The Journal of Immunology

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J Immunol 2012; 189:5985-5994; Prepublished online 16 November 2012;
doi: 10.4049/jimmunol.1201090
http://www.jimmunol.org/content/189/12/5985

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Converse 4-1BB and CD40 Ligand Expression Pattern Delineates Activated Regulatory T Cells (Treg) and Conventional T Cells Enabling Direct Isolation of Alloantigen-Reactive Natural Foxp3$^+$ Treg

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Natural regulatory T cells (nTreg) play a central role in the induction and maintenance of immunological tolerance. Experimental transplant models and recent clinical trials demonstrate that nTreg can control alloreactivity. To upgrade Treg-based cell therapies to a selective suppression of undesired immune reactions, only the transfer of Ag-specific nTreg represents the appropriate therapeutic option. However, Ag-specific nTreg are present at extremely low frequencies in the periphery, and so far appropriate surface markers for their precise identification are missing. In this study, we demonstrate that activated nTreg and activated conventional T cells differ in their 4-1BB and CD40 ligand (CD40L) expression signatures, allowing a clear dissection from each other. Based on the expression of 4-1BB and absence of CD40L expression, human alloantigen-reactive Foxp3$^+$ nTreg can be directly isolated from MLR cultures with high purity. Alloantigen-reactive 4-1BB$^+$CD40L$^-$ nTreg were characterized by a completely demethylated Treg-specific demethylated region and showed alloantigen-specific suppressive properties superior to polyclonal Treg. Importantly, isolated 4-1BB$^+$CD40L$^-$ nTreg maintain the nTreg phenotype and alloantigen-reactivity after in vitro expansion. Our results offer the possibility to simultaneously analyze Ag-specific nTreg and conventional T cells, and to establish cellular therapies with Ag-specific nTreg aiming at a specific inhibition of unwanted immunity. ***The Journal of Immunology*****, 2012, 189: 5985–5994.**

Natural CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) have been shown to control autoimmune responses, as well as inflammation and tissue destruction, by their ability to suppress the function of many cell types of the immune system (1). Consequently, the adoptive transfer of Treg is regarded as a promising treatment option for undesired immune reactions such as autoimmunity, graft rejection, or graft-versus-host disease (GvHD) (2). Successful prevention or control of alloantigen-specific immunity by Treg has been demonstrated in a variety of animal models (3), and pioneering studies in humans have been initiated (4, 5).

Because polyclonal Treg may also limit responses to foreign or tumor Ags (6–8), an adoptive transfer of large numbers of polyclonal Treg for the treatment of aberrant alloimmune responses could be detrimental. This risk could be circumvented with the use of Ag-specific Treg. Because the efficiency of Treg-based immunosuppression critically depends on Ag specificity (9–11), drastically lower numbers of Ag-specific Treg would be needed. However, to establish safe and efficient cellular therapies with Ag-specific Treg, it will be a prerequisite to use methods that enable an unequivocal and direct identification and isolation of activated Treg versus conventional CD4$^+$ T cells. Particularly after in vitro expansion cultures, which pose the risk for outgrowing conventional T cells (Tcon) or nonspecific Treg, the clear identification of Ag-specific Treg is critical. Recently, strategies for the isolation of activated CD4$^+$CD25$^+$CD127^{low} Treg after in vitro stimulation have been introduced involving the use of the surface markers CD121a/b and latency-associated peptide (12), and CD69 in combination with CD71 (9). However, in the former study, only polyclonal CD3/CD28 stimulations were evaluated, and in the latter study, the proposed activation marker profile does not discriminate between Treg and Tcon. A prepurification of polyclonal Treg would be essential to isolate Ag-specific Treg. In addition, the delineation of naturally occurring Treg (nTreg) and induced Treg (iTreg) characterized by lower functional stability (13) is currently not possible.

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Received for publication April 12, 2012. Accepted for publication October 9, 2012.

This work was supported by the ONE Study (European Union Seventh Framework Programme Grant 260687); the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich (SFB) 650, SFB TR36, and SFB 633; the Bundesministerium für Bildung und Forschung STThera (FZK: 01GU0802); and the European Sixth Framework Programme (LSH-2002-2.3.0-5 and LSH-2003-1.2.4-6).

A.S., M.F., and S.K. designed and performed research, analyzed data, and wrote the paper; J.K., C.R., M.F., J.D., P.W., H.D., B.M., and C.L. performed research and analyzed data; N.M. and A.T. designed research, analyzed data, and wrote the paper.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CD40L, CD40 ligand; GvHD, graft-versus-host disease; iTreg, induced regulatory T cell; mDC, myeloid dendritic cell; NOG, NOD. Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} mice; nTreg, natural regulatory T cell; P/I, PMA/ionomycin; Tcon, conventional T cell; Treg, regulatory T cell; Tresp, T responder cell; TSDR, Treg-specific demethylated region.

We and others have shown previously that CD40L, a member of the TNF superfamily, is a highly suitable activation marker for Tcon (14, 15). In a similar manner, Wolfl et al. (16) demonstrated that 4-1BB, (CD137), a member of the TNFR superfamily (17), serves as a specific activation marker for CD8$^+$ T cells (16). More recently, 4-1BB has been reported to be one of the target genes of the Treg-specific master transcription factor Foxp3 (18) and to be upregulated after polyclonal activation of murine Treg (19).

In this study, we demonstrate that a fast upregulation of 4-1BB combined with the absence of CD40L expression is an activation marker signature highly specific for CD4$^+$CD25$^+$Foxp3$^+$Helios$^+$ Treg in the course of short-term activation and enables direct access to Ag-specific Treg from cell mixtures. We have used this strategy to directly isolate live human alloantigen-reactive 4-1BB$^+$ CD40L$^-$Foxp3$^+$ Treg with high purity. Isolated cells were characterized by superior alloantigen-specific suppressive properties and fully retained functional stability after in vitro expansion. The use of the converse 4-1BB and CD40L expression signature as a specific marker of activated Treg will offer the opportunity to treat unwanted immune reactions with specific Treg avoiding the risk of a general immune suppression. Moreover, it allows simultaneous characterization and analysis of Treg and Tcon with same specificity in one sample.

Materials and Methods

Reagents and flow cytometry

Toxic shock syndrome toxin-1 (TSST1), PMA, brefeldin A, and ionomycin were purchased from Sigma. The following mAbs were used in this study: anti-human—CD1c FITC (Miltenyi Biotec), CD4-PE-Cy7, CD19-allophycocyanin, CD25-allophycocyanin,CD69-allophycocyanin-Cy7, Foxp3-A488, 4-1BB–PE, IL-2–allophycocyanin, and IFN-γ–allophycocyanin (BD Pharmingen); CD14-Cy5 (TM1) and CD4-Cy5 (TT1), both conjugated in our laboratory; Vβ2-FITC, Vβ17-FITC (Beckman Coulter), Helios-A647 or Pacific Blue, HLA-A2–PE, and CD40L-Pacific blue were from BioLegend; blocking anti–CD40 Ab (G28.5) was generated in our laboratory; anti-mouse—CD4-PE-Cy7 and CD69-FITC (BD Pharmingen); 4-1BB–PE, and Foxp3-allophycocyanin or FITC from eBioscience; and KJ1-26–allophycocyanin (conjugated in our laboratory). Cells were incubated with Abs for 10 min at 4°C in the dark and washed once. Intracellular stainings were performed with Foxp3 staining protocol according to manufacturer's instructions (eBioscience). Data were acquired on a LSRII flow cytometer (BD). Analysis was performed using FlowJo software (Tree Star).

