Bone Marrow Transplantation (2018) 53:1568–1577
https://doi.org/10.1038/s41409-018-0205-6

ARTICLE

Foxp3 expression in induced T regulatory cells derived from human umbilical cord blood vs. adult peripheral blood

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Received: 26 January 2018 / Revised: 9 April 2018 / Accepted: 11 April 2018 / Published online: 9 May 2018

Abstract

Foxp3 is essential for T regulatory cell (Treg) function. Broad complex-Tramtrack-Bric-a-brac domain (BTB) and Cap'n'collar (CNC) homology 1, transcription factor 2 (BACH2) stabilizes Treg immune homeostasis in murine studies. However, little is known regarding what role, if any, BACH2 may have in Foxp3 regulation in human-induced Treg (iTreg). We examined Foxp3 expression and regulation comparing iTreg differentiated from umbilical cord blood (UCB) vs. adult blood (AB) naive $CD4^+$ T-cells. Foxp3 expression was higher in UCB vs. AB-derived iTreg, and was sustained during 21-day expansion in vitro. The number of $Foxp3^+$ iTreg generated from UCB vs. AB naive $CD4^+$ T-cells was higher in iTreg differentiation conditions. In addition, UCB iTreg were more potent in suppressing T-cell proliferation compared to AB iTreg. Naive UCB $CD4^+$ T-cells highly expressed BACH2 protein compared to AB. Putative transcriptional BACH2 binding sites were identified at the Foxp3 promoter, using BACH2 consensus sequence. Cross-linking chromatin immunoprecipitation (ChIP) showed that BACH2 binds to the Foxp3 proximal promoter in UCB iTreg, but not AB iTreg. BACH2 was transcriptionally active, as shRNA-mediated BACH2 knockdown resulted in reduction of Foxp3 gene transcription in UCB $CD4^+$ T-cells. In summary, BACH2 serves to stabilize robust Foxp3 expression in UCB $CD4^+$ T-cell-derived iTreg.

Introduction

Early clinical experience identifies third party allogeneic natural or thymus T regulatory cells (nTreg or tTreg) to be safe and effective in acute graft vs. host disease (aGVHD) prophylaxis [1]. However, low numbers of tTreg in human peripheral blood as well as the low proliferative potential of tTreg remain significant challenges for broader clinical applications [2, 3]. Inducible Treg (iTreg) can re-establish tolerance in settings where tTreg are decreased or defective [4, 5]. However, instability in the expression of Forkhead box P3 (Foxp3) transcription factor, which is essential for iTreg differentiation and function, poses a significant barrier to iTreg clinical application to date [6, 7].

Foxp3 is a member of the forkhead/winged-helix family of DNA binding transcription factors and is the master regulator for the development and maintenance of regulatory T cells. Deletion or mutation of the Foxp3 gene in either mice or humans results in severe autoimmune diseases, which are attributable to Treg deficiency [8, 9]. Activated protein 1 (AP-1) [10], Nuclear factor of activated T-cells 1 (NFAT1) [11], Nuclear factor-κB (NF-kB) [12], Small mothers against decapentaplegic 2 (smad2) [12], smad3 [11], and signal transducer and activator of transcription 5 (STAT5) [13] all have been identified as regulators of Foxp3 expression. Despite these advances in understanding these multiple Foxp3 transcription partners, the regulation of Foxp3 expression in human $CD4^+$ T-cells remains not fully elucidated.

Broad complex-Tramtrack-Bric-a-brac domain (BTB) and Cap'n'collar (CNC) homology 1, basic leucine zipper transcription factor 2 (BACH2) is a bZip protein that acts as a transcriptional repressor or activator. Murine BACH2 deletion studies reveal that BACH2 promotes in vitro TGF-β-induced $Foxp3^+$ $CD4^+$ iTreg generation by suppressing differentiation into effector T cells [14, 15]. However, the role of BACH2 in human TGF-β-induced $Foxp3^+$ $CD4^+$ iTreg differentiation has not been fully examined.

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41409-018-0205-6) contains supplementary material, which is available to authorized users.

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To address this gap, studies summarized herein were conducted to determine whether BACH2 has a role in regulating Foxp3 expression in iTreg derived from human naive $CD4^+$ T-cells. We observed that naive umbilical cord blood (UCB) $CD4^+$ T-cells highly express BACH2 mRNA and protein expression when compared to naive AB $CD4^+$ T-cells. Knockdown of UCB naive $CD4^+$ T-cells with BACH2 shRNA resulted in significantly decreased Foxp3 mRNA and protein expression. Further, BACH2 shRNA-treated UCB naive $CD4^+$ T-cells exhibited reduced expression of CTLA-4 known to be associated with Foxp3 expression [16]. Putative transcriptional BACH2 binding sites were identified at the human Foxp3 promoter using BACH2 consensus sequence. Cross-linking chromatin immunoprecipitation (ChIP) demonstrated that BACH2 binds to the Foxp3 promoter in UCB $Foxp3^+$ $CD4^+$ iTreg, but not in AB $Foxp3^+$ $CD4^+$ iTreg. BACH2 was noted to be transcriptionally active, as significantly decreased Foxp3 gene transcription was measured in the human Foxp3 luciferase reporter (Luc) Jurkat recombinant cell line by luciferase quantification after transduction with BACH2 shRNA vs. scrambled shRNA.

