved in wound healing (**Fig. 6f**). These data point to distinct regulomes in CD161<sup>+</sup> Tregs imparting novel functions including wound-healing.

#### *CD161<sup>+</sup> Tregs are enriched in IBD, enhance wound healing and associate with reduced inflammation*

We confirmed by GSEA that activated CD161<sup>+</sup> Tregs were significantly enriched for wound healing genes, including soluble mediators, compared to other Treg populations (**Supplementary Fig. 7a-b**). Since CD161<sup>+</sup> Tregs expressed CCR9 and ITGA4 (**Fig. 3a** and **Supplementary Fig. 3a-b**), we determined if they are enriched in the bowel during

inflammation by comparing Treg populations in matched peripheral blood to colonic biopsies of healthy individuals and those with Crohn's disease (CD). There was mild enrichment of naïve and memory Tregs in colonic mucosa compared to blood. By contrast, there was a significant enrichment of CD161<sup>+</sup> Tregs in healthy colons, which was even more pronounced in patients with IBD (**Fig. 7a**). LLT1, the ligand of CD161, is expressed in actively inflamed areas<sup>31</sup>, which we speculated would drive cytokine expression in CD161<sup>+</sup> Tregs. We therefore tested the effect of CD161<sup>+</sup> Treg supernatants on wound healing using a human epithelial colorectal adenocarcinoma cell line (Caco-2 cells) to approximate bowel epithelium. Supernatants from activated CD161<sup>+</sup> Tregs increased and accelerated closure of the wound by almost two-fold compared to supernatant from memory Tregs or medium alone (**Fig. 7b-c**, **Supplementary Fig. 7c** and **Supplementary Movies 1-3**). Neutralization of IL-17 alone, IL17 together with IL-22, IL-4 or IL-10 impeded the wound healing capacity of supernatants of activated CD161<sup>+</sup> Tregs, implicating these cytokines as mediators of wound healing (**Fig. 7d**). Over-expression of BACH2 by lentiviral delivery in CD161<sup>+</sup> Tregs significantly inhibited production of IL-17 and IL-4, with a similar but not significant trend in IL-22 and IL-10 (**Fig. 7e** and **Supplementary Fig. 7d**), supporting the notion that the wound healing program of CD161<sup>+</sup> Tregs is dependent on reduced expression of the repressive BACH2 TF in these cells.

The suppressive function of CD161<sup>+</sup> Tregs and their ability to accelerate wound healing suggested that they may be beneficial in IBD. To explore this possibility, we examined RNA-seq from a large dataset of patients with CD (GSE57945) for expression of *KLRB1*, which encodes CD161. *KLRB1* correlated negatively with 15 genes previously shown to be upregulated in inflamed compared to uninfamed CD mucosa<sup>50</sup> (**Fig. 7f**). Of these, *CXCL1*, a clinical biomarker of CD, was significantly more highly expressed in inflamed versus uninfamed CD mucosa, while *KLRB1* expression was significantly higher in uninfamed CD mucosa (**Fig. 7g**). *KLRB1* is expressed on both Tregs, which are IL7R<sup>lo</sup>, and Th17 cells, that are IL7R<sup>hi</sup>. We used GSE57945 as a training dataset to test the performance of *KLRB1*, IL7R and *KLRB1*/IL7R ratio to distinguish inflamed from uninfamed CD. The receiver operating characteristic (ROC) curve for *KLRB1*/IL7R was highly significant (AUC=0.77; p-value<0.0001) and performed well in comparison with *CXCL1*, slightly better than *KLRB1* and substantially better than IL7R alone (**Fig. 7h**) when predicting inflamed versus uninfamed mucosa. We used two additional transcriptome datasets from CD mucosa as validation datasets. In all cases the *KLRB1*/IL7R predictor was higher in uninfamed CD mucosa (**Fig. 7i**) and distinguished inflamed from uninfamed mucosa (AUC range 0.63 to 0.79; **Fig. 7j**), suggesting that tissue infiltration with CD161<sup>+</sup> Tregs is associated with lower inflammation in CD. These data indicate that CD161<sup>+</sup> Tregs are enriched in colonic mucosa,

particularly in IBD, where they suppress inflammation and produce soluble factors that accelerate epithelial barrier healing, having overall a beneficial effect on outcomes.

## Discussion

Tregs are necessary for the prevention of autoimmunity and are currently under investigation as a cell therapy intended to prevent or ameliorate autoimmune diseases or transplant rejection<sup>2</sup>. Understanding biologically important and clinically relevant Treg populations is key to elucidating disease mechanisms and tailoring immunotherapy appropriately. The ability of Tregs to express TFs of other lineages, such as GATA3, IRF4, T-bet and STAT3<sup>8-11</sup> for appropriate licensing and biological function suggests functional heterogeneity or “division of labor” among Tregs. There is evidence that inappropriate high expression of alternate TFs, such as T-BET, can reprogram Tregs to carry out inflammatory functions<sup>7</sup> and this may be true of other lineage-associated TFs.

Here we have delineated the CD161<sup>+</sup> Treg population in humans, showing that it is a distinct, *bona fide* retinoic-acid dependent Treg sub-population enriched in the lamina propria. We show that it is highly suppressive, can enhance wound healing through soluble mediators and, importantly, is associated with regions of gut that have lower inflammation in Crohn’s disease. Transcriptional control over the properties of these cells involves a TF network in which BACH2, ROR $\gamma$ t, RUNX1, FOSL2 and BATF, all play a role. We show that CD161 is expressed on a discrete population of human Tregs that are phenotypically CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>FOXP3<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup>CD161<sup>+</sup> and distinct from Tconv expressing CD161. Their TCR repertoire is unique and, importantly, distinct from Tconv and Th17 cells. Thus, it is highly unlikely that they are contaminating Tconv and we conclude that they are a distinct sub-population of Tregs with stable regulatory function.

Our data suggest that CD161<sup>+</sup> Tregs are either induced to develop or to specialize in the periphery. This is consistent with almost complete absence of CD161<sup>+</sup> Tregs in human cord blood<sup>24</sup> and the relatively small numbers in peripheral circulation. In mice, ROR $\gamma$ t<sup>+</sup> Tregs in the colon can either be induced to develop from naïve CD4<sup>+</sup> T cells by the local microbiota to suppress intestinal inflammation, although these cells do not produce IL-17<sup>51,52</sup>, or from thymic Treg émigrés following immunization with MOG and CFA<sup>26</sup>. We identified that human CD161<sup>+</sup> Tregs have a retinoic acid gene signature and that retinoic acid directly regulates CD161 expression through ATRA-RARA complex binding to *KLRB1*, the gene encoding CD161. Since the bowel is a retinoic acid-rich site, a key area for Treg generation and since CD161<sup>+</sup> Tregs expressed CCR9, a gut-homing chemokine receptor, we were not surprised to find enrichment of CD161<sup>+</sup> Tregs in the lamina propria, particularly in patients with IBD. This is consistent with previous data identifying inflamed bowel as a site of enrichment for FOXP3<sup>+</sup>IL-17<sup>+</sup> T cells<sup>53</sup>. Thus, CD161<sup>+</sup> Tregs appear to be either induced or migrate to lamina propria, potentially a key site for their function.