Cell preparation and culture

After informed consent, PBMCs from healthy adult donors were obtained by Ficoll-Hypaque (PAA) gradient centrifugation. CD4$^+$ T cells were enriched using anti-CD4 microbeads (Miltenyi Biotec) according to manufacturer's instructions. Subsequently, CD4$^+$CD25$^+$CD127^{low} polyclonal Treg and CD4$^+$CD25$^-$CD127$^+$ Tcon were sorted using a FACSAria (BD). Cells were cultured in RPMI 1640 medium (Life Technologies BRL) with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 50 μM 2-ME (Sigma), and 10% heat-inactivated Human AB Serum (PAA) at 37°C and 5% CO_2.

Allogeneic stimulation with myeloid dendritic cells or CD3-depleted PBMCs

CD1c$^+$CD14$^-$CD19$^-$ myeloid dendritic cells (mDC) were sorted on a FACSAria (BD). Alternatively, PBMCs were depleted of CD3$^+$ T cells using anti-CD3 microbeads. Cells from donor A were mixed with mDC or CD3-depleted PBMCs from donor B in a 5:1 or 1:1 ratio, respectively, and incubated for 16 h. Anti-CD40 mAb (G28.5) was added directly to the culture (14). For intracellular staining, brefeldin A was added 4 h before harvesting. Blocking anti-MHC II (TÜ-39; BD) was added at 20 μg/ml.

Isolation and culture of murine cells

All mice used in this study (wild-type C57BL/6, OVA-TCR$^{tg/tg}$ OT-II, and DO11$^{+/-}$ × RIP-mOVA mice) were bred and maintained in a specific pathogen-free environment. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} mice (NOG) were purchased from Taconic. Single-cell suspensions from lymph nodes and spleens were generated by mechanical disruption, and cells were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 50 μM 2-ME (Sigma), and 10% heat-inactivated FCS (PAA).

Treg expansion

Purified Treg populations were cultured in round-bottom 96-well plates (1 × 10^4 Treg/well) with X-VIVO15 medium (Lonza) supplemented with 5% heat-inactivated Human AB Serum, 500 U/ml IL-2, and 10 nM rapamycin (Sigma) for 2–3 wk. "Treg Expander Beads" (Miltenyi Biotec) were added once at the beginning of the culture at a bead-to-cell ratio of 4:1. Every second day, fresh medium was added and cultures were split if necessary. For further experiments, expanded Treg were counted and analyzed for viability.

CFSE labeling

Up to 1 × 10^7 cells/ml were labeled with 5 μM CFSE for 5 min at room temperature, followed by 2 washing steps with 10 ml RPMI supplemented with10% serum.

Alloantigen-specific in vitro suppression assay

Purified alloreactive Treg and polyclonal Treg were rested for 4 d in culture medium supplemented with IL-2 (50 U/ml). Subsequently, Treg were titrated and cultured for 6 d with 5 × 10^4 CFSE-labeled CD3$^+$ T responder cells (Tresp) and CD3-depleted irradiated allogeneic PBMCs, from the primary (specific) donor or from a control donor. To exclude competition for allostimulators, we adjusted allogeneic CD3-depleted PBMCs to each Treg ratio, to have a fixed 1:1 ratio between total T cells (Treg + Tresp) and allostimulators. Analyses were performed in duplicates or triplicates. CFSE dilution of proliferating Tresp was analyzed by flow cytometry; Tresp were precisely separated from Treg and allogeneic APCs by opposite HLA-A2 expression. As control, Tresp were cocultivated with allogeneic CD3-depleted PBMCs only. To compare efficiency and specificity of suppression, we used FACS-purified CD4$^+$CD25$^+$CD127^{low} polyclonal Treg from the same donor after 16-h stimulation with anti-CD3 and anti-CD28 Abs (BD) and resting for 4 d with 50 U/ml IL-2. Suppressive capacities of expanded alloreactive Treg were tested with the same experimental strategy.

In vivo suppression assay

Spleen and lymph node cell suspensions from C57BL/6 and OT-II mice were stained with anti-CD25 Fab fragments (pC61.5, coupled to biotin in our laboratory) and were enriched for CD25$^+$ cells by magnetic separation using anti-biotin beads (Miltenyi Biotec). Foxp3 staining of sorted CD25$^+$ cells was performed to prove that at least 75% of sorted cells were Foxp3$^+$ Treg. CD25$^+$ cells were stimulated for 20 h with 10 μg/ml plate-bound anti-CD3 Abs (17A2; BD Pharmingen) and 1000 U IL-2 (R&D Systems). After stimulation, cells were stained and sorted for CD4$^+$4-1BB$^+$ T cells using FACSDiva. A total of 3 × 10^5 CD4$^+$4-1BB$^+$ T cells from either C57BL/6 or OT-II mice were transferred (i.v.) into untreated C57BL/6 mice. One day later, 3 × 10^5 OVA-pulsed (1 μg/ml) CD11c$^+$ DCs from C57BL/6 mice and 3 × 10^5 CFSE-labeled (1 μM) OVA-TCRtg CD4$^+$ T cells (OT-II) were transferred into recipient mice. Five days after T cell transfer, mice were sacrificed and splenocytes were stained. Proliferation of OVA-specific activated CD4$^+$ T cells was determined by decrease of CFSE intensity on CD4$^+$Vα2$^+$Vβ5.1/5.2$^+$ cells.

Xeno-GvHD model

Human CD4$^+$ T cells were stimulated with splenocytes from immunodeficient NOG for 16 h. Xeno-reactive Treg (4-1BB$^+$CD40L$^-$) were then sorted, expanded, and rested before cell transfer. Irradiated (300 cGy) NOG recipients were either transferred with 2.5 × 10^6 human PBMCs only to induce acute GvHD with typical symptoms such as >10% weight loss, ruffled fur, hunched posture, and limited activity, or cotransferred with xeno-reactive Treg in a 1:10 ratio (Treg/PBMCs). Control groups were irradiated but received PBS or xeno-reactive Treg only. Constitution and weight of mice were determined every day, and mice with symptoms of severe acute GvHD (e.g., 20% weight loss) were sacrificed.

Analysis of 4-1BB expressions in human spleen sections

Frozen sections of spleen tissue were air-dried overnight, fixed in acetone for 10 min, and incubated with mouse anti–4-1BB mAb (BD) for 30 min, followed by rabbit anti-mouse Igs (Dako) and an alkaline phosphatase antiphosphatase alkaline complex (Dako) using Fast Red as chromogen. Next, slides were formalin fixed, immersed in sodium citrate buffer solutions, heated, and rinsed. After washing in TBS, samples were blocked using a commercial peroxidase-blocking reagent (Dako) and incubated for

30 min with anti-Foxp3 mAb (clone PCH101; eBioscience), followed by a secondary rabbit anti-rat Ab (Dako) and the EnVision peroxidase kit (Dako) developed in diaminobenzidine. Negative controls were performed by omitting the primary Abs.

Methylation analysis of Foxp3 Treg-specific demethylated region

DNA was isolated from sorted cell subsets, using a QiaAmp DNA Mini Kit (Qiagen). Demethylation of the Foxp3 Treg-specific demethylated region (TSDR) was determined according to previously published protocols (20).

