IMMUNITY
SUPPLEMENTAL INFORMATION

Immunomodulatory functions of BTLA and HVEM govern induction of extrathymic regulatory T cells and tolerance by dendritic cells

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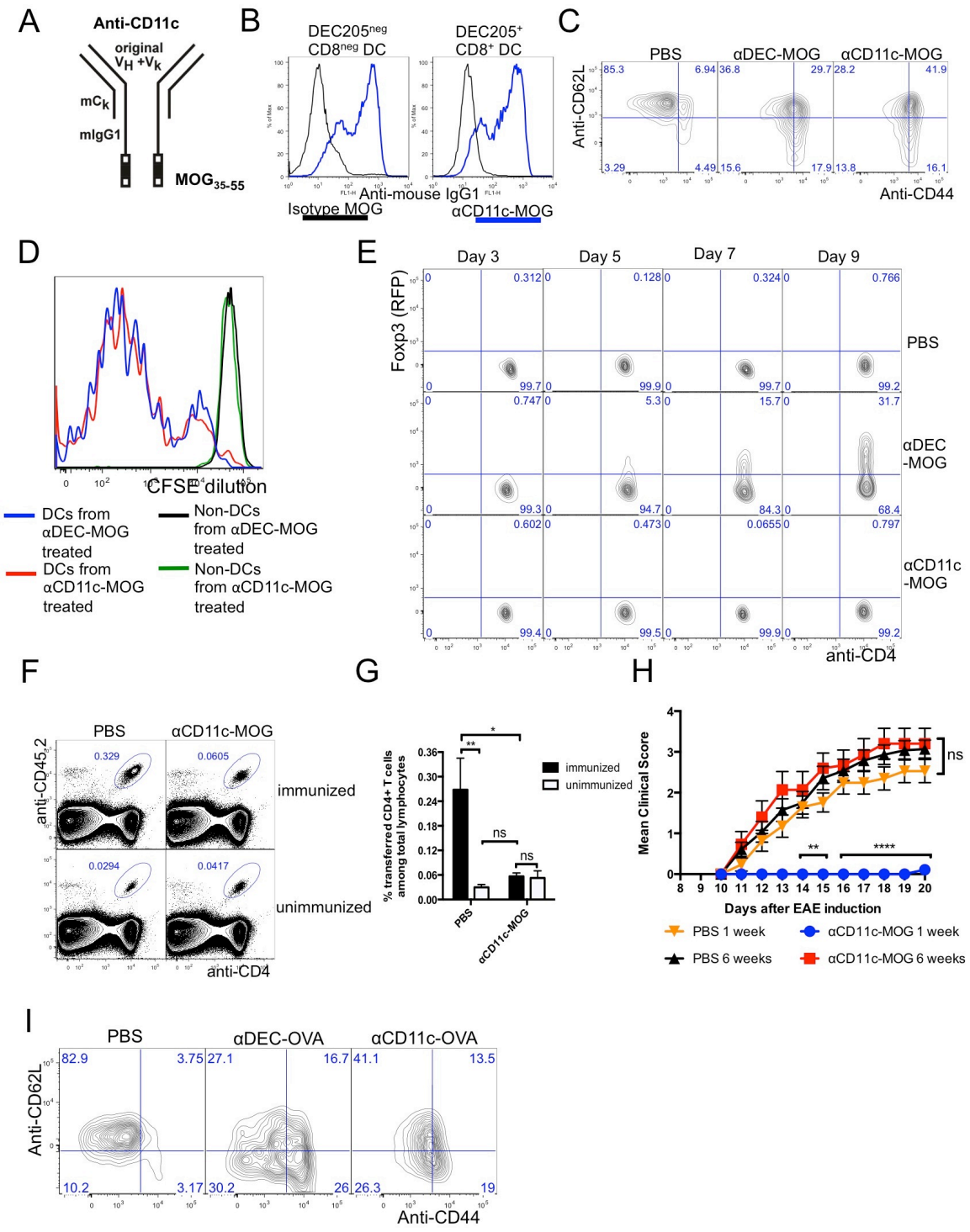