There are elements in the chromatin landscape of all three Treg populations we studied that are broadly accessible in all subsets. These include Treg lineage-associated loci, such as *FOXP3*, *CTLA4* and *IL2RA*, which are consistent with similar expression of these proteins in all the subsets. Indeed, the regulome of CD161<sup>+</sup> Tregs differed from memory Tregs by only ~500 OCRs, which is congruous with the relatively small transcriptional differences between them. The transcription factor circuitry explaining these differences is dominated by BACH2, which is itself expressed at lower levels in CD161<sup>+</sup> compared to memory Tregs. The importance of low BACH2 expression for the development and/or persistence of these Tregs was demonstrated by excess of the CD161<sup>+</sup> Treg subset in a patient with genetic haploinsufficiency of this TF. As BACH2 restricts expression of effector programs in T cells<sup>5</sup>, it is unsurprising that some de-repression by BACH2 imparts functional properties to Tregs that resemble those of conventional T cells, notably production of cytokines, without loss of suppressive phenotype. This is supported by the significantly diminished cytokine production evident on BACH2 over-expression in these Tregs. Of note, CCR9 is a classic BACH2-repressed gene<sup>5</sup> and one of the most differentially expressed genes in CD161<sup>+</sup> Tregs. Likewise, RORC, FOSL2 and BATF programs play important roles in Th17 lineage specification in conventional T cells<sup>48</sup> and their altered activity in CD161<sup>+</sup> Tregs appears mechanistically responsible for regulating genes involved in wound healing.

The role of CD161 itself in Tregs is noteworthy. Its cognate ligand, LLT1, is expressed on activated DCs and in actively inflamed areas<sup>31</sup>, so it is likely that that cross-linking of CD161 will occur in inflamed areas of bowel. Indeed, single nucleotide polymorphisms in LLT1 are associated with IBD in humans<sup>32</sup>. We report that CD161 cross-linking is co-stimulatory to CD161<sup>+</sup> Treg activation and synergizes with CD3/CD28-mediated signals to induce cytokine secretion. This is in agreement with previous data suggesting that CD161 interaction with its cognate ligand stimulates proliferation and cytokine secretion on other T lymphocytes<sup>28</sup> but contrasts with CD161 ligation on NK cells, which causes NK cell inhibition<sup>44,45</sup>. This suggests that any signal transduced by CD161 has effects that are cell-type and possibly context-dependent.

Although the correlation between CD161 expression and the Th17 program has been previously shown in Tconv<sup>30</sup>, it was unexpected to find the transcriptomes of activated CD161<sup>+</sup> Tregs to encode a complex cytokine cocktail. Since IL-17 can have pro-inflammatory functions associating it with multiple autoimmune diseases<sup>54</sup> it is possible that their expression of IL-17 could denote a pro-inflammatory function. **Indeed, some murine studies suggest that RORγt<sup>+</sup> Tregs can convert to pathogenic cells that promote autoimmunity and cancer<sup>55,56</sup>.** Our data, however, indicate that actively IL-17-producing Tregs continue to remain highly suppressive and we found no evidence that CD161<sup>+</sup> Tregs are a transitional or unstable population. **This is consistent with some studies in which adoptive transfer of IL-17**

producing Foxp3<sup>+</sup>RORγt<sup>+</sup> T cells in mice supports a stable and suppressive effect on gut inflammation<sup>57</sup>. The cytokine profile of CD161<sup>+</sup> Tregs could denote alternative functions in addition to cellular suppression and one such function of Tregs is a potential role in wound healing<sup>58</sup>. In fact, several of the cytokines produced by CD161<sup>+</sup> Tregs have established roles in wound healing, especially in the gut. Multiple studies have shown that IL-17 can promote repair of damaged intestinal epithelium and protect from excessive inflammation in colitis models<sup>59,60</sup>. Indeed, IL-17 blockade exacerbates colitis in both human and mouse models<sup>61,62</sup>. The same “protective” role has also been described for IL-22 by studies showing its role in inducing intestinal epithelial regeneration and protection from GvHD<sup>63</sup> as well as dampening inflammation in models of IBD<sup>64</sup> and ulcerative colitis<sup>65</sup>. In conclusion, these data would support the notion that enhancing CD161<sup>+</sup> Tregs at the site of inflammation, for example, by cell therapy, may prove beneficial, in IBD.



**Author contributions:** G.A.M. designed and performed experiments, analyzed data and wrote the manuscript. N.P. and P.P. provided patient samples and clinical and scientific input. S.K. designed the CyTOF panel, analyzed and interpreted data, provided scientific input and wrote the paper. G.L. provided scientific input, supervised the project and wrote the manuscript. B.A. conceptualised the study, supervised the project and wrote the manuscript. All other authors performed experiments, analyzed data and/or provided scientific input.

**Acknowledgements:** The authors wish to thank patients that contributed samples towards this study. This work was supported by the Wellcome Trust (grant 097261/Z/11/Z to B.A. and WT101159 to N.P.), the Crohn's & Colitis Foundation of America CCFA (A.L.), the Purdue Cancer Center (M.K.), British Heart Foundation (G.L.) and National Heart, Lung, and Blood Institute (grant 5K22HL125593-02 to M.K.). Research was also supported by the National Institute for Health Research (NIHR) Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. This research was supported [in part] by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, the National Institute of Diabetes and Digestive and Kidney Diseases and the National Heart, Lung and Blood Institute of the National Institutes of Health. We thank John O'Shea (National Institutes of Health) for his support and for providing access to ATAC-seq, the National Heart, Lung and Blood Institute DNA Sequencing and Genomics Core for performing single cell sequencing experiment and acknowledge the assistance of Matt Arno (Genomics Centre, King's College London) with gene expression microarray studies. In addition, the authors thank Ewy Mathé (Ohio State University) for critically reading the manuscript.

**Competing financial interests:** The authors have no competing interests to declare.

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## Figure legends

**Figure 1. CD161<sup>+</sup> Treg are a discrete population of memory Tregs.** (a) viSNE plots of CD4<sup>+</sup> T cells clustered using surface and intracellular markers. Shown are heatmaps for expression of indicated markers. White arrow highlights expression of CD161 within Tregs; (b) overlaid contour plots of T cell subsets, coloured by density, to highlight sub-populations of Tregs and Tconv; (c) 2D minimum spanning tree showing population nodes of CD4<sup>+</sup> T cells. Node size represents cell number and color CD161 median intensity. Grouped together are naïve (circled in orange), memory (circled in yellow) and CD161<sup>+</sup> (circled in purple) Tregs, as well as populations of naïve (circled in black), memory (circled in black) and CD161<sup>+</sup> (circled in red) Tconv; (a-c) show representative data from  $n=3$  experiments; (d) representative spectratype histograms from  $n=3$  experiments showing percentage of unique CDR3 sequences (templates) versus CDR3 length for the three highest (BV05, BV06 and BV07) and one of the lowest (BV19) TCRBV families contributing to the overall TCR repertoire in the indicated populations; (e) average Morisita-Horn Similarity Index of total TCRVB repertoire between the T cell populations (cumulative data from  $n=3$  experiments); and (f) dendrogram showing linkage distance based on the Morisita-Horn Similarity Index.

**Figure 2. CD161<sup>+</sup> Tregs have classical features of *bona fide* Tregs.** (a) number of differentially expressed genes between the sub-populations of freshly isolated Tregs; (b-c) Gene Set Enrichment Analysis (GSEA) plots for genes associated with other CD161<sup>+</sup> cells (b) and core human Treg signature genes (c) comparing freshly isolated memory to CD161<sup>+</sup> Tregs; (d) FOXP3 expression by sub-populations of Tregs and Tconv, showing representative flow cytometry plots (left) and cumulative data (mean + sem; right); (e) mean percentage CpG methylation of conserved CpGs (with chromosomal coordinates) at the *FOXP3* TSDR, *IL2RA* and *CTLA4* loci of naïve, memory, CD161<sup>+</sup> Tregs and Tconv of 3 male donors.  $n=3$  independent experiments in a-e; \*\*\*\* $p<0.0001$ .