Bisulfite-specific PCR and pyrosequencing

Genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen). Bisulfite conversion, PCR, and pyrosequencing were performed by Varionostic GmbH (Germany). Forty nanograms bisulfite-converted DNA was used for PCR with primers for *CD40LG*: forward: 5′-GAGAGA-GATGGAGAGAGAG-3′; reverse: 5′-biotin-ATACACTCCAAAACATA-

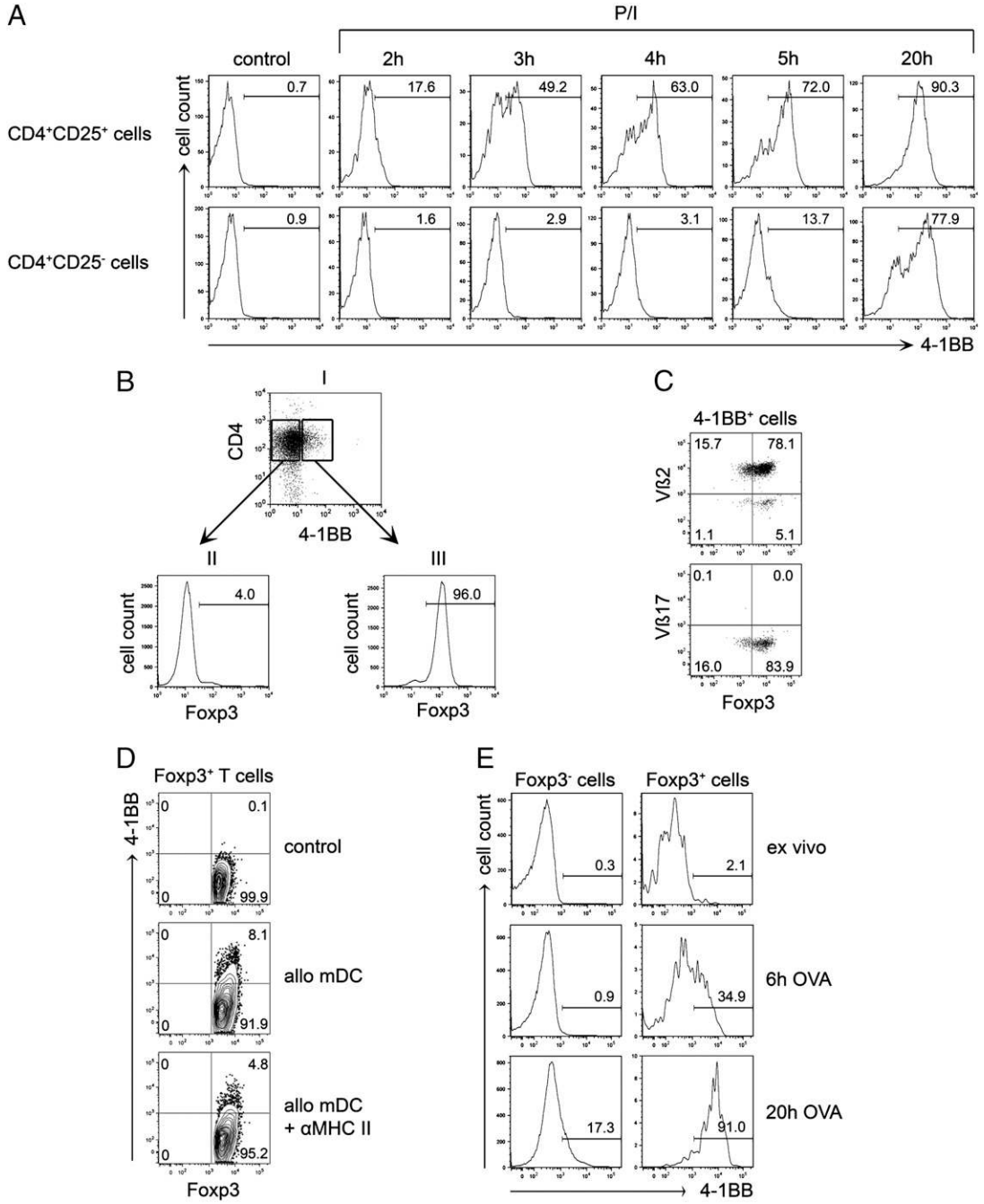

FIGURE 1. Detection and isolation of activated Treg by expression of 4-1BB. (**A**) Short-term kinetic of 4-1BB expression on human $CD4^+$ T cells. FACS-sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stimulated with P/I, fixed, stained for 4-1BB–PE, and analyzed by flow cytometry. Control cells were left in medium for 4 h. Numbers in the histograms indicate percentages of $4\text{-}1BB^+$ T cells at different time points after activation. (**B**) $4\text{-}1BB^+$ T cells after short-term stimulation are mainly Treg. Human $CD4^+$ T cells were stimulated for 3.5 h with P/I and FACS sorted according to 4-1BB expression (I). Analysis of Foxp3 in $4\text{-}1BB^-$ (II) and $4\text{-}1BB^+$ T cells (III) was performed. (**C**) 4-1BB expression on Treg is TCR dependent. PBMCs were stimulated for 3.5 h with TSST1 and FACS sorted for $CD4^+$ $4\text{-}1BB^+$ T cells. Subsequently, cells were stained intracellularly with anti-Foxp3 and anti-Vβ2 (*upper row*) or anti-Vβ17 (*lower row*) mAbs. (**D**) 4-1BB expression among $Foxp3^+$ cells was analyzed after 16-h stimulation of human $CD4^+$ T cells with allogeneic mDC (*middle panel*) and could be partially blocked by anti-MHC II Abs (*lower panel*). (**E**) Selective 4-1BB expression on Treg after peptide stimulation. Peripheral blood cells from OT-II mice were stained for CD4, 4-1BB, and Foxp3 directly ex vivo or after either 6 or 20 h stimulation with OVA-peptide. Percentages of $4\text{-}1BB^+$ T cells are indicated after gating on $Foxp3^-$ (*left column*) and $Foxp3^+$ (*right column*) $CD4^+$ T cells. (A–E) Representative data out of at least three independent experiments are shown.

TAAAACTA-3′. The PCR product was then sequenced with standard pyrosequencing procedures using the following sequencing primers: primer 1, 5′-AGAGAGATTGAAAGAGAA-3′; primer 2, 5′-TGTATTATT-TATGGAAAGAT-3′; and primer 3, 5′-AAGTTTTTATGGAGTAGG-3′. CpG analysis was done with Pyro Q-CpG software (Biotage).

Quantitative PCR

RNA from purified cell populations was isolated using an RNeasy Plus kit (Qiagen) and converted into cDNA using TaqMan reverse transcriptase (Applied Biosystems) according to the manufacturer's protocol. Transcript levels of Helios were normalized to GAPDH. Primers were as follows: Helios forward: 5′-GGACCCATTCTGTGGGTAAACCTCAC-3′; Helios reverse: 5′-CCATAGGAGGTACATGGTGACTCATG-3′; GAPDH forward: 5′-TGCACCACCAACTGCTTAGC-3′; GAPDH reverse: 5′-GGCATGGACTGTGGTCATGAG-3′. All PCRs were performed using RealMasterMix SYBR ROX (5 Prime) and measured with Mastercycler EP Realplex (Eppendorf).

Statistical analysis

Statistical analyses were performed using the paired, nonparametric, two-tailed Wilcoxon test.

Results

Specific expression of 4-1BB on activated Foxp3$^+$ Treg

First, we tracked the kinetic of 4-1BB expression on sorted human peripheral blood CD4$^+$CD25$^+$ Treg and CD4$^+$CD25$^-$ Tcon (Supplemental Fig. 1) after in vitro stimulation with PMA/ionomycin (P/I). Within 4 h after stimulation, 4-1BB was upregulated on the majority of Treg, whereas Tcon did not start to express 4-1BB until 5 h after stimulation (Fig. 1A). At 20 h after stimulation, both Treg and Tcon expressed high levels of 4-1BB. Next, we wanted to demonstrate that activated Treg could be identified and isolated by sorting 4-1BB$^+$CD4$^+$ T cells without preselecting CD4$^+$CD25$^+$ Treg. After stimulation of CD4$^+$ T cells with P/I for 3.5 h, almost all 4-1BB$^+$ cells expressed Foxp3, whereas 4-1BB$^-$ cells did not (Fig. 1B). To evaluate the specificity of Treg 4-1BB expression with respect to TCR engagement, we performed stimulation with the superantigen TSST1 that crosslinks distinct TCR β-chains with MHC class II molecules. The majority of TSST1-stimulated CD4$^+$4-1BB$^+$ T cells expressed Foxp3 and were almost exclusively positive for TSST1-reactive Vβ2, but not Vβ17, which in contrast with Vβ2 is not crosslinked by TSST1 (Fig. 1C). Next, human CD4$^+$ T cells were stimulated with peripheral blood-derived allogeneic mDC and analyzed for 4-1BB expression among Foxp3$^+$ T cells. In line with our previous experiments, there was a substantial upregulation of 4-1BB on Treg after allogeneic stimulation that could be blocked by anti-MHC class II Abs (Fig. 1D). To test whether 4-1BB upregulation also identifies murine Treg, we analyzed 4-1BB expression on peripheral blood-derived T cells from OT-II mice stimulated with $OVA_{323–339}$ peptide. Confirming our results with human cells, we observed that at early time points after stimulation with OVA-peptide, 4-1BB was selectively upregulated on murine Foxp3$^+$ Treg. Murine Foxp3$^-$ Tcon expressed 4-1BB only later (Fig. 1E). Altogether, our data demonstrate that after stimulation of total PBMCs or CD4$^+$ T cells by superantigen, allogeneic cells, or antigenic peptide, surface expression of 4-1BB can be used to detect activated Foxp3$^+$ Treg within a defined time window.