Taken together, these observations of higher BACH2 expression in human UCB naive $CD4^+$ T-cells and the regulation of Foxp3 by BACH2 in UCB naive $CD4^+$ T-cell-derived iTreg provides further clarification for the underlying molecular mechanisms contributing to the reported immune tolerance of UCB graft T-cells. UCB graft T-cells, in contrast to adult donor mobilized peripheral blood and bone marrow grafts, allows successful allogeneic transplantation across ≥2 loci HLA disparity without required T depletion, eliciting low GVHD incidence and severity while maintaining strong graft vs. malignancy effects [17–20]. The regulation of Foxp3 by BACH2 may contribute to the observed robust and sustained Foxp3 expression in UCB iTreg in studies summarized herein, and may contribute to the observed enhanced number and suppressive function of these UCB naive $CD4^+$ T-cell-derived iTreg [21, 22].

Materials and methods

Naive $CD4^+$ T-cell isolation and iTreg generation, and expansion

Naive $CD4^+$ T-cells were isolated by Miltenyi auto MACS (Auburn, CA, USA) with sequential $CD45RO^{neg}$ and $CD4^+$ selection per manufacturer's instructions. The isolated naive $CD4^+$ T-cells were activated in vitro with CD2/3/28 monoclonal antibody-coated dynabeads (beads to cell ratio, 1:2; Miltenyi) with added IL-2 (100 U/ml, Miltenyi) and with 5 ng/ml transforming growth factor-β (TGF-β; Peprotech, Rocky Hill, NJ) in 48-well culture plates at 5×10^5 cells per well. The culture medium in all experiments consisted of X-VIVO 15 (Lonza, Walkersville, MD) with 10% heat-inactivated human serum albumin (Gemini Bioproduct, Sacramento, CA).

Western blot

Western blotting for UCB and AB naive $CD4^+$ T-cells and differentiated iTreg were performed following standard techniques [23].

In vitro suppression assay

In vitro suppression assays were performed as previously described [24]. $CD25^{high}$ FACS-sorted iTregs were used for suppression assay. Responder naive $CD4^+$ T-cells and Mitomycin C-treated T-cell-depleted PBMC were prepared from a healthy donor. Carboxyfluorescein succinimidyl ester (CFSE)-labeled naive $CD4^+$ T-cells were plated at a 1:1 ratio with Mitomycin C-treated T-depleted PBMC and varying concentrations of day 4 AB and UCB iTreg. Soluble anti-CD3 (2 μg/ml) (clone HIT3a, BD Bioscience) mAb was added. CFSE dilution was examined by FACS.

FACS analysis

Stimulated cells were harvested and fixed after surface staining and permeabilized with Fixation/Permeabilization kit according to the manufacture's protocol (Miltenyi). Appropriate Ab were used for surface staining as described in the Supplementary Information. FACS percentages were multiplied by enumerated viable nucleated cells at each cell harvest day to calculate absolute numbers of specified iTreg populations during TGF-β induction and in vitro expansion.

BACH2 shRNA knockdown in day 0 naive $CD4^+$ T-cells and day 4 iTreg

BACH2 knockdown experiments were performed by spinoculation transduction method as described [25]. Technical details are described in the Supplementary Information files.

Identification of putative transcriptional BACH2 binding sites

The 2.5 Kb sequence centered on the −1 exon of human Foxp3 was downloaded from NCBI at http://www.ncbi.nlm.nih.gov/sites/entrez. The sequence was analyzed for known putative transcription factor binding sites using Blast (Basic Local Alignment Search Tool) at https://blast.ncbi.