**Figure 3. CD161 expression is regulated by retinoic acid.** (a) GSEA for ATRA-regulated genes in human Tregs comparing freshly isolated memory to CD161<sup>+</sup> Tregs. Classic ATRA-regulated genes within the leading edge of core enriched genes (see **Supplementary Fig. 4a**) are annotated. In bold are gut homing receptors; (b) expression of CD161 and CCR9 on Tregs cultured with and without ATRA for 2 days; shown are representative flow cytometry plots (left) and cumulative data from  $n=3$  experiments (right); p-values indicate comparisons with 2 $\mu$ M ATRA; (c) representative assay of ALDH activity in DCs from  $n=3$  independent experiments; (d) representative flow cytometry plots (left) and cumulative data (right) from  $n=3$  independent experiments showing CD161 expression on Tregs before (fresh) and after 5 days either with medium alone or co-culture with DCs in the presence or absence of the pan-RAR inverse agonist, BMS493; (e) RARA ChIP-qPCR for binding sites in *KLRB1* and *CCR9*, showing percentage of input; shown are representative examples from  $n=2$  independent experiments. Bar charts show mean + sem throughout; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

**Figure 4. CD161<sup>+</sup> Tregs are regulatory both *in vitro* and *in vivo*.** (a-b) *In vitro* Treg suppression assay showing representative CFSE dilution histograms of Tconv co-cultured with and without Tregs (a) and cumulative IC<sub>50</sub> of memory and CD161<sup>+</sup> Tregs from  $n=4$  experiments (b). Note that only one out of the 4 naïve Treg donors tested reached 50% suppression, therefore the mean IC<sub>50</sub> was not calculated for the naïve Treg population. (c) Xeno-graft versus host disease with and without 2:1 PBMC : Treg injection, showing survival plots (left panel; \*  $p<0.05$  compared to PBMC alone) and clinical severity (right panel). Shown is one experiment from two independent experiments carried out with  $n=5$  mice in each group. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\*\* $p<0.0001$ .

**Figure 5. CD161 ligation is co-stimulatory and induces cytokine production from CD161<sup>+</sup> Tregs.** (a) differentially expressed genes following 4h stimulation of CD161<sup>+</sup> Tregs

with anti-CD3/CD28; **(b)** venn diagram of transcriptional differences between freshly isolated and *in vitro* anti-CD3/CD28-activated CD161<sup>+</sup> and memory Tregs; **(c)** heatmaps of differentially expressed genes common to fresh and activated cells (lower panel) and specific to the activated condition (right panel), with cytokine genes highlighted in inset. Data in **a-c** are from 3 independent experiments; **(d)** concentration (pg/ml) of stated cytokines in supernatants of naïve, memory and CD161<sup>+</sup> Tregs after 3 days of polyclonal activation with anti-CD3/CD28 (cumulative data from *n*=6 experiments); **(e)** suppression assay showing cell trace violet dilution in proliferating Tconv cultured alone or in co-culture with stated populations of Tregs (Treg: Tconv ratio of 1:2). Shown are representative plots (left) and cumulative data from *n*=3 independent experiments (right); **(f)** concentrations of stated cytokines in supernatants of FACS-sorted CD161<sup>+</sup> Tregs stimulated for 3 days with either  $\alpha$ CD3+ $\alpha$ CD28+IgG2 or  $\alpha$ CD3+ $\alpha$ CD28+ $\alpha$ CD161-coated magnetic beads (cumulative data from *n*=4 experiments). Bar charts shown mean + sem throughout. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, \*\*\*\**p*<0.0001.

**Figure 6. Genome-wide chromatin landscapes define the regulatory circuitry of CD161<sup>+</sup> Tregs.** **(a-b)** heatmap showing signal intensity of each ATAC peak and clustering of peaks into three groups **(a)** with representative examples of open chromatin regions (OCRs) from the three clusters **(b)** - highlighted in blue are OCRs corresponding to ATAC peaks in the heatmap; **(c)** transcription factor (TF) footprints enriched relative to background in each cluster of ATAC peaks, with corresponding TF family indicated; **(d)** GSEA plots for BACH2-, ROR $\gamma$ t-, BATF-, FOSL2- and RUNX1-regulated genes, comparing memory to CD161<sup>+</sup> Tregs; **(e)** percentage of differentially expressed genes (DEG) between CD161<sup>+</sup> and memory Tregs that can be explained by each TF, with Fisher Exact test *p*-values indicated; **(f)** CD161 expression on CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup> Tregs of healthy age and sex-matched donors (*BACH2*<sup>WT/WT</sup>) and a patient with BACH2 haploinsufficiency (*BACH2*<sup>WT/L24P</sup>); shown are representative flow cytometry plots (left) and data from two independent experiments (right); **(g)** Venn diagram showing shared and unique DEGs regulated by TFs (see also **Supplementary Fig. 6e**) (left) and corresponding function for those DEGs (right). Data for **Fig. 6a-e and g** are from *n*=3 independent experiments.

**Figure 7. CD161<sup>+</sup> Tregs accelerate wound healing and are associated with lower inflammation in IBD.** **(a)** enrichment of Treg sub-populations in colons relative to blood of healthy subjects and patients with Crohn's disease (CD) (*n*=3 paired samples per group). Bar charts show mean + sem; **(b-c)** wound healing assay showing growth of Caco-2 cells cultured with medium alone or medium supplemented with culture supernatants (snt) of activated memory or CD161<sup>+</sup> Tregs. Shown are representative still images captured over time (0, 72, 96 and 120h) **(b)** and percentage of open wound over time from *n*=3 independent experiments; **(d)** Fraction of open wound at the end of culture of Caco-2 cells in the presence of culture supernatants from activated CD161<sup>+</sup> Tregs in the presence of blocking antibodies to stated cytokines or isotype control. Shown are cumulative data from *n*=3 independent experiments. **(e)** concentration (pg/ml) of stated cytokines in supernatants of CD161<sup>+</sup> Tregs transduced, or not, with control lentivirus or lentivirus encoding BACH2 (cumulative data from *n*=6 experiments); **(f)** correlation matrix of indicated transcripts in bowel specimens of patients with active CD; **(g)** violin plots showing distribution of CXCL1 (left) and KLRB1 (right) expression in inflamed and uninfamed biopsies of CD; **(h)** ROC curves showing performance of CXCL1, IL7R, KLRB1 and KLRB1/IL7R ratio to discriminate inflamed (*n*=157) versus uninfamed CD or non-IBD tissue (*n*=56). AUC and *p*-values are indicated. **(h-i)** KLRB1/IL7R ratio in transcriptomes of inflamed and uninfamed sites of CD **(h)** and AUC + 95% CI of KLRB1/IL7R ROC curves to distinguish the two **(i)**. In **i**, area of circles indicates sample size. Source data for **f-h**: GSE57945; **i-j**: GSE20881 and Häsler *et al.*, 2016. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, \*\*\*\**p*<0.0001.

## Supplemental Figure and Table legends

**Supplementary Fig. 1. CD161<sup>+</sup> Tregs are a discrete population of memory Tregs.** (a) heatmap showing signal intensity of selected markers for each node of the populations shown in Fig. 1c; (b-e) clustering of populations of Tconv and Tregs by single cell RNA-seq (scRNAseq) showing tSNE plot of 2636 cells from  $n=3$  independent donors separated into six major (clusters 0-6) and two minor (clusters 7-8) clusters (b), feature plots showing expression of three Treg markers (IL2RA encoding CD25, IL7R encoding CD127 and FOXP3 encoding FOXP3), KLRB1 (encoding CD161) and two naïve/memory markers (SELL encoding CD62L and CCR7 encoding CCR7) (c), heatmap showing top 20 discriminatory genes per cluster with representative genes of each cluster indicated (d) and population clustering (e) based on expression of the transcripts shown in c. Clusters in b are color-coded and labelled according to expression of the markers shown in e. (f) Clustering of Tconv and Treg sub-populations using protein expression data sourced from CyTOF in Fig 1a incorporating the same markers as in (e) and also including CD45RA and CD45RO. Clustering of sub-populations in (f) is similar to that from scRNAseq (e). (g-h) viSNE plots (g) and 2D minimum spanning tree (h) of CD4<sup>+</sup> T cells clustered following staining with anti-CD4, anti-CD25, anti-CD127, anti-CD45RA and anti-CD161 for flow cytometry. Shown in g are heatmaps for expression of indicated markers, with arrow indicating CD161 expression in Tregs. Nodes size in h represents cell number and color CD161 median intensity. Grouped together are naïve (circled in orange), memory (circled in yellow) and CD161<sup>+</sup> (circled in purple) Tregs, as well as populations of naïve (circled in black), memory (circled in black) and CD161<sup>+</sup> (circled in red) Tconv. (g-h) show representative data from  $n=4$  experiments. (i) representative example of FACS sorting strategy for naïve (orange), memory (yellow) and CD161<sup>+</sup> Tregs (purple); (j) proportions of each Treg sub-population in healthy human donor peripheral blood (cumulative data from  $n=10$  donors); (k) representative (upper panels) and cumulative (lower panels) expression of TCR V $\alpha$ 24-J $\alpha$ 18 and V $\alpha$ 7.2 in total CD4<sup>+</sup> T cells and CD161<sup>+</sup> Tregs. Both cell types show minimal expression of these invariant TCR chains. Data from  $n=3$  independent experiments; bars show mean + sem. (l) Contribution of the three highest (BV05, BV06 and BV07) and one of the lowest (BV19) TCRBV families to the overall TCR repertoire in naïve, memory and CD161<sup>+</sup> Tregs as well as CD161<sup>+</sup> and CD161<sup>-</sup> Tconv cells; data from  $n=3$  independent experiments; bars show mean + sem. (m) average percentage of TCR sequences either unique or shared among naïve, memory and CD161<sup>+</sup> Tregs, CD161<sup>+</sup> and CD161<sup>-</sup> Tconv cells (cumulative data from  $n=3$  experiments).