4-1BB expression identifies in vivo activated Treg

To assess whether 4-1BB is also expressed on Treg in vivo, we analyzed cryosections of normal human spleen and detected 4-1BB$^+$ Treg (Fig. 2A). In an analogous manner, 4-1BB$^+$ Treg were present among murine spleen and lymph node cells (Fig. 2B), probably reflecting self-Ag–driven homeostatic proliferation of Treg (21). Moreover, 4-1BB expression could be detected ex vivo on Foxp3$^+$ T cells present at the site of autoimmune inflammation, such as synovial fluid of patients with rheumatoid arthritis (Fig. 2C) and in draining lymph nodes of diabetic DO11$^{+/-}$ × RIP-mOVA mice (22). These mice express membrane-bound OVA as a self-Ag in the pancreas, and the local activation of OVA-specific CD4$^+$ T cells leads to diabetes in these mice. 4-1BB$^+$ OVA-specific Treg were selectively accumulated in pancreatic lymph nodes (Fig. 2D) as compared with peripheral lymph nodes and spleen. These data indicate that 4-1BB is expressed on Treg during homeostatic self-Ag–driven proliferation, as well as at sites of inflammation in vivo.

A converse expression signature of 4-1BB and CD40L delineates activated Treg and Tcon independent of time

Although we could show that 4-1BB is upregulated on Foxp3$^+$ Treg after activation, the restricted time window during which 4-1BB was selectively expressed on Treg compared with Tcon represents a limitation for the use of this technology to isolate pure Treg. The unambiguous identification of Tcon would be critical to avoid contamination with Tcon. We and others have previously shown that CD40L upregulation is characteristic for activated CD4$^+$ Tcon (14, 15). Therefore, the simultaneous assessment of CD40L and 4-1BB expression may permit the identification of activated 4-1BB$^+$ Treg with the exclusion of activated CD40L$^+$ Tcon. Six

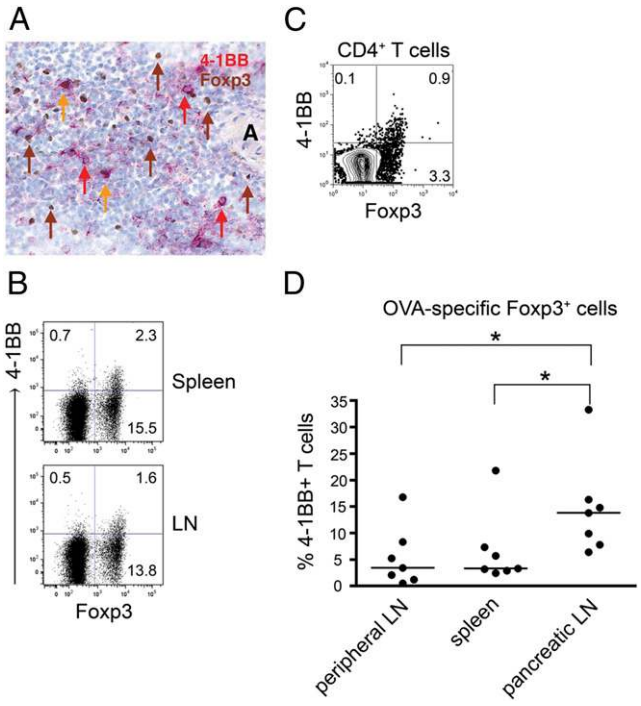

FIGURE 2. Ex vivo 4-1BB expression identifies in vivo activated Treg. (**A**) 4-1BB is expressed ex vivo in human spleen. Cryosections of human spleen from trauma patients (n = 5) were stained with anti–4-1BB (red) and anti-Foxp3 (brown) mAbs. 4-1BB and Foxp3 double-positive cells (indicated by yellow arrows) were observed predominantly in the T cell areas surrounding splenic arterioles (original magnification ×400). (**B**) 4-1BB is expressed ex vivo on mouse Treg in secondary lymphoid organs. Spleen and peripheral lymph node (LN) cells from C57BL/6-mice were stained directly ex vivo for 4-1BB, Foxp3, and CD4, and analyzed by flow cytometry. (**C**) 4-1BB$^+$ Treg are present ex vivo at sites of autoimmune inflammation. Synovial fluid cells of patients with rheumatoid arthritis were analyzed for 4-1BB expression on Foxp3$^+$ T cells. (**D**) Increased percentage of 4-1BB–expressing Treg at sites of autoimmune inflammation. Cells from spleen, peripheral, and pancreatic LNs of DO11$^{+/-}$ × RIPmOVA mice (n = 7) were stained and analyzed ex vivo. The frequency of 4-1BB expression among OVA-specific (KJ1-26$^+$) CD4$^+$Foxp3$^+$ Treg is shown. (A–C) Representative data of one experiment out of three are shown. *$p \leq 0.0156$ as calculated by Wilcoxon test.

hours after polyclonal stimulation or 16 h after stimulation with allogeneic mDC, $CD4^+$ T cells expressing 4-1BB, but not CD40L, were almost exclusively $Foxp3^+$ (Fig. 3A, 3B). On the contrary, activated $CD40L^+$ T cells comprise only a minor fraction of $Foxp3^+$ T cells. This is in line with epigenetic analyses of the methylation status of CpGs at the 3′-enhancer of the *CD40LG* locus (23) in 4-$1BB^+CD40L^-$ T cells and $CD4^+CD25^-$ Tcon (Treg-depleted). 4-$1BB^+CD40L^-$ T cells are hypermethylated in this region, whereas Tcon are hypomethylated (Supplemental Fig. 2). We also tested whether other commonly used activation markers such as CD69 could replace 4-1BB as a Treg-specific signature. Strikingly, Foxp3 expression was detectable only by alloantigen-activated 4-$1BB^+CD40L^-$ T cells as compared with alloantigen-activated $CD69^+CD40L^-$ T cells (Fig. 3C), thus clearly demonstrating that a combined analysis of CD69 and CD40L fails to specifically define activated Treg. In summary, these results validate that the combination of CD40L and 4-1BB enables clear distinction of activated Tcon from activated $Foxp3^+$ Treg.