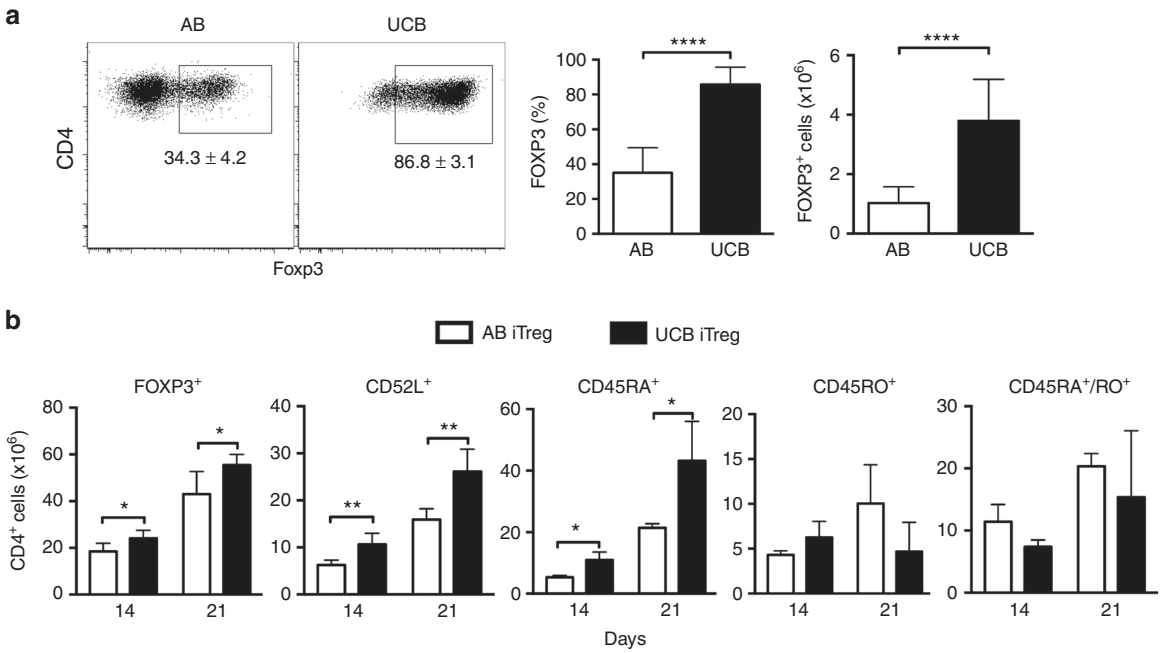

Fig. 1 TGF-β-induced Foxp3$^+$ regulatory T cells from AB and UCB naive CD4$^+$ T-cells in vitro. **a** Foxp3$^+$ iTregs were generated from AB and UCB naive CD4$^+$ T-cells. Foxp3 expression and absolute number of Foxp3$^+$ CD4$^+$ T-cells was measured day 4 induction (AB, $n = 9$; UCB, $n = 12$). Data are from 4 to 6 independent experiments. **b** Absolute number of AB and UCB Foxp3$^+$, CD62L$^+$, CD45RA$^+$, and CD45RO$^+$ iTreg cells were calculated at day 14 and 21 in the presence of IL-2. Data are from two individual experiments ($n = 5$). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, unpaired Student t-test

nlm.nih.gov/Blast.cgi. The Foxp3 proximal promoter was examined for putative NFAT1 [NGGAAAHH] and AP-1 [TGAYTMMK] binding sites, with particular attention to regions adjacent to the NFAT1 binding sites that may be partial AP-1 sites not scored by the algorithm. These regions were aligned manually and examined for similarity to the known consensus sequence for BACH2 [TGASTCAY]. Only those sequences with 2 or fewer mismatches exceeding 75% similarity to the BACH2 consensus site were considered further.

Cross-linking ChIP assay

ChIP assays were performed according to the cell signaling protocol (https://www.cellsignal.com/contents/resources/protocols/resources-protocols), as previously described [26]. UCB and AB naive CD4$^+$ T-cells were stimulated with TGF-β in conditions summarized above. After 4 days of stimulation cells were examined by ChIP. Between 1.0×10^7 and 1.5×10^7 cells were treated with 1% formaldehyde to cross-link protein to DNA. Protein-DNA lysates were mixed with 1:50 ratio of rabbit monoclonal BACH2 antibody (Cell Signaling, Boston, MA) then immune-precipitated with protein A/G Agarose Beads (D3T3G, Cell Signaling). After digestion of proteins by Proteinase K treatment, DNA was purified by DNA isolation spin column (Cell Signaling) and examined for the presence of the Foxp3 promoter by RT-PCR.

Luciferase assay

The human Foxp3 luciferase reporter (Luc) Jurkat recombinant cell line was used (BPS Bioscience, San Diego, CA). Overall, 1×10^6 cells were transduced by scrambled or BACH2 shRNA. After 16 h, cells were stimulated with CD2/3/28 monoclonal antibody-coated dynabeads with IL-2 (100 U/ml) with 5 ng/ml TGF-β in 96-well culture plates at 1×10^4 cells per well. Luciferase activities were measured after 24 h using the ONE-step^{TM} luciferase assay system kit (BPS Bioscience).

Statistical analysis

Statistical comparative analyses were performed using the Student's t-test (Prism 6 software-GraphPad, La Jolla, CA). Data are presented as the mean ± standard deviation, SD. A p value of < 0.05 was considered significant.

Results

Absolute number and Foxp3$^+$ expression in iTreg derived from UCB vs. AB naive CD4$^+$ T cells

Initial experiments revealed that TGF-β-induced Foxp3 expression was 2.5-fold higher in iTreg derived from UCB naive CD4$^+$ T-cells as compared to AB naive CD4$^+$ T-cells

(86.8 ± 3.1 vs. 34.3 ± 4.2, $n = 12$; $p < 0.001$) (Fig. 1a). In addition, the absolute number of Foxp3$^+$ iTreg from UCB vs. AB naive CD4$^+$ T-cells at day 4 TGF-β induction was fourfold higher (3.8×10^6 vs. 1.0×10^6) (Fig. 1a). Expansion of Foxp3$^+$ iTreg cells was notable for significantly higher absolute numbers of Foxp3$^+$ UCB iTreg compared to AB iTreg at day 14 and 21 (Fig. 1b). Absolute number of UCB iTreg expressing CD62L$^+$ and CD45RA$^+$ was significantly higher during 21 day in vitro expansion (Fig. 1b; Supplementary Figure S2).

Phenotypic and functional characterization of UCB and AB iTreg

Further studies were performed to determine whether UCB vs. AB iTreg may exhibit similar or differing suppressive function and/or expression of surface molecules associated with T-cell activation or suppression. Expressions of CD25 and CTLA-4 were significantly higher on UCB Foxp3$^+$ iTreg compared to AB Foxp3$^+$ iTreg (Fig. 2a). Next, surface molecules associated with suppressive functions of Treg were examined, including OX-40 and CD39; higher expression was noted on UCB Foxp3$^+$ iTreg vs. AB. In contrast, the surface expression of checkpoint inhibitors and other surface molecules associated with T-cell activation or suppression including: PD-1, ICOS, LAG-3, TIM-3, NRP-1, and TIGIT were similar or slightly diminished on UCB Foxp3$^+$ iTreg compared to AB (Fig. 2b). In addition, results of suppression assay revealed that AB iTreg (CD25^{high} FACS-sorted iTreg cells) exerted significantly lower suppressive function compared with UCB iTreg, with only ~60% suppression (60.6 ± 6.7) when mixed with responder cells at a 1:1 ratio, and dropping to ~30% suppression (29.9 ± 3.0) at 10:1 ratio (Fig. 2c). In contrast, UCB iTreg exerted significantly enhanced suppressive function with ~90% inhibition (89.9 ± 1.8) at a ratio of 1:1, and ~70% inhibition (70.6 ± 1.9) at a ratio of 10:1 (Fig. 2c). Furthermore, UCB iTregs showed enhance suppressive function in a limiting dilution assay utilizing equivalent numbers of UCB and AB Foxp3$^+$ cells (Supplementary Figure S3). In summary, day 4 iTreg derived from UCB naive CD4$^+$ T-cells demonstrated higher CTLA-4, CD25, OX-40, CD39 expression and suppressive function vs. AB with equivalent expression of checkpoint inhibitors and other surface molecules associated with T-cell activation or suppression.