**Supplementary Fig. 2. Transcriptome and methylome of CD161<sup>+</sup> Tregs.** (a) principal component analysis (PCA) 2D mapping of variance in expression of transcripts in freshly isolated Naïve, Memory and CD161<sup>+</sup> Tregs ( $n=3$ ); (b-d) scatter plots showing correlation between mean gene expression of freshly isolated Treg sub-populations; (e-f) GSEA plots for core human Treg signature genes comparing freshly isolated naïve to memory Tregs (e) and to CD161<sup>+</sup> Tregs (f); (g) schematic representation of *FOXP3*, *IL2RA* and *CTLA4* gene loci showing the target sequence and conserved CpGs assayed for methylation analysis in Fig. 2e; (h) mean region methylation percentage of *FOXP3* TSDR, *IL2RA* and *CTLA4* loci from 3 male donors (data from  $n=3$  independent experiments).

**Supplementary Fig. 3. Regulation of CD161 by retinoic acid.** (a) heatmap of leading edge (core enriched) genes from GSEA for ATRA-regulated genes comparing freshly isolated memory to CD161<sup>+</sup> Tregs (Fig. 3a). Data from  $n=3$  independent experiments. (b) CCR9 surface protein expression on memory and CD161<sup>+</sup> Tregs; shown are representative flow cytometry plots (left) and cumulative data from  $n=3$  independent experiments (right). (c) CD161 expression on Tregs co-cultured for 5 days with DCs with and without BMS493 at increasing concentrations. Shown are representative plots (left) and cumulative data from  $n=3$  independent experiments. (d-e) sequence logo for RARA DNA motif (d) and schematic representation of the *KLRB1* and *CCR9* gene loci ( $\pm 5$ kb) showing the predicted binding sites



of RARA (e); red arrowhead indicates the binding site for *CCR9* selected for analysis in ChIP-qPCR. Bars show mean + sem throughout the figure. \* $p < 0.05$ , \*\* $p < 0.01$ .

**Supplementary Fig. 4. CD161<sup>+</sup> Tregs are highly regulatory.** (a-b) cumulative mean percentage suppressive function of freshly isolated naïve, memory and CD161<sup>+</sup> Tregs from Fig. 4a ( $n=4$  experiments) (a) and representative IC<sub>50</sub> calculation (b; dashed arrows show the IC<sub>50</sub> for CD161<sup>+</sup> and Memory Tregs). (c) cumulative mean percentage suppressive function of memory and CD161<sup>+</sup> Tregs after in vitro expansion for two weeks ( $n=4$  experiments). (d) suppression of Tconv proliferation when in direct contact with Tregs or when separated by a transwell at a Treg:Tconv ratio of 1:1 (cumulative data from  $n=4$  independent experiments); (e) suppressive function of Tregs under neutral (null) conditions or in the presence of blocking antibodies directed against CD161, PDL1, TGFβRII or IL10R, all at a Treg:Tconv ratio of 1:2 (cumulative data from  $n=3$  independent experiments); (f) suppressive function of CD161<sup>+</sup> Tregs under neutral (null) conditions or in the presence of Th1 and Th17 skewing conditions, all at a Treg:Tconv ratio of 1:2 (cumulative data from  $n=3$  independent experiments); (g) expression of perforin, granzyme A and B in sub-populations of Tregs, with NK cells as positive control; shown are representative flow cytometry plots for each marker (left) and cumulative data from  $n=3$  independent experiments (right). Bar charts show mean + sem throughout the figure. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

**Supplementary Fig. 5. CD161<sup>+</sup> Tregs produce cytokines on activation.** (a) differentially expressed genes following 4h stimulation of memory and naïve Tregs with anti-CD3/CD28; (b) volcano plot showing significant genes differentially expressed in CD161<sup>+</sup> Tregs after 4h activation with anti-CD3/CD28; differentially expressed genes are shown in blue; red indicates significantly upregulated cytokine genes that are differentially expressed compared to memory Tregs (see Fig. 5c); orange indicates other cytokine genes of interest upregulated in activated CD161<sup>+</sup> Tregs. a-b are from 3 independent experiments; (c-d) cell sorting of IL-17<sup>+</sup> and IL-17<sup>-</sup> CD161<sup>+</sup> Tregs by surface IL-17 capture. Shown are (c) representative flow cytometry plots showing double staining for intracellular and extracellular (captured on the surface) IL-17 with (right) and without (left) fixation and permeabilization and (d) sorting strategy using IL-17 surface capture and post-sort purity for the IL-17<sup>+</sup> and IL-17<sup>-</sup> fractions of CD161<sup>+</sup> Tregs.

**Supplementary Fig. 6. Global analysis of Treg regulomes.** (a) open chromatin regions (OCRs) at prototypical Treg gene loci; (b) genomic distribution of ATAC peaks (promoter, intragenic or intergenic regions) in the three groups shown in Fig. 6a; (c) p-values for transcription factor (TF) motifs shown in each cluster in Fig. 6c; (d) expression of key TFs participating in gene regulation in CD161<sup>+</sup> Tregs. Shown are normalized signal intensity (mean + sem) from  $n=3$  microarrays for each Treg population; (e) integrated TF network showing contribution of each TF to DEGs and overlap between them (fold difference in expression of each TF is also indicated next to the TF).

**Supplementary Fig. 7. CD161<sup>+</sup> Tregs accelerate wound healing.** (a-b) GSEA plots for wound healing associated genes (a) and wound healing associated soluble mediators (b) comparing activated CD161<sup>+</sup> to memory Tregs. Data from  $n=3$  independent experiments; (c) wound closure rate of Caco-2 cells cultured in the presence of either medium alone or medium supplemented with culture supernatants (snt) from activated memory or CD161<sup>+</sup> Tregs. Shown are cumulative data from  $n=3$  independent experiments. Bar chart shows mean + sem; \* $p < 0.05$ .

**Supplementary Table 1. List of differentially expressed genes (DEGs).** Listed are DEGs in comparisons of freshly isolated Tregs to each other (tab 1) and transcriptional changes in each population when comparing CD3/CD28-activated cells to freshly isolated cells (tab 2).

**Supplementary Table 2. Expression of killer cell lectin like receptors (Klr) in wild-type mouse Tregs.** Shown are RPKM values ( $n=6$ ) for Klr gene transcripts, Foxp3 and Actin. Data from Feng *et al.*, 2014, GSE58905.