4-$1BB^+CD40L^-$ T cells are highly enriched in Ag-reactive nTreg

$CD25^+Foxp3^+$ Treg have been shown to be a mixture of natural thymus-derived Tregs, as well as peripherally iTreg. Recently, the transcription factor Helios, a member of the Ikaros gene family, has been shown to be selectively expressed on nTreg, but not on iTreg (24). Furthermore, it has been demonstrated that nTreg, but not iTreg, are characterized by complete demethylation of defined CpG islands in the enhancer region of the *FOXP3* locus, a region defined as TSDR (13). Because nTreg might represent the most suitable candidates for adoptive therapy, we assessed Helios expression as well as the TSDR methylation status in different T cell subsets defined by 4-1BB and CD40L expression after allogeneic stimulation. In contrast with CD40L-expressing subsets, 4-$1BB^+$ $CD40L^-$ T cells showed complete TSDR demethylation (Fig. 4A) and high Helios expression (Fig. 4B, 4C), which was even higher compared with isolated $CD25^+$ Treg or gated $Foxp3^+$ T cells. Finally, we analyzed expression of effector cytokines in the different subsets. 4-$1BB^+CD40L^-$ T cells obtained after P/I stimulation did not express IL-2 and/or IFN-γ, or only expressed marginal levels. Both 4-$1BB^+CD40L^+$ and 4-$1BB^-CD40L^+$ T cells expressed high amounts of both cytokines (Fig. 4D). Interestingly, a considerable frequency of effector cytokine expression could be detected among $CD40L^+$ T cells coexpressing Foxp3. These data clearly show that only 4-$1BB^+CD40L^-$ T cells are highly enriched in activated nTreg.

Alloantigen-reactive 4-$1BB^+CD40L^-$ T cells display potent suppressive function in vitro

With respect to other reports showing efficient immunosuppression by Ag-specific Treg populations (9–11), we examined the suppressive capacity of alloantigen-reactive 4-$1BB^+CD40L^-$ T cells in comparison with polyclonal $CD25^+$ $CD127^{low}$ Treg that were characterized by similar frequencies of Foxp3 and Helios (data not shown). Polyclonal Treg were able to partially suppress proliferation of Tresp when used in ratios as high as 1 to 8. Lower ratios of polyclonal Treg starting with 1 to 32 were not sufficient to mediate suppression of Tresp activation (Fig. 5A). In contrast, alloantigen-reactive 4-$1BB^+CD40L^-$ T cells (allo-reactive Treg) exhibited superior suppression of Tresp proliferation up to 90% at

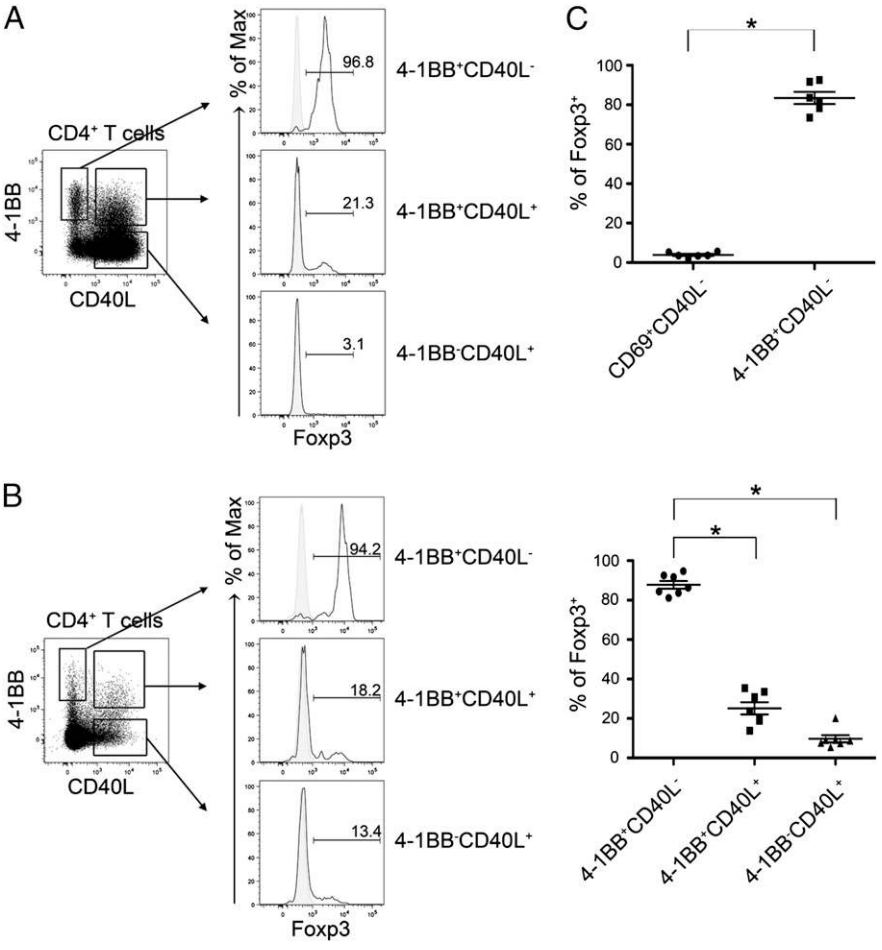

FIGURE 3. Expression of 4-1BB and lack of CD40L defines activated $Foxp3^+$ Treg. Human T cells were polyclonally stimulated with P/I for 6 h (**A**) or with allogeneic CD3-depleted PBMCs for 16 h (**B**) and stained for CD4, 4-1BB, and CD40L. Frequencies of Foxp3 expression are shown on subsets gated according to 4-1BB and CD40L expression. One representative experiment is shown in histograms; the diagram summarizes seven independent experiments after 16-h allostimulation. Gray graphs represent $Foxp3^-$ $CD4^+$ cells. $*p \leq 0.0156$ as calculated by Wilcoxon test. (**C**) Foxp3 expression was analyzed on gated $CD69^+CD40L^-$ and 4-$1BB^+CD40L^-$ T cells after 16 h of alloantigen stimulation with allogeneic CD3-depleted PBMCs. Graph summarizes six independent experiments. $*p \leq 0.0313$ as calculated by Wilcoxon test.