Expression of BACH2 and NFAT1 in UCB naive CD4$^+$ and Foxp3$^+$ CD4$^+$ T-cells

Given previous work pointing to a role for BACH2 in murine Treg function [14], and the known association of NFAT1 with Foxp3 function in human Treg [27], further studies were conducted to examine the mRNA and protein levels of these transcription factors in UCB and AB naive CD4$^+$ T-cells and iTreg. Expression of BACH2 mRNA was noted to be significantly higher in UCB vs. AB naive CD4$^+$ T-cells at baseline (day 0) and similar after 4 days (96 h) induction in iTreg differentiation (Supplementary Figure S4). NFAT mRNA expression was slightly lower both in UCB naive CD4$^+$ at baseline and day 4 iTreg compared to AB (Supplementary Figure S4). Similar to mRNA measurements, protein levels of BACH2 in UCB naive CD4$^+$ T-cells were also noted to be significantly higher than AB naive CD4$^+$ T-cells at baseline (day 0) and NFAT1 protein expression was lower in UCB naive CD4$^+$ T-cells compared to AB (Fig. 3a). In day 4 iTreg (with or without TGF-β added), NFAT1 expression was similar in AB and UCB. However, significantly higher BACH2 protein levels were noted in day 4 iTreg derived from UCB naive CD4$^+$ T-cells vs. AB (Fig. 3b) regardless of addition of TGF-β in iTreg differentiation conditions. Taken together, BACH2 mRNA and protein expressions are significantly higher in UCB vs. AB naive CD4$^+$ T-cells at baseline (day 0) and in day 4 iTreg. Further, TGF-β added to iTreg culture conditions has no effect on BACH2 expression.

Knockdown of BACH2 in UCB CD4$^+$ naive T-cells results in decreased Foxp3 and CTLA-4 expression

To address the potential role for BACH2 transcriptional regulation in UCB naive CD4$^+$ T-cells, BACH2 shRNA knockdown was performed. UCB naive CD4$^+$ T-cells were lentiviral transduced with GFP-tagged scrambled or BACH2 shRNA virus and GFP$^+$ CD4 T-cells were sorted for analysis (Fig. 4a; Supplementary Figure S5a, b). BACH2 shRNA transduction resulted in ~60% reduction in BACH2 expression in UCB naive CD4$^+$ T-cells compared to scrambled shRNA (Fig. 4b) and western blot results confirmed FACS analysis (Supplementary Figure S5c). Expression of Foxp3 in day 4 iTreg differentiated from UCB naive CD4$^+$ T-cells transduced with BACH2 shRNA dropped significantly, e.g., ~40% (scrambled; 81.7 ± 3.4 % vs. BACH2; 45.7 ± 3.6 %, $p < 0.001$) (Fig. 4c). As a consequence of BACH2 shRNA knockdown, levels of CTLA-4 protein expression in BACH2 transduced UCB CD4$^+$ T-cells was also noted to significantly decrease by twofold (Fig. 4c). In addition, mRNA levels of Foxp3 and CTLA-4 dropped significantly (Supplementary Figure S5d). In addition, suppressive activity was diminished after BACH2 knockdown (Fig. 4d). As shown in Fig. 4e, Foxp3 expression progressively increased in scrambled shRNA transduced UCB naive CD4$^+$ T-cells, whereas Foxp3 expression in BACH2 shRNA transduced UCB naive CD4$^+$ T-cells remained significantly lower over a 3-day time period (Fig. 4e). Interestingly, we did not see BACH2 effects in AB-derived iTregs (Supplementary Figure S6). In