Figure Supplementary 1 (associated with Figure 1) **(A)** Diagram of αCD11c-MOG to deliver MOG_{35-55} to $CD11c^+$ DCs. **(B)** Targeting with αCD11c-MOG *in vivo*. Overlaid histograms show anti-mouse IgG staining intensity in $DEC205^+CD8^+CD11c^+$ splenic DCs

and DEC205^{neg}CD8^{neg}CD11c^{+} splenic DCs isolated from C57BL/6 mice 30 minutes after treatment with either αCD11c-MOG or isotype control as indicated. Results represent one of two similar experiments. **(C)** Activation markers. CD45.2^{+} Foxp3^{neg}CD25^{neg} T cells were isolated from 2D2 TCR tg *Foxp3^{RFP}* mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with αDEC-MOG, αCD11c-MOG or PBS. Plots show anti-CD62L and anti-CD44 staining intensity as indicated in the transferred CD4^{+} T cells among splenocytes analyzed by flow cytometry after 21 days. Results represent one of two similar experiments. **(D)** Proliferation induced by DCs targeted with αDEC-MOG and αCD11c-MOG *in vivo*. CD11c^{+} DCs and CD11c^{neg} non-DCs were isolated from the spleens and lymph nodes of mice treated 24 hours prior with αDEC-MOG, αCD11c-MOG or PBS and cultured *in vitro* with CFSE labeled 2D2 TCR tg CD4^{+} T cells. Overlaid histograms show CFSE fluorescence intensity after 7 days in culture. Results represent one of two similar experiments. **(E)** Absence of early transient induction of Foxp3 expression in T cells activated by all CD11c^{+} DCs. CD45.2^{+} Foxp3^{neg}CD25^{neg} T cells were isolated from 2D2 TCR tg *Foxp3^{RFP}* mice and then adoptively transferred into CD45.1^{+} recipient mice and analyzed by flow cytometry at indicated days after treatment with αDEC-MOG, αCD11c-MOG or PBS. Plots show Foxp3 (RFP) expression and anti-CD4 staining intensity in the transferred CD45.2^{+} lymphocytes. Results represent one of three similar experiments. **(F-G)** T cells initially activated by CD11c^{+} DCs become unresponsive to subsequent re-challenge with antigen. CD45.2^{+} CD4^{+} T cells were isolated from 2D2 TCR tg mice and then adoptively transferred into CD45.1^{+} recipient mice treated with αCD11c-MOG or PBS. 8 days later the recipients were either immunized or not immunized with MOG_{35-55} in CFA + *Pertussis* Toxin *(*PT*)* and then lymphocytes were analyzed by flow cytometry after another 6 days. **(F)** Plots show anti-CD45.2 and anti-CD4 staining intensity in lymphocytes from spleens.

Results represent one of two similar experiments. **(G)** Graphs show percentages of transferred $CD4^+$ T cells among all $CD4^+$ T cells (n=3 mice per group pooled from two independent experiments). **(H)** $CD11c^+$ DCs induce a transient protection from EAE. Multiple groups of mice were treated with either αCD11c-MOG or PBS either 1 week or 6 weeks before immunization with MOG_{35-55} in CFA + *Pertussis* Toxin *(*PT*)*. Graphs show mean EAE disease scores in indicated groups (n=10 mice per group pooled from two independent experiments). **(I)** Activation markers. $CD45.2^+$ $Foxp3^{neg}CD25^{neg}$ T cells were isolated from OTII TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into $CD45.1^+$ recipient mice that were then treated with αDEC-OVA, αCD11c-OVA or PBS. Plots show anti-CD62L and anti-CD44 staining intensity as indicated in the transferred $CD4^+$ T cells among splenocytes analyzed by flow cytometry after 7 days. Results represent one of 2 similar experiments. **(G and H)** Graphs show mean +/- standard error of mean (SEM), * P< 0.05, ** P< 0.01 and **** P< 0.0001 determined by one-way or two-way ANOVA. **(C, E, F, I)** Numbers in quadrants indicate corresponding percentages.