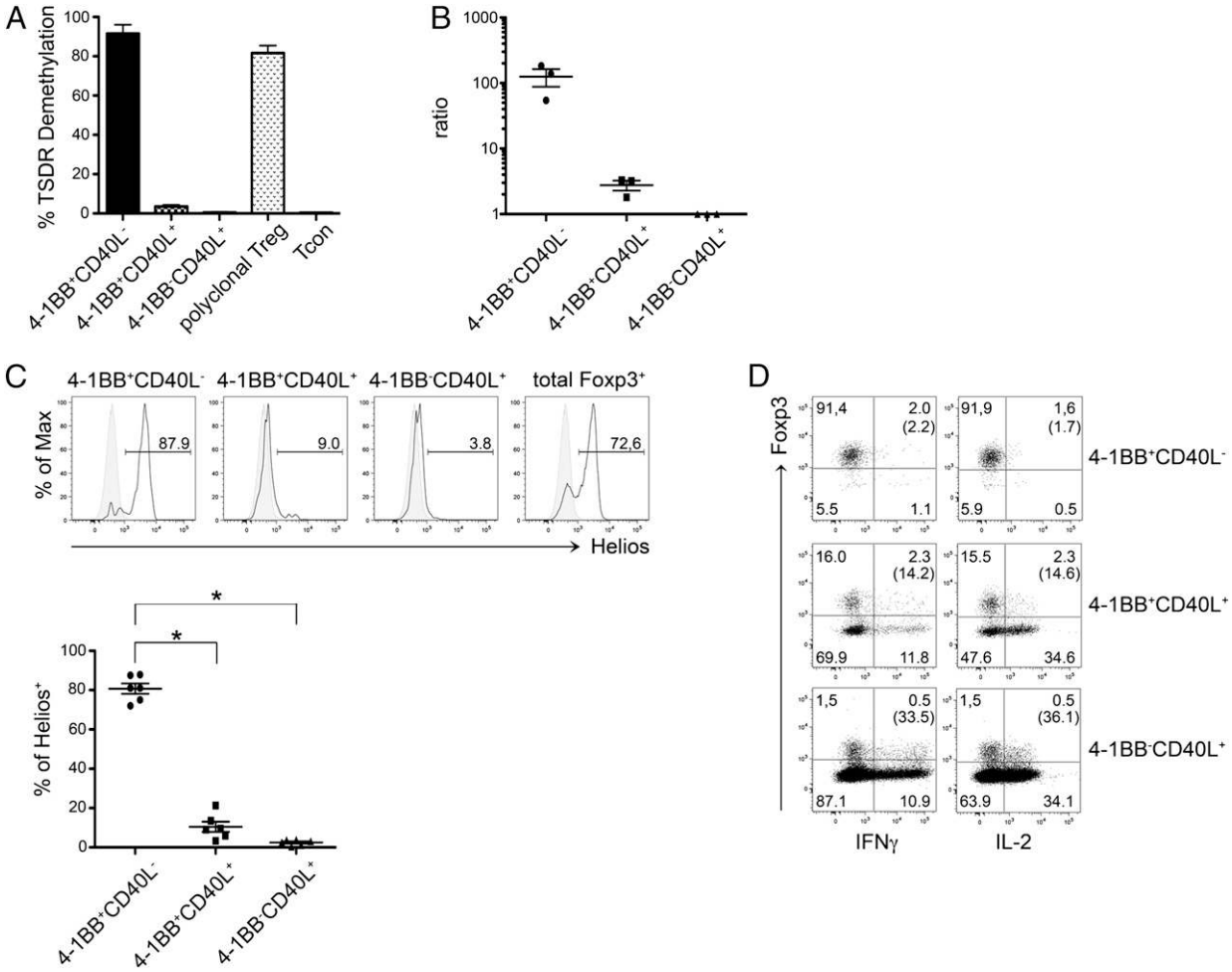

FIGURE 4. Alloantigen-reactive 4-1BB$^+$CD40L$^-$ Treg are nTreg. Alloantigen-activated T cell subsets were sorted by FACS based on 4-1BB and CD40L expression. (**A**) Methylation pattern of an evolutionarily conserved region of the *FOXP3* locus (TSDR) was analyzed for all sorted cell subsets. Total CD4$^+$ CD25$^-$ T cells (Tcon) and CD4$^+$CD25$^+$ T cells (polyclonal Treg) were purified from the same donor and used as controls. Percentage of demethylation at CpG methylation sites of the TSDR was measured for each purified cell population ($n = 3$). (**B**) RNA was purified from sorted cell populations and analyzed by real-time PCR for the expression of Helios ($n = 3$). Expression of GAPDH was used as reference for normalization. (**C**) Helios protein expression was examined by intracellular staining of purified cells. One representative experiment is shown in histograms; the diagram summarizes six independent experiments. *$p \leq 0.0313$ as calculated by Wilcoxon test. (**D**) Expression of intracellular IL-2 and IFN-γ was measured on cell subsets gated according to 4-1BB and CD40L expression after 6-h P/I stimulation. Numbers in parentheses indicate percentages of potentially cytokine-producing cells among Foxp3$^+$ T cells. One representative experiment out of three is shown.

a 1:32 ratio. Tresp proliferation could be suppressed up to 35% at ratios of 1:256 and up to 20% at ratios of 1:1024. When the allo-reactive Treg were stimulated with a random control donor, the efficiency of Tresp suppression was similar between polyclonal and allo-reactive Treg in almost all ratios (Fig. 5B). These data demonstrate that 4-1BB$^+$CD40L$^-$ T cells isolated after allostimulation are highly enriched for alloantigen-reactive Treg exerting very potent suppression potential. Furthermore, the binding of an mAb to 4-1BB does not alter the in vitro suppressive properties of isolated Treg by blocking or activating this functionally relevant molecule (25–27).

Activation-induced expression of 4-1BB and lack of CD40L is a stable feature of nTreg after in vitro expansion

A different approach to obtain Ag-specific Treg is to sort polyclonal Treg and stimulate them in vitro repetitively with specific Ags or alloantigens. However, contamination with nonspecific T cells or Tcon may be a limitation of such protocols. Accordingly, we tested whether 4-1BB and CD40L expression signature discriminates activated Treg and Tcon also after in vitro expansion. At first, we demonstrated that alloantigen-reactive 4-1BB$^+$CD40L$^-$ T cells can be efficiently expanded in vitro up to 300-fold within 3 wk, even starting with cell numbers as low as 1×10^4 (Fig. 6A). In contrast with alloantigen-reactive Tcon, alloantigen-reactive Treg retained coexpression of Foxp3 and Helios (Fig. 6B). We then investigated re-expression of 4-1BB and CD40L after alloantigen-specific restimulation in alloantigen-reactive Treg, CD40L$^+$ Tcon, and polyclonal Treg, all derived and expanded from the same donor (Fig. 6C). Expanded alloantigen-reactive Treg displayed a significant 4-1BB re-expression capacity without CD40L expression. In contrast, alloantigen-reactive Tcon expressed 4-1BB and CD40L after restimulation. As expected, polyclonal Treg exhibited low frequency of activation (0.3–1.0% among Treg) with both allogeneic donors (Fig. 6C). Again, we tested the functional potential of the expanded Treg in an in vitro suppression assay (Fig. 6D). Expanded alloantigen-reactive Treg were still able to mediate effective inhibition of Tresp proliferation in response to the primary allogeneic donor, in contrast with polyclonal Treg. When activated with CD3-depleted PBMCs of a control donor, expanded al-

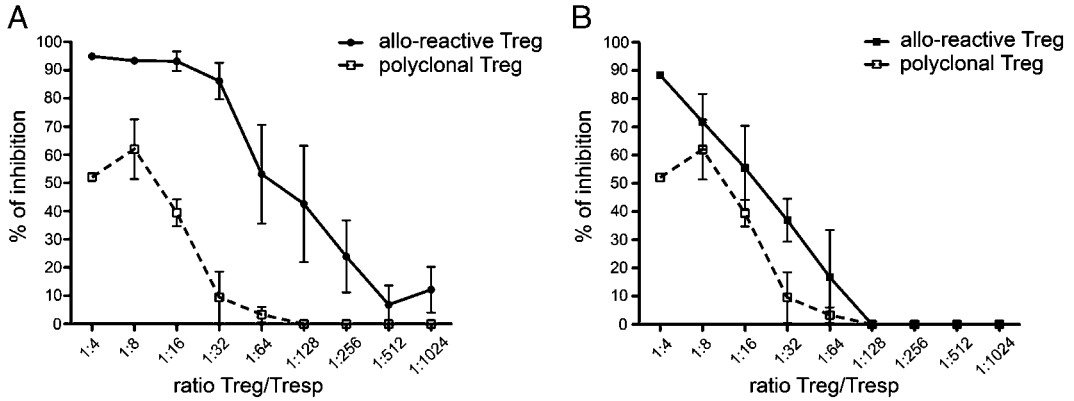

FIGURE 5. Superior suppressive capacities of alloantigen-reactive Treg. The suppressive functions of isolated alloantigen-reactive 4-1BB$^+$CD40L$^-$ Treg and polyclonal CD25$^+$CD127^{low} Treg were compared using a 6-d in vitro CFSE dilution assay. Graphs summarize the results of four independent experiments and show percentage of inhibition of Tresp proliferation mediated by the Treg populations after stimulation with specific (**A**) or control (**B**) allogeneic CD3-depleted PBMCs.

loantigen-reactive Treg suppressed Tresp to a similar extent as polyclonal Treg. Altogether, these data show that expression of 4-1BB and lack of CD40L is a stable feature of nTreg after activation and can be used to select alloantigen-reactive nTreg after in vitro expansion and to exclude contaminations of Tcon, nonspecific T cells, and iTreg.