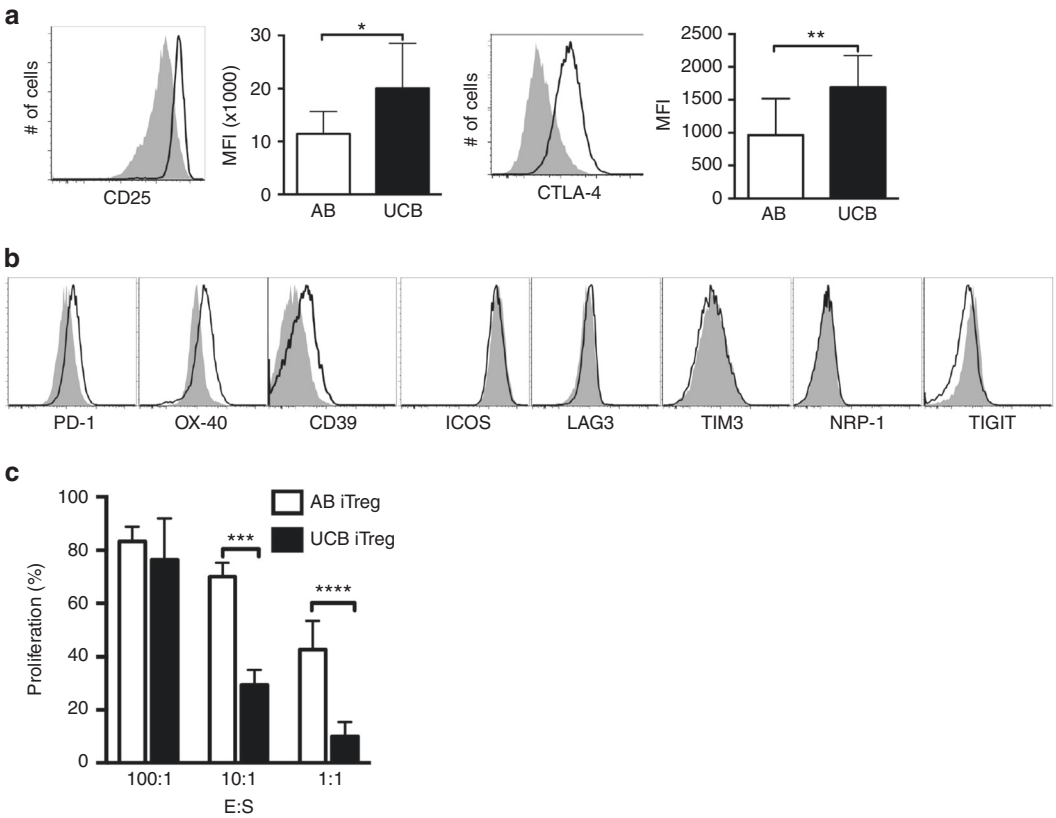

Fig. 2 Analysis of phenotype and suppressive capacity of AB and UCB Foxp3$^+$ iTreg. **a** CD25 and CTLA-4 expression were measured from AB (shaded) and UCB (open) Foxp3$^+$ iTregs (AB, $n = 12$; UCB, $n = 15$). **b** Surface inhibitory molecules on Foxp3$^+$ iTreg were analyzed on AB (shaded) and UCB (open) iTregs. **c** Suppression assay of iTregs. Suppressive function was assessed as described in Methods. Data are from two independent experiments ($n = 4$–5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, unpaired Student *t*-test

summary, knockdown of BACH2 using shRNA in human UCB naive CD4$^+$ T-cells significantly reduces Foxp3 and CTLA-4 expression in UCB iTreg.

Identification of putative transcriptional BACH2 binding sites

On the basis of the results of BACH2 shRNA knockdown in UCB naive CD4$^+$ T-cells demonstrating significant reduction in Foxp3 expression, additional studies were performed to determine whether BACH2 could potentially bind to the proximal region of the exon −1 promoter region of the human Foxp3 promoter region. BACH2, AP-1, and NFAT1 consensus DNA binding sequences were compared with the promoter region of human Foxp3 [27]. Sequence analysis of the human Foxp3 promoter region confirmed known AP-1 binding sites, which were >75% similar to the BACH2 consensus binding sites (Fig. 5a). The proximal Foxp3 −1 exon region also contained putative NFAT1 binding sites (Fig. 5a). Overall, this analysis identified sequences containing BACH2 consensus DNA binding sites that were located within the promoter region of human Foxp3.

Cross-linking ChIP assay

On the basis of the observed higher expression of BACH2 in UCB naive CD4$^+$ T-cells vs. AB, higher Foxp3 expression in day 4 UCB iTreg, and the presence of BACH2 consensus binding sites in the human Foxp3 promoter, studies were next conducted to determine whether BACH2 binds to the human Foxp3 promoter in UCB CD4$^+$ iTreg. AB and UCB naive CD4$^+$ T-cells were differentiated into iTreg as described above. iTreg were collected on day 4 and processed for ChIP assays. As shown in Fig. 5b, binding of BACH2 to the Foxp3 promoter region was significantly higher in UCB Foxp3$^+$ iTreg vs. AB. These results were confirmed using standard gel electrophoresis (Fig. 5c).

Luciferase assay at the Foxp3 promoter region

As BACH2 binding was confirmed at the human Foxp3 promoter in UCB iTreg by cross-linked ChIP with formaldehyde DNA-protein linkage, studies were conducted to determine whether BACH2 is transcriptionally active at the Foxp3 promoter. Luciferase activity was measured using