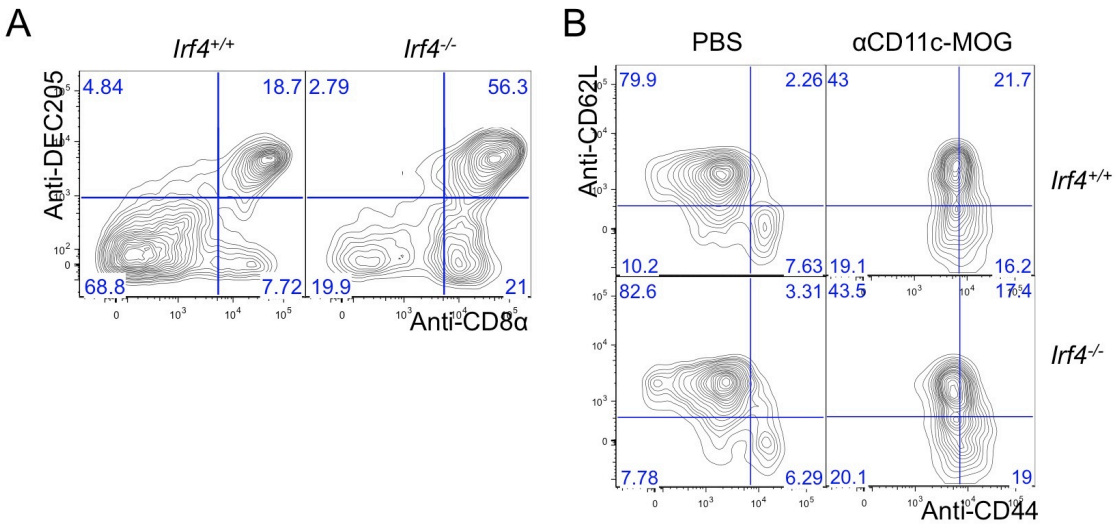

Figure Supplementary 2 (associated with Figure 2) (A) Increased population of DEC205^{+}CD8^{+}CD11c^{+} DCs in *Irf4*$^{-/-}$ mice. Plots show anti-DEC205 and anti-CD8α staining intensity in splenic CD11c^{+} MHCII^{+} DCs from *Irf4*$^{-/-}$ and *Irf4*$^{+/+}$ mice. Results represent one of two similar experiments. **(B)** Similar activation of T cells in *Irf4*$^{-/-}$ and *Irf4*$^{+/+}$ mice. CD45.1^{+} Foxp3^{neg}CD25^{neg} T cells were isolated from 2D2 TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into CD45.2^{+} *Irf4*$^{-/-}$ and *Irf4*$^{+/+}$ recipient mice treated with αCD11c-MOG or PBS. Plots show anti-CD62L and anti-CD44 staining intensity as indicated in transferred CD4^{+} T cells among splenocytes analyzed by flow cytometry after 21 days. Results represent one of two similar experiments. **(A and B)** Numbers in quadrants indicate corresponding percentages.