**Supplementary Table 3. Open chromatin regions (OCRs) from ATAC-seq.** Listed are genes with OCRs conforming to cluster 1 (naïve Tregs-specific), cluster 2 (shared between CD161<sup>+</sup> and memory Tregs) and cluster 3 (CD161<sup>+</sup> Tregs-specific).

**Supplementary Table 4. List of conjugated antibodies used for mass cytometry.**

**Supplementary Table 5. Gene lists used for gene set enrichment analyses.**

**Supplementary Movies 1-3. Wound healing assay.** Movies show growth of Caco-2 cells cultured with medium alone (**Supplementary Movie 1**) or medium supplemented with culture supernatants (snt) of activated memory (**Supplementary Movie 2**) or CD161<sup>+</sup> Tregs (**Supplementary Movie 3**). Time-lapse images were recorded from 0-120h.

## Online methods

### T-cell separation, sorting, and flow cytometry

Human PBMCs (peripheral blood mononuclear cells) and T cells subsets were purified from either leukodepletion cones (Blood Transfusion Service, London) or fresh blood of healthy volunteers. Tregs were isolated by initially enriching with CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotec), cells were subsequently stained with mouse anti human CD4 (OKT4), CD127 (eBioRDR5), CD45RA (HI100, all from eBiosciences) and CD25 in PE (both 2A3 and M-A251) and CD161 (DX12, all from BD Biosciences); PE-labelled cells were then captured using anti-PE MicroBeads (Miltenyi Biotec) to enrich for Tregs, pre-sorting. Cells were FACS sorted for surface markers to separate naïve (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>+</sup>CD161<sup>-</sup>), memory (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup>CD161<sup>-</sup>), and CD161<sup>+</sup> (CD4<sup>+</sup>CD25<sup>hi</sup>CD127<sup>lo</sup>CD45RA<sup>-</sup>CD161<sup>+</sup>) Tregs. Sorting of naïve Tconv (CD4<sup>+</sup>CD25<sup>-</sup>CD45RA<sup>+</sup>CD161<sup>-</sup>), total Tconv (CD4<sup>+</sup>CD25<sup>-</sup>) and Th17 (CD4<sup>+</sup>CD25<sup>-</sup>CD45RA<sup>-</sup>CD161<sup>+</sup>) cells was performed using the same panel. Intracellular staining for FOXP3 (PCH101, eBioscience) was carried out using the Foxp3 / Transcription Factor Staining Buffer Set Kit (eBioscience) according to manufacturer's instructions. For intracellular staining of cytokines and transcription factors cells were activated for 4 hours with PMA (50 ng/mL) and ionomycin (1 mM, both from Sigma) with the addition of Brefeldin A (3 µg/mL; eBioscience) before staining. Additional staining of Tregs were further stained for invariant chains TCRV $\alpha$ 24-J $\alpha$ 18 (6B11) and TCR V $\alpha$ 7.2 (3C10, both from Biolegend), perforin (dG9, eBioscience), granzyme A (GB11, Biolegend) and granzyme B (CB9, Biolegend) using appropriate fluochrome-conjugated antibodies. Flow cytometry data were acquired on an LSR Fortessa (BD) and subsequently analyzed using FlowJo version 10.1 (TreeStar Inc.).

### Mass Cytometry (CyTOF)

2x10<sup>6</sup> CD4<sup>+</sup>CD127<sup>lo</sup> cells were isolated using RosetteSep™ Human CD4<sup>+</sup>CD127<sup>low</sup> T Cell Enrichment Cocktail (STEMCELL Technologies) before extracellular and intracellular staining with metal conjugated antibodies. For full list of both antibody panels refer to **Supplementary Table 4**. CyTOF-2 mass cytometer (Fluidigm) was used for data acquisition and beads (Ce140) were used for normalization<sup>1</sup>. 320,000 cells were proportionally sampled from all individuals to perform automated clustering. Data were initially processed and analysed using Cytobank<sup>1</sup>; CD4 sample “cleanup” was performed by gating on intact (191Ir<sup>+</sup> DNA stain), no beads (Ce140<sup>-</sup>), live (103Rh<sup>+</sup>), no B cells CD19<sup>-</sup>CD20<sup>-</sup> (Nd142), no neutrophils CD15<sup>-</sup>CD123<sup>-</sup> (Eu151), CD34<sup>-</sup> (Er166), CD45<sup>+</sup>(Y89), CD3<sup>+</sup> (Sm154) and CD4<sup>+</sup> (Nd145) T cells. Mass-cytometry complex data was analysed using viSNE, in combination with SPADE, to identify distinct subpopulations<sup>2,3</sup> using the following parameters: CCR6 (141Pr), CD45RA (143Nd), CCR4 (149Sm), CD161 (150Nd), CD103 (152Sm), CD62L (153Eu) Helios (156Gd), CCR7 (159Tb), Tbet (160Gd), CD95 (161Dy), CXCR3 (163Dy), CD45RO (164Dy), GATA3 (167Er), CCR9 (168Er), CD25 (169Tm), Foxp3 (171Yb), CXCR4 (173Yb), HLA-DR (174Yb), CD127 (176Yb). viSNE and SPADE plots were generated using Cytobank Inc. (CA, USA). When indicated in the figure legend, figures were overlaid for demonstration purpose.

### T cell culture

Unless indicated otherwise, cells were cultured in X-Vivo 15 with Gentamycin and PR (Lonza) supplemented with Penicillin-Streptomycin Glutamine (PSG, Thermo Fisher) and 5% human AB serum (Biosera), henceforth shortened to X-Vivo 5% HS; cells were then seeded at 10<sup>6</sup>cell/ml.

For the evaluation of the effect of ATRA on CD161 expression, 250,000 total Tregs were isolated and stimulated for 48h with 1:1 ratio of  $\alpha$ CD3/CD28 Dynabeads in X-Vivo 5% HS supplemented with 100IU/ml of IL-2 (Proleukin, Novartis), in the presence of 0, 0.02, 0.2 or 2µM ATRA (Sigma-Aldrich); cells were then stained for CD4, CD161 and CCR9 and the

effects of ATRA on expression of CD161 (stained with antibody clone DX12 from BD) and CCR9 (stained with antibody clone L053E8 from Biolegend) was evaluated by flow cytometry.

For Treg expansion to facilitate *in vivo* models sorted memory and CD161<sup>+</sup> Tregs were cultured in X-Vivo 5% HS supplemented with 1,000 IU/ml IL-2 and stimulated with  $\alpha$ CD3/CD28 Dynabeads at a 1:1 ratio for 2-3 rounds of expansion of 10-14 days each. Beads were removed by magnetic adherence following each round of stimulation and fresh  $\alpha$ CD3/CD28 Dynabeads (1:1 ratio) added. After the last round of expansion, beads were removed and the cells were rested for 2 days before injection. Cell viability was close to 100% prior to each *in vivo* experiment.

To evaluate the effects of CD161 crosslinking on cytokine production, CD161<sup>+</sup> Tregs were cultured for 3 days in X-Vivo 5% HS, supplemented with 100 IU/ml IL-2 and stimulated 1:1 with microbeads from the T cell activation/expansion kit (Miltenyi Biotec) loaded with anti-CD3 and anti-CD28 following manufacturer's instructions with the addition of anti-CD161 (191B8, Miltenyi) or IgG2a (eBM2a eBioscience).