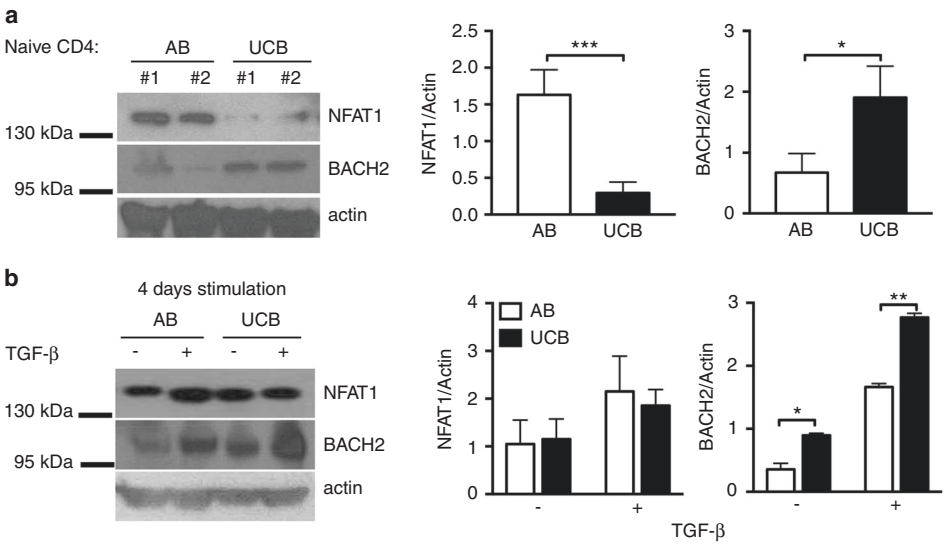

Fig. 3 BACH2 and NFAT1 expression in AB and UCB naive and TGF-β-induced Foxp3$^+$ iTreg. **a** BACH2 and NFAT1 protein expression in AB and UCB naive CD4$^+$ T-cells was measured by western blot. **b** BACH2 and NFAT1 protein expression in day 4 induced AB and UCB Foxp3$^+$ iTreg was measured by western blot. The image intensities for western blots were normalized to β-actin. Images are representative of multiple western blots. Data are from multiple experiments ($n =$ 4–8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired Student *t*-test

the human Foxp3 luciferase reporter (Luc) Jurkat recombinant cell line (BPS Bioscience, San Diego, CA). Jurkat cells expressed BACH2 protein (Supplementary Figure S7a). BACH2 expression was significantly reduced after transduction with BACH2 shRNA but not in scrambled shRNA (Fig. 6a; Supplementary Figure S7b). As shown in Fig. 6b, TCR stimulation increases luciferase expression in these Foxp3 reporter cells. BACH2 shRNA treatment significantly diminished luciferase expression compared to scrambled shRNA-treated cells (Fig. 6b).

Discussion

On the basis of comparative FACS analyses, assessment of iTreg suppressive function, ChIP of the human Foxp3 promoter, shRNA knockdown, and luciferase measurements, the findings outlined in this report strongly support BACH2 as a critical regulator of Foxp3 expression in iTreg differentiated from UCB naive CD4$^+$ T-cells. Increased BACH2 expression in UCB naive CD4$^+$ T-cells may serve as a strong promoter of Foxp3 expression in UCB iTreg. During iTreg generation in vitro using standard differentiation conditions including TGFβ, we noted that low levels of BACH2 in AB naive CD4$^+$ T-cells were insufficient to upregulate Foxp3 and to stably sustain its expression during 21-day iTreg expansion.

As robust and sustained Foxp3 expression is required for Treg lineage maintenance and suppressive function [28], instability of Foxp3 in iTreg compared with tTreg [7] has to date severely limited the clinical development of iTreg for GVHD prophylaxis. It has been demonstrated that instability of Foxp3 expression in iTreg limits the utility of adoptively transferred iTreg as a source of cellular therapy for the abrogation of GVHD [6]. However, the low frequency of tTreg in human peripheral blood provides a strong rationale for further study of the generation and expansion of large numbers of stable Foxp3-expressing iTreg for adoptive therapy use as GVHD prophylaxis, as high numbers of Treg are needed to facilitate tolerance after allogeneic stem cell transplantation. Our studies demonstrated the ability to generate ~$1–4 \times 10^9$ iTreg with a 21-day expansion protocol from individual UCB units, which would expectedly meet the needs of human clinical application, despite the noted variance in iTreg cell yields between different UCB units (data not shown).

We observed significantly upregulated expression of CD25 and CTLA-4, two well-described molecules that contribute to Treg function, on UCB Foxp3$^+$ iTreg [29, 30]. Expression of other surface inhibitory and exhaustion molecules including PD-1 [31], ICOS [32], LAG-3 [33], TIM-3 [34], NRP-1 [35, 36], and TIGIT [37] did not differ between AB and UCB Foxp3$^+$ iTreg. However, enhanced expression of OX-40 [38] and CD39 [39] on UCB iTreg, both of which may also contribute to the enhanced suppressive function of UCB iTreg was observed, and warrants further investigation. Whether UCB iTreg maintain robust Foxp3 expression, appropriate trafficking to inflammatory sites, and suppressive function in inflammatory conditions in vivo are also important factors to consider in future studies. Of relevance to future in vivo studies, we observed significantly higher sustained surface expression of CD62L required for entry into lymph nodes [40], on UCB iTreg during 21 day in vitro expansion. Although tTreg have been shown in murine models and early human trials of GVHD prophylaxis [1] to exert favorable suppressive effects, caution must be taken to draw firm conclusions regarding efficacy of iTreg cell therapy without further understanding of the in vivo stability of Foxp3 expression, as contextual cues may influence what transcriptional regulatory proteins