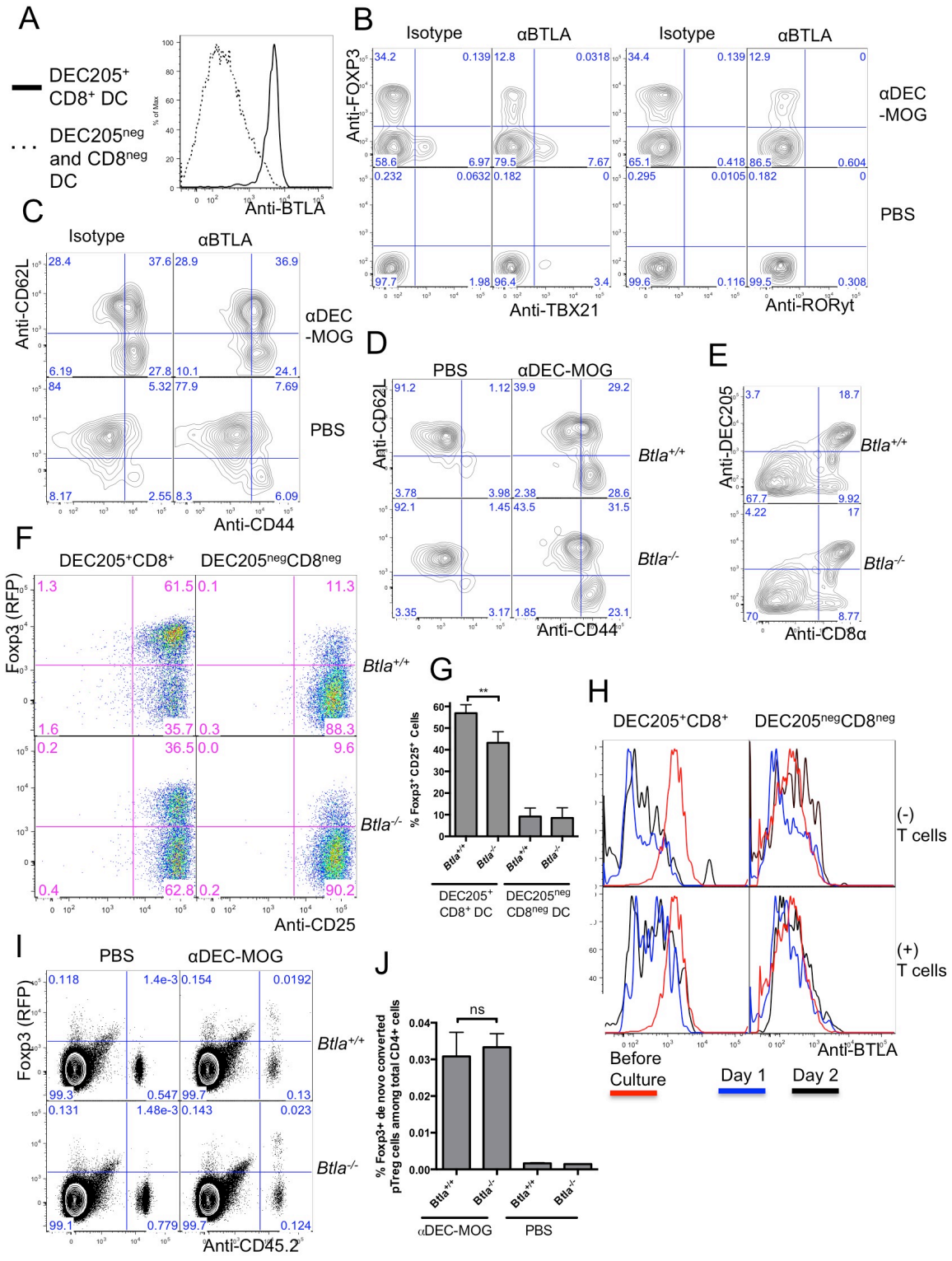

Figure Supplementary 3 (associated with Figure 3) (A) BTLA is specifically expressed in DEC205^{+}CD8^{+}CD11c^{+} DCs. Overlaid histograms show anti-BTLA staining

intensity in indicated gated populations of $CD205^{+}CD8^{+}$ cells and the $CD8^{neg}$ as well as $DEC205^{neg}$ cells among $CD11c^{+}$ DCs in peripheral lymph nodes. Results represent one of three similar experiments. **(B)** Transcription factors in T cells following BTLA blocking *in vivo*. $CD45.2^{+}$ $Foxp3^{neg}CD25^{neg}$ T cells were isolated from 2D2 TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into $CD45.1^{+}$ recipient mice treated with αDEC-MOG or PBS. Additionally, recipients were treated twice with αBTLA or isotype control. Plots show anti-FOXP3 and anti-TBX21 **(left panel)** or anti-ROR-γt **(right panel)** intracellular staining intensity in transferred $CD4^{+}$ cells among splenocytes after 10 days. Results represent one of two similar experiments **(C)** Activation markers in T cells following BTLA blocking *in vivo*. Cells were transferred and mice treated as in (B). Plots show anti-CD62L and anti-CD44 staining intensity as indicated in transferred $CD4^{+}$ T cells among splenocytes analyzed by flow cytometry after 21 days. Results represent one of three similar experiments. **(D)** Activation markers in T cells in *Btla*$^{+/+}$ and *Btla*$^{-/-}$ recipients. $CD45.1^{+}$ $Foxp3^{neg}CD25^{neg}$ T cells were adoptively transferred into $CD45.2^{+}$ *Btla*$^{+/+}$ and *Btla*$^{-/-}$ recipient mice that were treated with αDEC-MOG or PBS. Plots show anti-CD62L and anti-CD44 staining intensity as indicated in transferred $CD4^{+}$ T cells among splenocytes analyzed by flow cytometry after 9 days. Results represent one of three similar experiments. **(E)** Similar numbers of $DEC205^{+}CD8^{+}CD11c^{+}$ DCs in *Btla*$^{+/+}$ and *Btla*$^{-/-}$ mice. Plots show anti-DEC205 and anti-CD8α staining intensity in splenic $CD11c^{+}$ DCs from *Btla*$^{-/-}$ or *Btla*$^{+/+}$ mice. Results represent one of three similar experiments. **(F and G)** *Btla*$^{+}$ $DEC205^{+}CD8^{+}CD11c^{+}$ DCs induce iTreg cells *in vitro***.** Naïve 2D2 $Foxp3^{neg}CD25^{neg}$ T cells were co-cultured with sorted *Btla*$^{+/+}$ and *Btla*$^{-/-}$ $DEC205^{+}CD8^{+}CD11c^{+}$ and $DEC205^{neg}CD8^{neg}CD11c^{+}$ DCs as indicated in the presence of MOG_{35-55} and TGF-β. **(F)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in cultured $CD4^{+}$ T cells analyzed by flow cytometry after 4 days as indicated.