#### Treg suppression assay

Cryopreserved Tconv cells (CD4<sup>+</sup>CD25<sup>-</sup>) were used as target cells throughout. These were isolated by negative selection of CD4<sup>+</sup> T cells followed by positive selection of CD25<sup>+</sup> T cells using miniMACS CD4<sup>+</sup>CD25<sup>+</sup> T Regulatory Cell Isolation Kit (Miltenyi Biotec, UK) according to manufacturers' instructions. Tconv cells were obtained from the CD25<sup>-</sup> negative fraction. Cryopreserved Tconv were thawed and labelled with either 2.5 $\mu$ M Carboxyfluorescein diacetate, succinimydil ester (CFSE) (Molecular Probes, USA) or 1 $\mu$ M CellTrace Violet (CTV) (Molecular Probes, USA) according to manufacturers' instructions. Tconv cell viability was routinely greater than 95% prior to suppression assay. Suppression assays were conducted in X-Vivo 5% HS and U-bottom 96-well plates incubated at 37°C, 5% CO<sub>2</sub> for 5 days, at constant Tconv cell number (10<sup>5</sup> cells) and Treg:Tconv ratio of 1:1 or 1:2, as indicated. Where indicated, Treg numbers were titrated to result in a Treg:Tconv ratio of 1:1 to 1:32 ratio. Cells were stimulated with  $\alpha$ CD3/CD28 Dynabeads (bead:cell ratio of 1:40) and CFSE/CTV dilution was assessed by flow cytometry on day 5. Percentage of suppression and IC50 were calculated as previously described<sup>4</sup>. Where indicated, Treg suppression was also evaluated in the presence of either 10 $\mu$ g/ml  $\alpha$ CD161 (BD Pharmingen), 10 $\mu$ g/ml  $\alpha$ PDL-1 (eBioscience), 10 $\mu$ g/ml  $\alpha$ IL-10R (Sigma), 1 $\mu$ g/ml  $\alpha$ TGFBRII (R&D) or in the presence of Th1 (40ng/mL IL-12 (Biolegend) and 5 $\mu$ g/mL  $\alpha$ IL-4 (R&D)) or Th17 (40ng/mL IL-1 $\beta$  (R&D) and IL-6, 10ng/mL TGF- $\beta$ 1 (both from Biolegend), 50ng/mL IL-23 and 5 $\mu$ g/mL  $\alpha$ IL-4 and  $\alpha$ IFN $\gamma$ , all from R&D) skewing conditions.

For transwell suppression assay the HTS Transwell-96 Permeable Support with a 0.4 $\mu$ m Pore Polycarbonate Membrane system (Corning) was used. Suppression was tested at a 1:1 Treg:Tconv ratio and Tconv were seeded in the lower compartment of the transwell plate, while Tregs were seeded on to the upper compartment. Cells in both the upper and lower chambers were stimulated with  $\alpha$ CD3/CD28 Dynabeads (bead:cell ratio of 1:40). As reference of standard suppression, Tregs and Tconv were also co-seeded in the lower compartment.

#### Gene expression analysis

200,000 naïve, memory and CD161<sup>+</sup> Tregs were either sorted from fresh blood directly into Trizol LS (Ambion) for baseline genetic profile or cells were polyclonally activated post-sorting for 4h with Dynabeads Human T-Activator CD3/CD28 (ratio 1:1) and then lysed in Trizol LS. RNA was isolated using RNeasy mini kit (QIAGEN) and Ovation PicoSL WTA System V2 (NuGEN Technologies) was used for reverse transcription and cDNA amplification steps. Fragmentation and labelling was performed using the Encore Biotin

Module (NuGEN Technologies); all kits were employed following manufacturer's instructions. Samples were run on GeneChip® Human Gene 1.0 ST Array (Affymetrix Ltd). Data analysis was performed using Partek Genomic Suite™ (Partek Incorporated). Thresholds for significance were set at 1.5-fold difference at  $p < 0.05$  for freshly isolated cells and 2-fold difference at  $p < 0.05$  for *in vitro* activated cells.

Gene set enrichment analysis (GSEA) was performed using GSEA version 2.2.2<sup>5</sup>. Datasets used for GSEA are shown in **Supplementary table 1**. Core human Treg genes were sourced from<sup>6</sup>; genes associated with CD161<sup>+</sup> cells were obtained from<sup>7</sup>. The ATRA-regulated gene list was generated by microarray from human Tregs treated, or not, with ATRA (Scottà *et al.* unpublished data). Genes regulated by RORC, BATF, and FOSL2 were curated from comparison of wild type and single gene knock-out Th17 cells from<sup>8</sup>(GSE40918), RUNX1 from wild type and *Cbfbeta* knockout Tregs<sup>9</sup> (GPL1261) and BACH2 from wild type and *Bach2* knockout Tregs<sup>10</sup>(GSE45975), all at a significance threshold of 1.5-fold change and  $p < 0.05$ . Mouse gene symbols were converted to human homologs using the BioMart data-mining tool in ensemble: <http://www.ensembl.org/biomart/martview>. General wound healing associated genes were curated from published gene lists<sup>11-13</sup>, the “resolve wound healing and fibrosis-related genes” dataset (<http://www.resolve-whfg.appspot.com/list/Human/>) and “wound healing RT2 profiler PCR array” (Qiagen). Wound healing associated soluble mediators were defined as the online dataset for “cytokines in wound healing (R&D Systems)” supplemented with cytokines and other soluble mediators from the general wound healing associated genes list.

#### Single cell RNA-sequencing

The single cell sequencing experiment was performed using the 10X Genomics' Chromium Single Cell 3' gene expression V2 kit following the manufacturer's instruction. CD4<sup>+</sup>CD25<sup>+</sup> T cells were freshly isolated from peripheral blood of 3 healthy donors. Cells were captured for each sample and libraries were sequenced on the Illumina HiSeq 3000 instrument. Raw reads from the 3 samples were combined and processed using 10x Genomics Cell Ranger v2.1<sup>14</sup>. The result was summarized into an expression matrix with the unique molecular identifier (UMI) count for every cell and every gene. Genes expressed in < 3 genes and cells with < 200 genes detected or > 5% of the total UMI count in mitochondrial genes were removed, resulting a final matrix comprising 2,636 cells and 15,357 genes. Dropouts were imputed using DrImpute<sup>15</sup>. We analyzed data using the R package Seurat<sup>16</sup> with default parameters if not specified. Top 15 principal components were used to compute the tSNE plot. Resolution was set to 0.4 for clustering.

#### Methylation analysis

For CpG methylation analysis 250,000 cells from each desired population were FACS sorted. CpG methylation analysis was determined by pyrosequencing of bisulphite-modified genomic DNA. Methylation analysis was conducted by EpigenDx, as previously described by<sup>17</sup>. CpG methylation of *FOXP3* TSDR (ADS783FS2), *IL2RA* (ADS4564FS) and *CTLA4* (ADS3074FS2) loci shown in **Supplementary Fig. 2g** were evaluated.

#### TCRBV sequencing

For TCRBV sequencing 250,000 cells from each population were FACS sorted. Amplification and sequencing of TCRB CDR3 was performed by Adaptive Biotechnologies using the immunoSEQ Platform<sup>18,19</sup>. Analysis was performed using ImmunoSEQ analyser for spectratyping analysis and clone sharing among samples; to assess the overlap in TCR composition between populations, the Morisita–Horn similarity index<sup>20</sup> was calculated using R-Studio. Only productive (in frame and without included STOP codon) amino acidic (VJ) sequences were analysed; the number of both common and specific sequences for each combination of populations was used to calculate the percentage of unique and shared sequences within the different populations.

### DCs generation, MLR and CD161 induction

From PBMCs, CD14<sup>+</sup> cells were isolated with CD14 microbeads (Miltenyi Biotec) and DCs generated by culturing for 5 days in X-Vivo 5% HS supplemented with 50ng/ml GM-CSF (Peprotech) and 800U/ml IL-4 (R&D). Maturation of the DCs was achieved by culturing for further 48h with 50ng/ml GM-CSF, 800U/ml IL-4, 10ng/ml of IL-1 $\beta$ , IL-6 (eBioscience), TNF- $\alpha$  (Biolegend) and 1 $\mu$ g/ml of PGE2 (BioVision) and LPS (Sigma-Aldrich). To assess the capacity of the DCs to produce ATRA the ALDEFLUOR kit (STEMCELL Technologies) was used to stain the cells for ALDH. Mixed lymphocyte reaction (MLR) was performed by co-culturing DCs with total Tregs for 5 days in the presence or absence of 1 $\mu$ M pan RAR inverse agonist (BMS493, Tocris Bioscience).