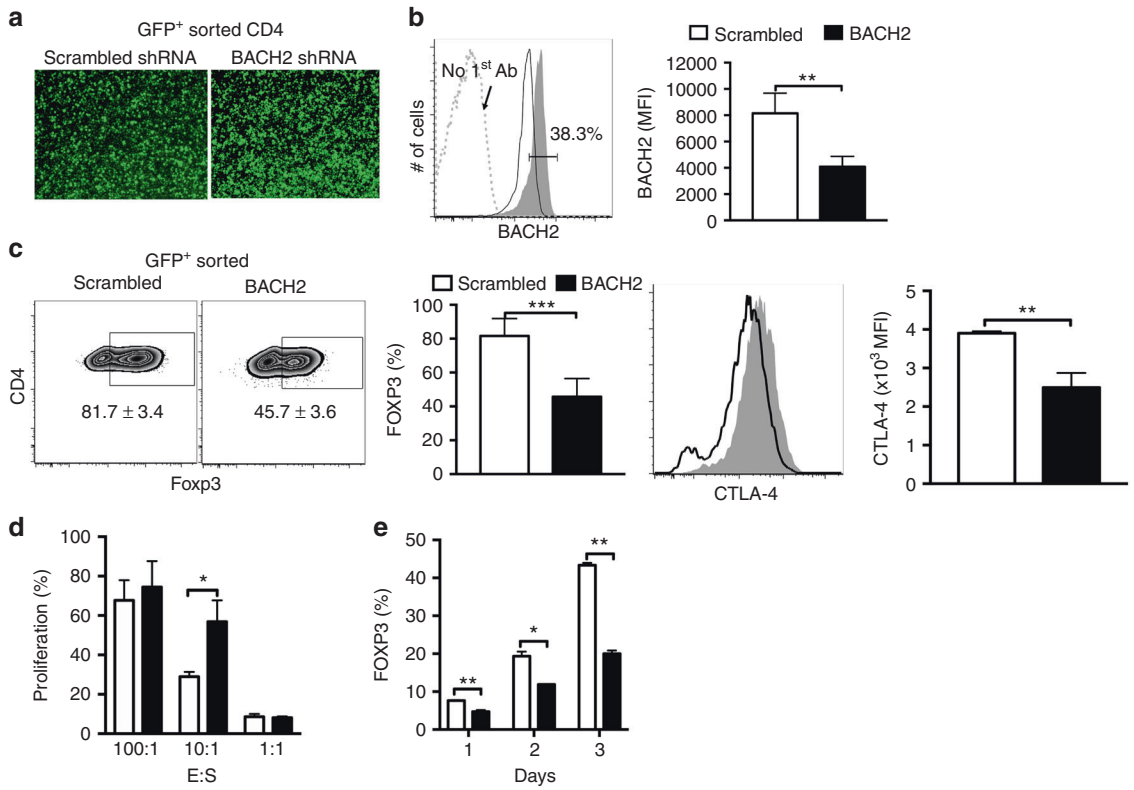

Fig. 4 BACH2 knockdown results in significantly reduced Foxp3 and CTLA-4 expression in UCB iTreg. **a** GFP fluorescence of GFP$^+$ lentiviral transduced CD4 T-cells after FACS purification. **b** BACH2 expression in day 4 GFP$^+$ UCB CD4 was measured after scrambled (shaded) and BACH2 (open) shRNA treatment. Data presented are from three independent experiments ($n = 7$–8). **c** Expression of Foxp3 in day 4 GFP$^+$ UCB iTreg was analyzed after transduction with scrambled (shaded) and BACH2 (open) shRNA. CTLA-4 measured by FACS analysis. Data are represented from three different experiments ($n = 7$–8). **d** Suppression assay BACH2 knockdown UCB iTregs. CFSE dilution measured at 3 days after stimulation. **e** Kinetic of Foxp3 expression in UCB iTreg treated with either scrambled or BACH2 shRNA transduced UCB naive CD4$^+$ T-cells. Data are from two independent experiments ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired Student t-test

may be recruited to the Foxp3 complex [41], thereby contributing to iTreg plasticity in vivo.

BACH2 was first characterized as an important transcriptional regulator to control B-cell development [42]. Further, inhibition of BACH2 was shown to suppress IL-2 expression in UCB CD4$^+$CD45RA$^+$ T-cells [43]. Recently, a human disorder termed 'BACH2-related immunodeficiency and autoimmunity' (BRIDA), caused by heterozygous mutations in BACH2, has been described [44]. In addition, recent murine gene deletion studies revealed that BACH2 is a critical transcription factor for TGF-β-induced Foxp3$^+$ regulatory T-cell function [14, 15]. Roychoudhuri et al. [14] found that BACH2 regulates immune responses in murine T-cell differentiation by repressing genes associated with effector cell differentiation. Studies summarized herein identify that BACH2 regulates human UCB iTreg development via direct transcriptional activity at the Foxp3 promoter. The factors regulating BACH2 expression in developing and mature CD4$^+$ T-cells, as well as a better understanding of the molecular mechanisms that establish a Treg-specific transcriptional program, remain only partially defined. Also, whether UCB naive CD4$^+$ T-cells may have a distinctive gene profile [45] underlying the observed higher BACH2 expression compared to adult naive CD4$^+$ T-cells remains to be determined. Nevertheless, our data support the view that BACH2 is a critical transcription factor that interacts with and regulates Foxp3, thereby governing iTreg vs. effector T-cell differentiation and function in vitro.

Foxp3 associates with a large number of transcription co-factors [27] and is modified by histone acetylation [46] to maintain Treg lineage and suppressive function. Our findings reveal a significant loss of Foxp3 expression in BACH2 shRNA transduced UCB naive CD4$^+$ T-cells. To determine further effects of reduced Foxp3 expression in stimulated UCB CD4$^+$ T-cells, expression levels of CTLA-4 were examined in BACH2 shRNA transduced UCB CD4$^+$ T-cells. Our data showed a significant loss of CTLA-4 expression under conditions of reduced Foxp3 expression. These findings are consistent with prior work supporting the concept that CTLA-4 is associated with Treg transcriptional signature [47]. It has been reported that Foxp3 regulates