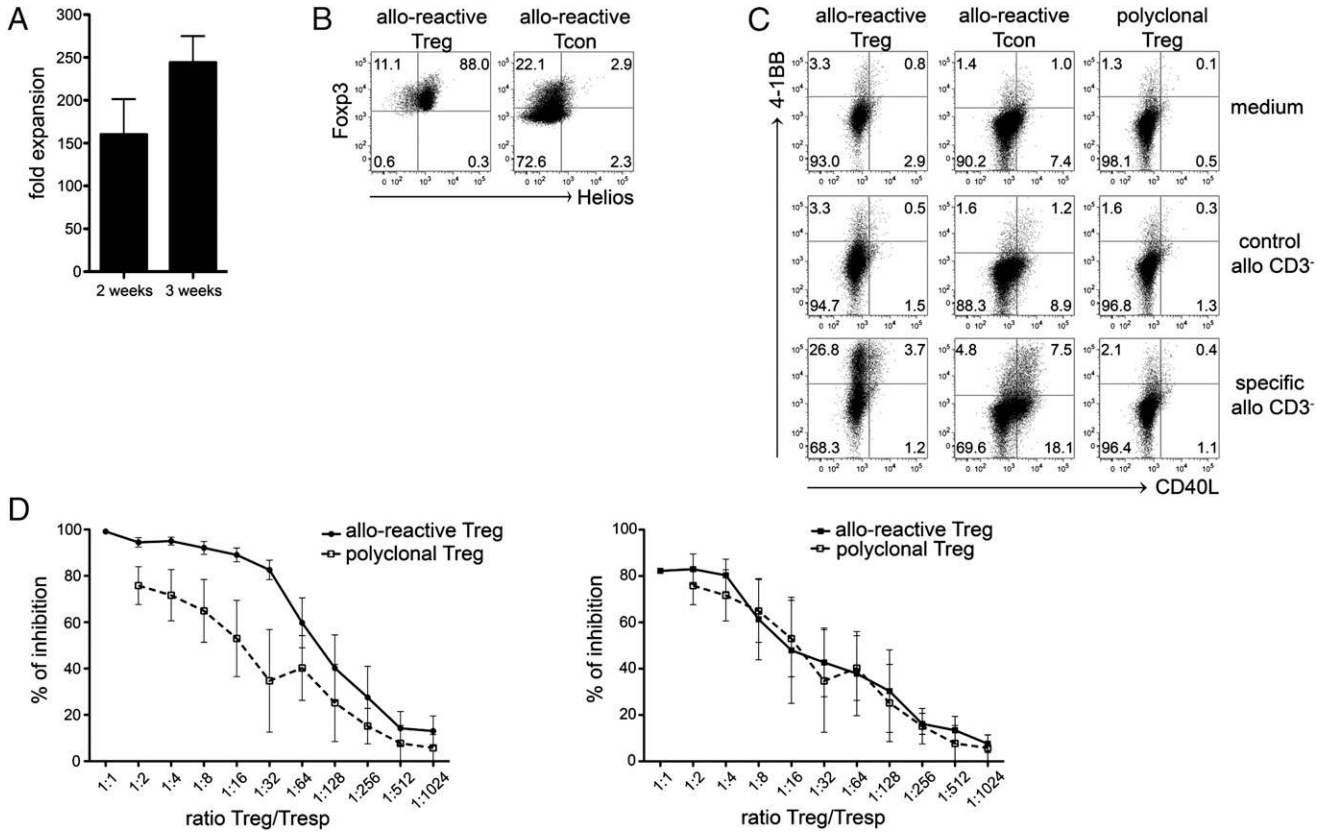

FIGURE 6. Upregulation of 4-1BB and lack of CD40L expression is a stable characteristic of nTreg also after in vitro expansion. (**A**) Expansion rates of alloantigen-reactive 4-1BB$^+$CD40L$^-$ Treg. After alloantigen stimulation, 4-1BB$^+$CD40L$^-$ Treg were isolated by FACS and expanded with "Treg Expander Beads" for 2 or 3 wk under optimized conditions (X-VIVO 15 medium, 500 U/ml IL-2, 10 nM rapamycin, 5% Human AB Serum). The mean fold change in cell number was calculated and summarized (n = 5). (**B**) Alloantigen-reactive Treg (4-1BB$^+$CD40L$^-$) and Tcon (CD40L$^+$) were simultaneously expanded for 2 wk and phenotypically analyzed for intracellular Foxp3 and Helios protein expression. (**C**) Alloantigen-reactive Treg (4-1BB$^+$CD40L$^-$) and Tcon (CD40L$^+$) and polyclonal Treg (CD25$^+$CD127^{low}), all isolated from the same donor, were restimulated after 2 wk expansion culture either with specific (*lower row*) or control (*middle row*) allogeneic CD3-depleted PBMCs for 16 h, as unstimulated control (*upper row*) cells were left in medium only. CD40L and 4-1BB were stained and analyzed by FACS. (**D**) The suppressive capacities of expanded alloantigen-reactive 4-1BB$^+$CD40L$^-$ Treg and polyclonal CD25$^+$CD127^{low} Treg were compared using a 6-d in vitro CFSE dilution assay. The graphs summarize the results of four independent experiments and show percentage of inhibition of Tresp proliferation mediated by the Treg populations after stimulation with specific (*left panel*) or control (*right panel*) allogeneic CD3-depleted PBMCs.

Activated nTreg isolated according to 4-1BB expression retain their suppressive function in vivo

Next, we wanted to clarify whether Treg isolated according to 4-1BB expression retain their Ag-specific immunosuppressive capacity also in vivo. To this aim, wild-type C57/Bl6 hosts were injected with C57/Bl6- or OT-II Treg that had been polyclonally activated in vitro and sorted according to 4-1BB expression. Subsequently, OVA-pulsed dendritic cells and naive OT-II T cells were transferred into the same recipient mice. In contrast with wild-type 4-1BB$^+$ Treg, the transfer of activated 4-1BB$^+$ Treg from OT-II mice significantly suppressed the proliferation of CFSE-labeled naive OVA-specific OT-II T cells (Fig. 7A). This result demonstrates that direct isolation of 4-1BB–expressing nTreg with a 4-1BB–specific Ab does not affect the suppressive function of nTreg.

With respect to a potential use of human alloantigen-specific nTreg in cellular therapies, we finally wanted to assess the suppressive capacity of human Ag-specific nTreg in a relevant preclinical GvHD mouse model. We therefore stimulated human CD4$^+$ T cells with splenocytes from NOG, lacking T, B, and NK cells, and isolated human xeno-reactive Treg according to 4-1BB expression and lack of CD40L expression. After 2 wk of expansion and 4 d of resting time, the human xeno-reactive Treg were transferred into irradiated NOG mice together with human PBMCs. Control groups received only PBMCs, only xeno-reactive nTreg, or PBS.

Mice receiving only human PBMCs survived no longer than 18 d after cell transfer because of severe acute GvHD, whereas mice transferred only with xeno-reactive Treg remained healthy and did not suffer from any GvHD symptoms (Fig. 7B). When xeno-reactive Treg were transferred together with human PBMCs (ratio 1:10), 50% of the mice survived until day 26 after cell transfer and >30% of mice survived until the end of the experiment (day 34) without showing GvHD symptoms. In conclusion, these data demonstrate that 4-1BB staining and the following isolation do not alter the functional potential of human nTreg, a prerequisite for the implementation of the 4-1BB technology into therapeutic applications.

Discussion

In this article, we introduce a new technology that permits the direct identification and isolation of Ag-reactive natural Foxp3$^+$ Treg directly from complex cell mixtures based on the expression of 4-1BB and the absence of CD40L expression. Although 4-1BB is specifically expressed on Ag-activated Treg early during short-term stimulation, CD40L enables the specific and efficient exclusion of activated Tcon. Our data are in line with recent reports that identified 4-1BB as a target gene of the Treg-specific master transcription factor Foxp3 (18), suggesting that Foxp3 regulates the fast expression of 4-1BB. Moreover, 4-1BB has been characterized as a specific marker of TCR-induced activation of conventional CD4$^+$ (28) and CD8$^+$ T cells (17). In contrast, CD40L is an approved activation marker defining Ag-specific conventional CD4$^+$ T cells (14, 15). Notably, CD40L has been recently reported to be expressed on a subset of Treg after activation. However, CD40L$^+$ Treg rather resembled Tcon because they acquired typical Th cell functions (29). In line with these results, the suppressive capacities of CD40L expressing "Treg" seem to be less efficient and may reflect competition for growth factors (30). Activated nTreg that lack CD40L expression as we described in this article are characterized by methylated CpGs in the *CD40LG* locus and also by a complete lack of other effector function such as effector cytokine production.