Results represent one of three similar experiments. **(G)** Graphs show percentages of Foxp3+CD25+ iTreg cells as indicated, (n=4 per group pooled from three independent experiments). **(H)** BTLA expression is partially down-regulated on in vitro cultured DCs. DEC205^{+}CD8^{+}CD11c^{+} and DEC205^{neg}CD8^{neg}CD11c^{+} DCs were purified by sorting from C57BL/6 mice and cultured in vitro either in the absence or in the presence of 2D2 CD4^{+} T cells and MOG antigen as indicated. Overlaid histograms show BTLA expression on DCs analyzed as indicated. Results represent one of three similar experiments. **(I and J)** Similar pTreg cell conversion from *Btla*$^{+/+}$ and *Btla*$^{-/-}$ T cells. CD45.2^{+} Foxp3^{neg}CD25^{neg} T cells were isolated from *Btla*$^{+/+}$ and *Btla*$^{-/-}$ 2D2 TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with αDEC-MOG or PBS. **(I)** Plots show Foxp3 (RFP) expression and anti-CD45.2 staining intensity as indicated in the CD4^{+} T cells among splenocytes analyzed by flow cytometry after 9 days. Results represent one of two similar experiments. **(J)** Graphs show percentages of CD4^{+} T cells that were Foxp3 (RFP)$^{+}$ and CD45.2^{+} (n=4-5 mice per group pooled from two independent experiments). **(B, C, D, E, F and I)** numbers in quadrants indicate corresponding percentages. **(G and J)** Graphs show mean +/- SEM, n.s. not significant or ** P< 0.01determined by one-way ANOVA.