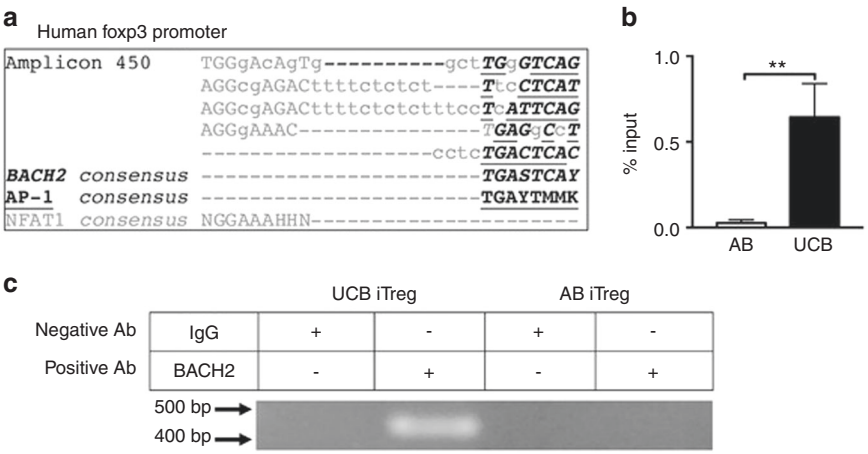

Fig. 5 BACH2 binds to the Foxp3 promoter in TGF-β-induced Foxp3$^+$ UCB iTregs. **a** A representative human Foxp3 sequence with the BACH2 binding site. Location of putative BACH2 DNA binding sites in the Foxp3 promoter with similarity to the AP-1 DNA binding consensus sequence, and their relative position to NFAT1 DNA binding sites and the 450 bp amplicon. BACH2 DNA binding sites within the Foxp3 promoter aligned with the AP-1 DNA consensus sequence (underlined), BACH2 DNA consensus sequence (italic), and distance from NFAT1 DNA binding sites (gray). **b** ChIP assays for Foxp3 in TGF-β-induced AB and UCB Foxp3$^+$ T cells. Data are from 3 to 5 individual samples. Each sample was analyzed in duplicate. Background was determined using the IgG control group. $^{**}p < 0.01$, unpaired Student *t*-test. **c** Gel image of PCR amplification of the Foxp3 promoter from BACH2 and IgG control samples

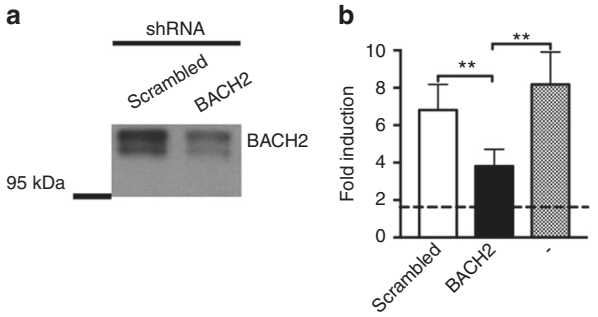

Fig. 6 BACH2 is transcriptionally active at the Foxp3 promoter. Foxp3 promoter activity measured by luciferase assay. **a** BACH2 expression after scrambled and BACH2 shRNA treatment of the human Foxp3 reporter cell line. **b** Luciferase activity of lentivirus transduced Foxp3 reporter (Luc) Jurkat recombinant cells measured after 24–36 h. Fold induction of luminescence is calculated based on media condition (dotted line). Data are from 8 to 10 individually tested samples. $^{**}p < 0.01$, unpaired Student *t*-test

CTLA-4 via interaction with NFAT1 [27]. Our data demonstrating a role of BACH2 in Foxp3 expression does not exclude the possibility that BACH2 may also indirectly affect Foxp3 expression through other interacting transcription factors that also participate in T-cell fate determination.

As the Foxp3 promoter is activated by IL-2 through STAT5 [48], it would appear that UCB naive CD4$^+$ T-cells are primed for the expression of the Foxp3 gene, as previous studies have shown that STAT5 binds to deacetylated histones 4 for trans-activation [49]. As UCB naive CD4$^+$ T-cells have reduced NFAT1 protein expression [50] and hypo-acetylated histone 3 and 4 at the Foxp3 promoter [51], a reasonable hypothesis may be that these cells have muted Th1 differentiation response and sustained Foxp3 gene expression under primary stimulation conditions (i.e., CD3 and CD28 co-stimulation) with high doses of IL-2 [52].

BACH2 robust expression in UCB naive CD4$^+$ T-cells may comprise one mechanism underlying the altered response of UCB naive CD4$^+$ T-cells in in vitro iTreg expansion culture compared with AB naive CD4$^+$ T-cells. These studies have elucidated a previously uncharacterized role for BACH2 in early human adaptive immune responses. Taken together, differing BACH2 regulation comparing UCB vs. AB naive CD4$^+$ T-cells has important implications for further elucidation of molecular mechanisms underlying neonatal tolerance and normal human T-cell repertoire development. These studies thus provide a basis for further in-depth analyses of BACH2 in UCB naive CD4$^+$ T-cell differentiation, as well as rationale for consideration of clinical development of UCB iTreg as potential adoptive cellular therapy for GVHD prophylaxis.

Acknowledgements We thank Daniel Zwick for editorial assistance and Dr. Bruce Torbett for valuable suggestions. This work was supported by the Abraham J. & Phyllis Katz Foundation, Atlanta, GA.

Author contributions J-sD, FZ, and MJL conducted experiments, literature search, developed study design, analyzed data, and wrote the manuscript. AYH, WJvHF, and MF contributed to the study design, data review and interpretation, as well as writing and approval of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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