Although in experimental murine systems analysis of Ag-specific Treg is easily performed using transgenic TCR-bearing Treg, similar assessments with human Treg are challenging so far, because of their low frequency. Therefore, we focused on the assessment of human alloantigen-reactive Treg, suspected to be present in feasible frequencies among peripheral blood cells (31). Moreover, alloantigen-specific suppression of graft rejection or of GvHD by Treg is regarded as one of the most prominent and promising applications of Ag-specific Treg in the field. A recent study introduced activation markers such as CD69 and CD71 for the isolation of alloantigen-reactive Treg (9). However, because the expression profiles of both markers do not differ between CD4$^+$CD25$^+$ Treg and CD4$^+$CD25$^-$ Tcon, a separation of Treg from Tcon before stimulation with alloantigen would be neces-

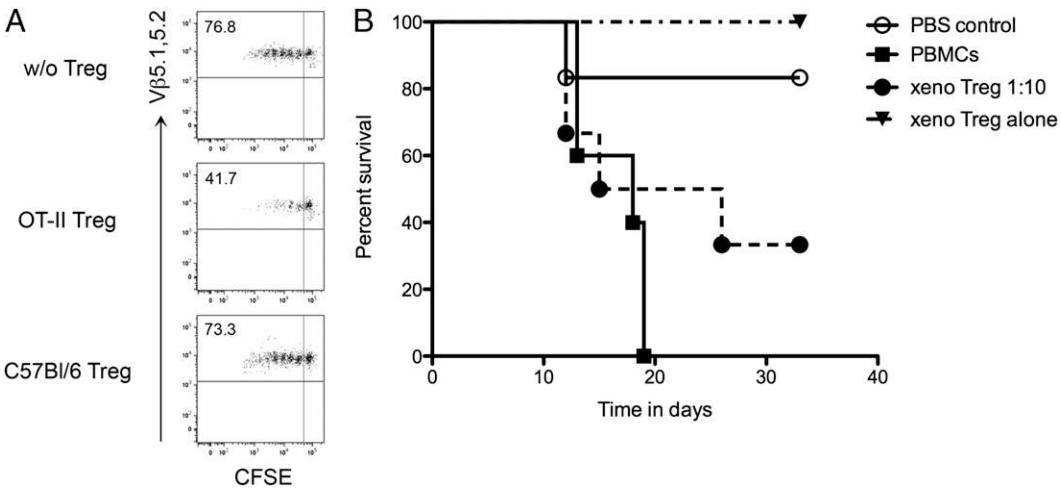

FIGURE 7. Isolated murine and human 4-1BB$^+$ Treg retain Ag-specific suppression capability in vivo. (**A**) Ag-specific suppression of sorted murine 4-1BB$^+$ Tregs in vivo. 4-1BB$^+$ Treg from C57BL/6 or OT-II mice were transferred into untreated C57BL/6 mice. One day later, CD11c$^+$ OVA-pulsed DC and CFSE-labeled naive OT-II CD4$^+$ T cells were coinjected into the same recipients. At day 5, proliferation was measured after gating on CD4$^+$, Vα2$^+$, and Vβ5$^+$ T cells. Numbers indicate the percentage of proliferated cells. Data from one experiment of three (n = 2 mice in each group) are shown. (**B**) Inhibition of xeno-GvHD by xeno-reactive human Tregs. Survival curves compare NOG mice receiving 2.5 × 10^6 human PBMCs ± expanded xeno-reactive Treg at 10:1 ratio (PBMCs/Treg). Control groups received PBS or xeno-reactive Treg only. Each group consisted of six mice.

sary. Because of low cell numbers of alloantigen-reactive Treg, an in vitro expansion will be indispensable for clinical applications either before or after their isolation. In this study, the converse 4-1BB and CD40L expression signature permitting the differentiation of activated nTreg from activated Tcon would enable purification of alloantigen-reactive natural Foxp3$^+$Helios$^+$ Treg also after expansion. Importantly, sorting of activated nTreg according to Ag-induced 4-1BB does not alter their functional properties. We assessed activated murine nTreg expressing a transgenic TCR or human xenoantigen-specific nTreg for their suppressive capacity in vivo. Similar to our in vitro assays, we did not observe any functional impairment of nTreg isolated after labeling with 4-1BB Abs, suggesting that at least 4-1BB–specific Abs do not affect the suppressive function of nTreg. Some studies have been undertaken to further analyze the role of 4-1BB costimulation for nTreg. Our results do not support other data that have recently reported that signals through 4-1BB may inhibit Treg (32). On the contrary, more reliable data have been published suggesting that signals through 4-1BB may serve in a rather agonistic way (33–35). The exact mechanisms particularly in in vivo systems may be difficult to assess because 4-1BB has been demonstrated to be a potent stimulator particularly for conventional CD8$^+$ T cells as well. Together with the fact that 4-1BB–deficient mice exhibit normal numbers of Foxp3$^+$ nTreg, these previous findings would suggest that although 4-1BB signaling can augment nTreg activation, the molecule itself has no major functional role for nTreg (36).

This may be relevant considering that 4-1BB signaling can modulate the activation of CD4$^+$CD25$^+$ Treg (34, 35). It has been shown that the peripheral Treg pool comprises a complex mixture of Treg subpopulations (37) with the major classification into thymus-derived nTreg and peripherally iTreg. As a major functional difference, a stable suppressive function has been reported to be characteristic only for nTreg correlating with the complete demethylation of an evolutionary conserved region in the Foxp3 gene, the TSDR (13, 38). In contrast, TGF-β–induced Foxp3 expression and the resulting suppressive phenotype of iTreg have been shown to be transient in vitro and in vivo (39–41). This correlates with findings that transferred alloantigen-reactive iTreg failed to prevent GvHD in contrast with nTreg (40). We demonstrate in this study that only 4-1BB$^+$CD40L$^-$ alloantigen-reactive T cells fulfill all known criteria characteristic for nTreg as compared with iTreg and Tcon. They are characterized by a fully demethylated TSDR and fail to express effector cytokines such as IFN-γ or IL-2 to any significant level. Furthermore, 4-1BB$^+$CD40L$^-$ alloantigen-reactive T cells express the transcription factor Helios. The exclusive expression of Helios by nTreg is controversially discussed; therefore, the selective usage of this marker to define nTreg has to be handled with care. Nevertheless, we observed Helios expression predominantly in the 4-1BB$^+$CD40L$^-$ T cell compartment, which fits well with the earlier mentioned specific nTreg characteristics. Moreover, we demonstrate in this study that nTreg exhibit a methylated region of the *CD40LG* locus correlating with their inability to upregulate CD40L expression in the course of short-term activation.

In conclusion, the use of the 4-1BB and CD40L expression signature as a marker for Ag-specific nTreg will open new therapeutic options, for example, for cell therapies with nTreg specific for dominant autoantigens such as insulin, GAD65, or Factor VIII relevant in defined immunopathological situations. Moreover, our method will enable for the first time, to our knowledge, a direct and simultaneous assessment of Ag-specific nTreg versus Tcon for diagnosis and prognosis of harmful immunity, for example, in transplantation, allergy, and autoimmune diseases where responsible Ags are defined.

Acknowledgments

We thank the Flow Cytometry Laboratory of the Berlin-Brandenburg Center for Regenerative Therapies for expert technical assistance and Dirk Busch, Julian Braun, and Abul Abbas for critical discussion and reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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