### RARA ChIP-qPCR

JASPAR <sup>21</sup> was used to scan the sequences of both *KLRB1* and *CCR9* gene loci ( $\pm$  5kb from gene body) for predicted binding sites of ATRA. 15x10<sup>6</sup> cells were cultured for 4h in X-Vivo, not supplemented with human serum, in the presence or absence of 2 $\mu$ M of ATRA (Sigma-Aldrich). RARA ChIP-qPCR was then performed. Briefly, cells were fixed with 16% Formaldehyde (ThermoFisher) and harvested in PBS containing protease inhibitors Aprotinin (MP biochemicals), Leupeptin (Bachem), both at 1 $\mu$ g/mL and 1mM Phenylmethylsulfonyl fluoride (PMSF, Sigma). Frozen cell pellets were lysed in SDS lysis buffer containing protease inhibitors and DNA sheared into lengths of 0.2-1Kbp using a Branson sonicator (11% power amplitude). Samples were resuspended in 2ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100 (Sigma), 1.2mM EDTA, 16.7mM Tris HCl pH8.1, 167mM NaCl in dH<sub>2</sub>O) containing protease inhibitors; 100 $\mu$ l of samples were taken as input DNA and stored for downstream qPCR. To reduce non-specific binding, samples were pre-cleared by incubation with salmon sperm DNA/protein A agarose-50% slurry (EMD Millipore). Chromatin immunoprecipitations (ChIPs) were carried out using 10 $\mu$ l/IP of RARA polyclonal antibody (Diagenode C15310155) and salmon sperm DNA/protein A agarose- 50% slurry. Slurry was then serially washed with Low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl pH 8.1, 150mM NaCl in dH<sub>2</sub>O) followed by High salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris HCl pH 8.1, 500mM NaCl in dH<sub>2</sub>O), LiCl wash buffer (0.25M LiCl (Sigma), 1% NP40 (ThermoFisher), 1% deoxycholate (ThermoFisher), 1mM EDTA, 10mM Tris HCl pH 8.1 in dH<sub>2</sub>O) and finally twice with TE buffer pH 8 before elution from the beads with elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>). Cross-linking was reversed with 5M NaCl/ml and heating to 65 $^{\circ}$ C for 4 hours; dissociated TFs and antibodies were digested by adding 0.5M EDTA, 1M Tris HCl (pH 6.5) and 20mg/ml Proteinase K (ThermoFisher) followed by overnight incubation at 55 $^{\circ}$ C. DNA was precipitated with isopropanol and re-dissolved in dH<sub>2</sub>O before purification with MinElute PCR Purification Kit (QIAGEN). Primers for *KLRB1* (forward: GTCCCCACCCACATACTT; reverse: AGAACAAATGAGCCTCCAGCA) and *CCR9* (forward: AGTTTCCCCTTATCCCAGCA; reverse: CAGTACCCGATAACAACACG) were designed and used for quantification by qPCR. Percentage of input, normalising the signal obtained from the ChIP against the input sample, was calculated.

### Cytokine secretion measurement

To evaluate the cytokine production of the different populations of Tregs after activation, 10<sup>6</sup> naïve, memory and CD161<sup>+</sup> Tregs were FACS sorted and activated for 3 days with 1:1 ratio of  $\alpha$ CD3 $\alpha$ CD28 Dynabeads and 100U/ml of IL-2. Cytokine protein levels were measured in the supernatant by either Th1/Th2/Th17 Cytometric Bead Array (CBA, BD) or LEGENDplex Human T helper Cytokine panel (Biolegend). To evaluate the effect of CD161 crosslinking on cytokine production 100,000 cells were stimulated for 3 days with ratio 1:1 microbeads coated with either  $\alpha$ CD3 $\alpha$ CD28IgG2 or  $\alpha$ CD3 $\alpha$ CD28 $\alpha$ CD161 (T Cell Activation/Expansion Kit, Miltenyi Biotec, as previously described <sup>7</sup>) and protein levels measured in the supernatant by LEGENDplex Human T helper Cytokine panel (Biolegend).

### IL-17 capture assay

$10^6$  CD161<sup>+</sup> Tregs were FACS sorted and then stimulated with PMA and Ionomycin for 5h. IL-17 producing CD161<sup>+</sup> Tregs were then labelled using the IL-17 Secretion Assay Kit (Miltenyi Biotec) according to manufacturer's instructions and then FACS sorted into IL-17 producing and non-producing cells. Cells were rested overnight and a standard suppression assay, including naïve and memory Tregs as controls, performed.

Simultaneous staining of surface and intracellular IL-17 was carried out on  $10^6$  CD4<sup>+</sup> T cells by incubating cells for an extra 2 hours in PMA/Io with supplementation of BD GolgiPlug, before washing and staining with surface IL-17 detection antibody (IL-17 Secretion Assay Kit, Miltenyi Biotec). Following extracellular staining and fixation/permeabilisation, intracellular staining was then performed with a second  $\alpha$ IL-17 antibody conjugated with a different fluorochrome. To validate the specificity of the double staining, staining with the antibody for intracellular IL-17 was also performed on cells without fixation/permeabilization step, but after surface staining with IL-17 Detection antibody; lack of double positive cells in the control sample demonstrated that the staining for intracellular IL-17 was specific.

### In vitro wound healing assay

250,000 Caco-2 cells were seeded into CytoSelect 24-Well Wound Healing Assay plates (Cell Biolabs) in DMEM supplemented with 10% FBS (both from GIBCO) and 1% MEM Non-essential Amino Acid (NEAA, Sigma-Aldrich) and grown until confluence for 7 days. Sorted memory and CD161<sup>+</sup> Tregs were stimulated for 3 days with 1:1 ratio of  $\alpha$ CD3 $\alpha$ CD28 Dynabeads and 100U/ml of IL-2 in complete RPMI (GIBCO) with 10% v/v HS and supernatant collected. Supernatant from the cells was then diluted 1:4 with fresh DMEM 10% FBS 1% NEAA and added to the Caco-2 cells, after insert removal. As negative control RPMI 10% HS diluted with 1:4 with DMEM 10% FBS 1% NEAA was used. Time-lapse image capture was recorded using Biostation CT (Nikon) over a period of 120h. Percentage of open wound was calculated using the "wound-healing" tool on NIS Elements Advanced Research Microscope Imaging Software (Nikon). For evaluation of the effect of cytokine blocking on wound healing either IgG1a (5 $\mu$ g/ml) together with IgG2b Isotype (5 $\mu$ g/ml),  $\alpha$ IL-17 (5 $\mu$ g/ml),  $\alpha$ IL-22 (5 $\mu$ g/ml),  $\alpha$ IL-4 (5 $\mu$ g/ml),  $\alpha$ IL-10R (10 $\mu$ g/ml),  $\alpha$ IFN $\gamma$  (5 $\mu$ g/ml),  $\alpha$ IL-17 (5 $\mu$ g/ml) together with  $\alpha$ IL-22 (5 $\mu$ g/ml) or  $\alpha$ IL-10R (10 $\mu$ g/ml) together with  $\alpha$ IL-4 (5 $\mu$ g/ml; all antibodies from R&D systems) were added to the wound healing assay; image capture was recorded at start and at 120h post culture.

### Patient biopsies

Six consecutive patients who attended the endoscopy department at King's College Hospital (London, UK) for a colonoscopy required in the context of their routine clinical care were consented for the study (REC 15/LO/1998). Three patients were recruited with an established diagnosis of Crohn's disease (CD), based on conventional clinical criteria, and three without any history of chronic inflammatory bowel disease (IBD), but with gastrointestinal symptoms or anaemia that required investigation; these latter patients were then considered as "healthy individuals" with no diagnosis of CD. Peripheral blood was collected in EDTA tubes on the day of attendance to endoscopy and prior to colonoscopy. During colonoscopy 2mm biopsies were collected from inflamed sites in patients with CD or randomly for those without a diagnosis of IBD. Endoscopic findings of inflammation and/ or normal macroscopic appearances were confirmed by histopathology.