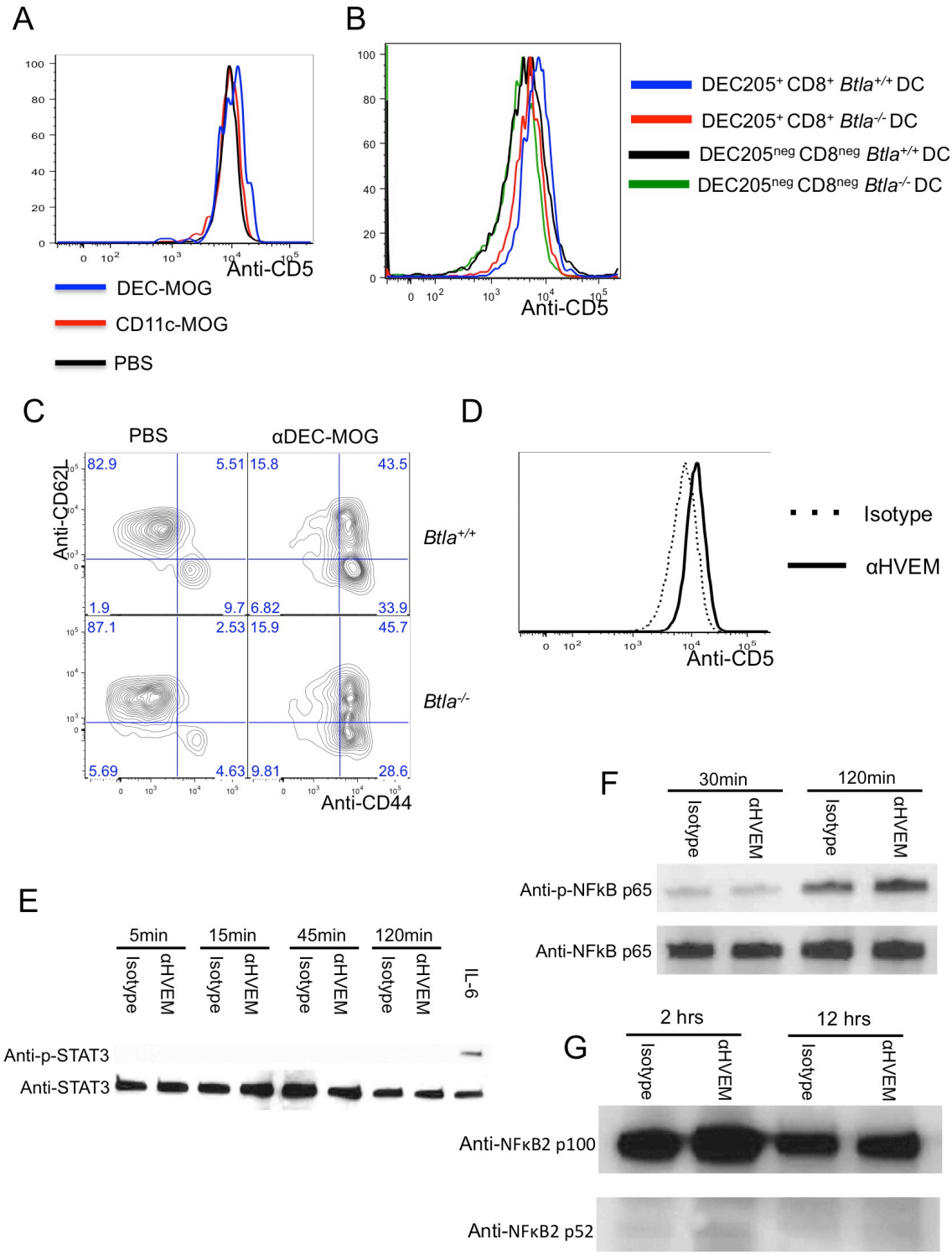

Figure Supplementary 4 (associated with Figure 4) (A) Stimulation by total CD11c^{+} DCs fails to increase CD5 expression in T cells. CD45.2^{+} Foxp3^{neg}CD25^{neg} T cells were

isolated from 2D2 TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with αDEC-MOG, αCD11c-MOG or PBS. Overlaid histograms show specific staining intensity with anti-CD5 in the transferred Foxp3^{neg} CD4^{+} T cells as indicated among splenocytes analyzed by flow cytometry after 9 days. Results represent one of three similar experiments. **(B)** BTLA^{+}DEC205^{+}CD8^{+}CD11c^{+} DCs increase CD5 expression in T cells *in vitro*. Naïve 2D2 Foxp3^{neg}CD25^{neg} T cells were co-cultured with sorted *Btla*$^{+/+}$ and *Btla*$^{-/-}$ DEC205^{+}CD8^{+}CD11c^{+} and DEC205^{neg}CD8^{neg}CD11c^{+} DCs as indicated in the presence of MOG_{35-55} and TGF-β. Overlaid histograms show specific staining with anti-CD5 in Foxp3^{neg} T cells analyzed by flow cytometry after 2 days as indicated. Results represent one of three similar experiments. **(C)** Activation markers in *Btla*$^{+/+}$ and *Btla*$^{-/-}$ T cells. CD45.2^{+} Foxp3^{neg}CD25^{neg} T cells were isolated from *Btla*$^{+/+}$ and *Btla*$^{-/-}$ 2D2 TCR tg *Foxp3*^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with αDEC-MOG or PBS. Plots show anti-CD62L and anti-CD44 staining intensity as indicated in transferred CD4^{+} T cells among splenocytes analyzed by flow cytometry after 9 days. Numbers in quadrants indicate corresponding percentages. Results represent one of two similar experiments. **(D)** Engagement of HVEM in polyclonal T cells induces CD5 upregulation. Naïve Foxp3^{neg}CD25^{neg} polyclonal CD4^{+} T cells from C57BL/6 mice were stimulated for 3 days *in vitro* in the presence of αCD3, αCD28 and also in the presence of either αHVEM or isotype control followed by cross-linking with a secondary reagent. Overlaid histograms show CD5 expression in T cells in either the presence or absence of HVEM engagement as indicated. Results represent one of two similar experiments. **(E-G)** STAT3 phosphorylation **(E),** NFkB p65 phosphorylation **(F)** and NFκB2 p100 processing **(G)**. Immunoblot analysis in lysates from naïve Foxp3^{neg}CD25^{neg} 2D2 CD4^{+} T cells that were stimulated for indicated times *in vitro* in the

presence of αCD3, αCD28 and also in the presence of either αHVEM or isotype control or with IL-6 as indicated followed by cross-linking with a secondary reagent. Results represent one of two similar experiments.