PBMC were isolated following standard isolation protocol and colonic Lamina Propria Mononuclear cells (cLPMC) were isolated after digestion of the colonic tissue as previously described<sup>22</sup>. Both PBMC and cLPMC were then stained for CD45, CD3, CD8, CD4, CD25, CD127, CD45RA and CD161. Total CD4<sup>+</sup> T cells were initially defined as CD45<sup>+</sup>CD3<sup>+</sup>CD8-

CD4<sup>+</sup> and Tregs subpopulations were then selected following standard gating strategy, as previously described. Treg populations were then compared in matched peripheral blood to colonic biopsy samples of healthy individuals and those with IBD.

#### Severe xeno-graft-versus-host-disease (GvHD) model

NOD/scid/IL-2R $\gamma^{-/-}$  (NOD.cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ) mice (NSG; The Jackson Laboratory) were used between 8-10 weeks of age. Animals were bred and maintained in the Biological Services Unit of King's College London. All mice were kept under specific-pathogen-free conditions, and procedures were conducted in accordance with institutional guidelines (PPL70/7302) and the Home Office Animals Scientific Procedures Act (1986). Sorted Memory and CD161<sup>+</sup> Tregs were cultured in X-Vivo 15 with Gentamycin and PR (Lonza) supplemented with 5% human AB serum (HS, Biosera), 1,000 IU/ml of IL-2 (Proleukin, Novartis) and stimulated with Dynabeads Human T-Activator CD3/CD28 (GIBCO) at a 1:1 ratio for 2-3 rounds of expansion (10–14 days each). After the last round of expansion, beads were removed and the cells were rested for 2 days before injection into the mice. Human PBMCs (10x10<sup>6</sup>) depleted of CD25<sup>+</sup> cells were injected intravenously to induce xeno-GvHD with or without *in vitro*-expanded Memory or CD161<sup>+</sup> Tregs (5x10<sup>6</sup>) at a 2:1 PBMC:Treg ratio. Mice injected with PBS alone were used as negative control. Mice were monitored for symptoms of xeno-GvHD over time and experiment was carried on until either a clinical score greater than 6 or a weight loss greater than 15% from the initial weight was reached. Clinical GvHD score was calculated by applying a modified scoring system (adapted from <sup>23</sup>; individual mice received a score of 0 to 2 for each criteria.

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	<5%	>5% to <10%	>10%
Posture	Normal	Hunching only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

#### Lentiviral preparation

Lentiviruses were prepared by transfecting HEK293T cells. 10<sup>6</sup> cells were plated in DMEM 10% FCS and 1% PSG and transfected with 15 $\mu$ g of psPAX2 (Addgene), 5  $\mu$ g of pMD2.G (Addgene) and 20 $\mu$ g of either Control or Bach2-expressing pLVX-EF1 $\alpha$ -IRES-ZzGreen plasmids (Clontech). The transfection mix was then added to an equal volume of CaCl<sub>2</sub> 0.5M. The resulting solution was added dropwise to an equal volume of 2x HEPES buffered saline (HBS; Fluka, Sigma-Aldrich Company Ltd., Dorset, England) to precipitate the DNA. After an incubation of 30 minutes at room temperature, the prepared solution was added evenly, drop wise, to the cells and the plate was incubated at 37°C overnight. Two collections of supernatant containing viral particles were performed at day 3 and 4 post transfection and concentration was performed by addition of PEG-it Virus concentration solution (SBI, System Bioscience, Mountain View, U.S.A) following manufacturer's instructions. After allowing 48 hours for virus precipitation, the solution containing the viral particles was spun at 1500g for 30mins at 4°C to obtain a viral pellet that was resuspended in 300 $\mu$ l of cold medium and stored at -80°C until used.



### CD161<sup>+</sup> Treg transduction

Sorted CD161<sup>+</sup> Tregs were stimulated for 3 days with  $\alpha$ CD3/CD28 dynabeads (1:1 cell to bead ratio) in RPMI 10% HS supplemented with 100U/ml of IL-2. Cells were then counted, washed and resuspended at 10<sup>6</sup>/ml. 100,000 cells were seeded in a 96 well plate and left overnight at 37°C; the following day 1X Transdux and Max Enhancer (TransDux Max kit, SBI) were added to each well in the presence or absence of 10 $\mu$ l of Control or Bach2 lentiviruses. 3 days post addition of the virus  $\alpha$ CD3/CD28 dynabeads were magnetically removed, cells fed with fresh medium and left at 37°C for a further 3 days. At the end of the transduction protocol, cells were counted, washed and resuspended at 10<sup>6</sup>/ml and stimulated for another 3 days with  $\alpha$ CD3/CD28 dynabeads (1:1 cell to bead ratio) in RPMI 10% HS supplemented with 100U/ml of IL-2. Supernatant was then collected and cytokine production was measured.

### CD161<sup>+</sup> Treg transfection

Sorted CD161<sup>+</sup> Tregs were stimulated for 3 days with  $\alpha$ CD3/CD28 dynabeads (1:1 cell to bead ratio) in RPMI 10% HS supplemented with 100U/ml of IL-2. Cells were then counted, washed and resuspended at 2x10<sup>6</sup>/ml. 100,000 cells were then seeded in a 96 well plate and transfected with either All star Negative Control siRNA or HS\_RORC\_7 Flexitube siRNA (both from Qiagen) following manufacturer's instruction; untransfected cells were used as baseline control. Cells were then cultured for 3 days at 37°C; supernatant was then collected and cytokine production measured.

### ATAC-Seq

ATAC-seq was performed according to published protocol <sup>24</sup> with minor modification as described in <sup>25</sup>. Paired-end libraries (50 cycles) were prepared according to ATAC-seq protocol (see above) with three biological replicates (i.e. cells from 3 different individuals) for each library. The sequencing was performed using Illumina 2000. To obtain the open chromatin regions, reads were aligned to hg19 using Bowtie v2.2.9 <sup>26</sup> with parameters [--maxins 175 --no-discordant --no-mixed]. Properly paired and uniquely mapped alignments were extracted. The open chromatin regions were identified using Homer findPeaks tool <sup>27</sup> with parameters [-region -size 500 -minDist 50 -tbp 0]. All the open chromatin peaks were merged using bedtools <sup>28</sup> to obtain a set of all potential peaks. For each replicate, the differential set of open chromatin regions for each pair of samples (i.e. memory vs naïve, memory vs CD161, naïve v CD161) were extracted using homer getDifferentialPeaks with parameter -F 2 and the set of all potential peaks. For each pair of samples, the common peaks among three replicates were extracted using bedtools multiinter. All the common differential open chromatin regions were merged using bedtools and displayed using seqMINER <sup>29</sup> with three clusters. To identify motifs that are enriched in each cluster, we used homer findMotifsGenome on all known motifs with parameter [-size given].

### Data analysis and statistical tools

Statistical analysis was carried out using GraphPad Prism 7 (GraphPad software Inc., USA). All measures of variance were expressed as mean  $\pm$  standard error of the mean (SEM) and for data comparison t-test, one- or two-way RM ANOVA were used as indicated. Data were considered statistically significant with p<0.05, p<0.01, p<0.001 or p<0.0001 and represented in figures as indicated. ROC curves were constructed in GraphPad Prism v7 (GraphPad software Inc) using the dataset from Haberman *et al.*, 2014 (GSE57945) as a training set, taking inflamed versus uninfamed Crohn's disease and healthy donor tissues as defined by the investigators. Test datasets were sourced from Noble *et al.*, 2010 (GSE20881) and Häsler *et al.*, 2016 <sup>30</sup>, classifying samples as either normal versus any inflammation (Noble *et al.*, 2010) or non-inflamed versus inflamed as defined by the investigators (Häsler *et al.*, 2016).

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