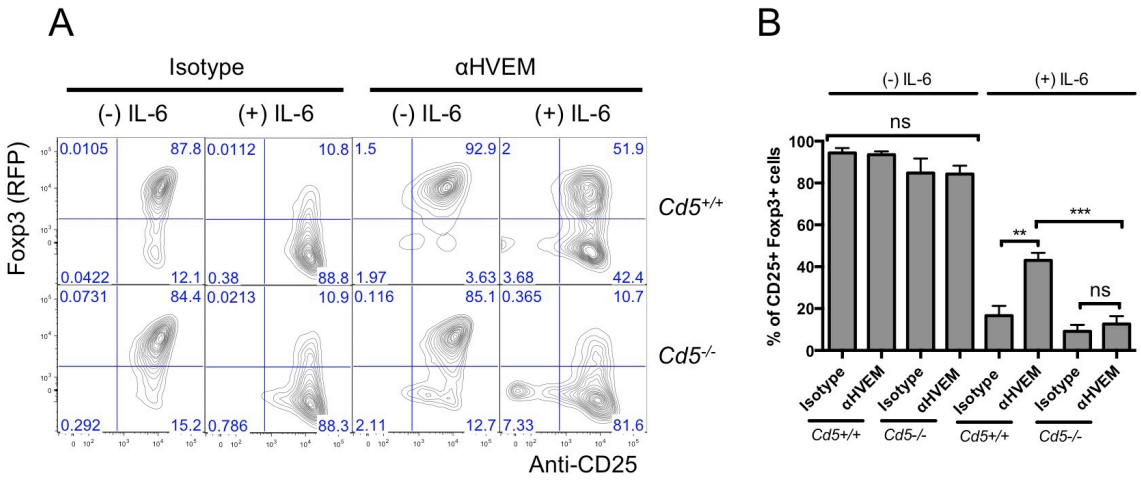

Figure Supplementary 5 (associated with Figure 5) (A and B) Engagement of HVEM in T cells induces CD5-dependent Treg induction. Naïve *Cd5*$^{+/+}$ and *Cd5*$^{-/-}$ Foxp3^{neg}CD25^{neg} polyclonal CD4^{+} T cells were cultured for 5 days *in vitro* in the presence of αCD3, αCD28, TGF-β and also in the presence of either αHVEM or isotype control followed by cross-linking with a secondary reagent and in either the presence or absence of IL-6 as indicated. **(A)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity. Numbers in quadrants indicate corresponding percentages. Results represent one of three similar experiments. **(B)** Graphs show percentages of Foxp3^{+}CD25^{+} cells in the indicated groups of cells (n=3-4 per group pooled from three independent experiments). Results represent mean +/- SEM, ** P< 0.01, *** P< 0.001 determined by one-way ANOVA.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Cd5$^{-/-}$ (Tarakhovsky et al., 1994), *Foxp3*^{RFP} (Wan and Flavell, 2005), anti-MOG TCR transgenic (2D2) (Bettelli et al., 2003), OTII TCR Tg (Barnden et al., 1998), *Batf3*$^{-/-}$ (Hildner et al., 2008), *Irf4*$^{fl/fl}$ (Klein et al., 2006), *Cd11c-Cre* (Caton et al., 2007) and *Btla*$^{-/-}$ (Watanabe et al., 2003) mice were previously described and are available from the Jackson Laboratory. All mice were bred on a CD45.1 or CD45.2 congenic C57BL/6 background. 6-8 week old sex and age-matched littermates were used for all experiments. All mice were maintained in our facility under specific pathogen free conditions and used in accordance with guidelines of the Saint Louis University Institutional Animal Care and Use Committee.

Chimeric antibodies

To make chimeric αCD11c-MOG and αCD11c-OVA antibodies, we applied the same general design we used before for αDEC-MOG (Hawiger et al., 2001; Hawiger et al., 2004). We cloned the V regions of αCD11c from total RNA obtained from the corresponding N418 hybridomas (Metlay et al., 1990) using 5`-RACE PCR kit (Invitrogen) following manufacturer's instructions. We then PCR-cloned the obtained anti-CD11c V heavy (5'-gaggtgcagctggtggagaaggggggaggcttggtgcagcctggaaagtccctgaaactctcctgtgcagcctctggattcaccttcagtgaatactggatgaactgggttcgccaggctccagggaaggggctggagtgggttggtgtaattaaatacaaatatagtaattatgcaacagaatatgcggagtctgtgaaaggcagattcaccatctcaagagacgactccaaaagcagcgtctacctgcaaatgaacaacttaagagccgaggacaccgccatttattactgtgcaagaacctgggagaactggtacttcgatttctggggccaggggacccaggtcaccgtctcctca) and kappa (5'-gacatccagatgacccagtctccatcatcactgcctgcctccctgggagacagagtcactatccattgtcaggccagtcag

gacattagcaattatttaacctggtaccagcagaaaccagggaaagctcctaagctcctgatctatgaaacaaataaattg gcagatggagtcccatcaaggttcagtggcagtggttctgggagagattattctttcactatcagcagcctggaatctgaaga tgttggatcttattactgtcaacattattatgactatcctcggacgttcggacctggcaccaagctggaaatcaaacgg) regions with the leader peptide and murine IgG1 kappa and heavy constant regions (Hawiger et al., 2001) also containing MOG sequence at their 3' ends (Hawiger et al., 2004) or OVA (Hawiger et al., 2010).

Chimeric antibodies were produced as previously described (Hawiger et al., 2001; Hawiger et al., 2004). Briefly, antibodies were expressed in A293 cells by transient transfection using calcium-phosphate method. Cells were grown in serum free DMEM supplemented with Nutridoma SP (Roche) and antibodies were purified on protein-G columns. Chimeric antibodies were injected in PBS intraperitoneally at 15μg/mouse (MOG-delivering antibodies) or 125 ng/mouse (OVA-delivering antibodies) as established previously (Hawiger et al., 2004; Hawiger et al., 2010).

Flow cytometry and antibodies used for staining

Anti-CD4 (GK1.5), anti-CD25 (PC-61), anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD5 (53-7.3), anti-mouse IgG1 (Poly 4060), anti-DEC205 (NLDC-145) anti-CD8α (53-6.7) anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD11c (N418), anti-CD19 (6D5), anti-BTLA (6A6), and anti-TBX21 (4B10) were from BioLegend. Anti-Foxp3 (FJK-16a) was from eBioscience. Anti-I-A^b (AF6-120.1) and anti-RORyt (Q31-378) were from BD. Cell sorting and analyses were performed on ARIA III, FACS CALIBUR, LSRII, and CANTO (BD). Zombie Aqua viability dye was from BioLegend. For intracellular staining cells were fixed and permeabilized using Fixation/Permeabilization buffers from eBioscience according to manufacturers' manual.

Adoptive transfers

Lymph nodes and spleen cells from 5-10 mice per each experimental group were pooled and $CD4^+$ T cells were enriched by depleting $CD8^+$, $B220^+$, $CD11c^+$, $CD11b^+$ and $NK1.1^+$ cells with magnetic microbeads (Miltenyi) and then cell sorted on ARIA III (BD). Cells were washed 3x with PBS and $5x10^6$ cells/mouse were injected intravenously in tail veins. In all experiments transferred T cells and recipients were congenically labeled based on CD45.1 and CD45.2 expression.

Antibody Injections

αBTLA (6A6) or isotype control (Armenian Hamster IgG) antibodies (BioXCell) in PBS were injected intraperitoneally 150μg/mouse 4 hours and 26 hours after chimeric antibodies injection.

EAE Model

To induce EAE mice were injected with 100ug of synthetic Myelin Oligodendrocyte Glycoprotein peptide (MOG_{35-55}, Yale Keck Protein Synthesis Facility) in Complete Freund's Adjuvant (Difco) subcutaneously in each flank. CFA was enriched with *Mycobacterium tuberculosis* (10 ml CFA + 40 mg *M. tuberculosis* from Difco). *Pertussis Toxin* (List Biological Laboratories Inc.) was injected 200ng per mouse in PBS intraperitoneally on days 0 and 2 after MOG_{35-55} injections. Clinical score of EAE was graded on a scale of 1-4 - 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness, abnormal gait; 3, complete hind limb paralysis; 4, complete hind limb paralysis and forelimb weakness or paralysis. Mice were scored daily. Each experimental group was scored in a blinded fashion. Spinal cords were extracted from the spinal columns of

experimental mice. The spinal cords were then mashed through 70 µm filters using a 5 ml syringe plunger and prepared for FACS analysis.

Cell cultures

Lymph node and spleen cells from 4-12 mice per each experimental group were pooled and $CD4^+$ T cells were enriched using magnetic microbeads (Miltenyi) and then cell sorted on ARIA III (BD). $0.25x10^6$/well T cells were cultured for 1-5 days in 96-well plates (Thermo-Fisher) with anti-CD3 (145-2C11), (1 µg/ml) and anti-CD28 (37.51) (1.5 µg/ml) in RPMI-1640 media containing 10% FBS, Penicillin-Streptomycin, L-Glutamine, b-Mercaptoethanol (Gibco), Sodium pyruvate (Gibco), recombinant IL-2 (200 units /ml), and in some experiments TGF-β (1-4 ng/ml) (all from BioLegend). Anti-CD3 was cross-linked by addition of anti-Armenian Hamster IgG (15 µg/ml) after 20 min incubation. In some experiments recombinant IL-4 (2ng/ml) or IL-6 (8ng/ml) were additionally added as indicated in the manuscript to T cells. Additionally, anti-HVEM (HMHV-1B18) (BioLegend) or Isotype control (Armenian Hamster IgG) (BioXCell) were added to some cultures at 6 µg/ml or as indicated in the manuscript and were cross-linked by addition of anti-Armenian Hamster IgG (15 µg/ml) after 20 min incubation. Alternatively LNs and spleens were dissociated in 5% FCS RPMI and incubated in the presence of collagenase D (Roche) and EDTA as described before (Hawiger et al., 2001; Hawiger et al., 2004), $CD11C^+$ DCs were enriched using microbeads (Miltenyi) and then sorted on ARIA III (BD). $0.35\text{-}0.7x10^5$/well DCs and $CD11c^{neg}$ cells were cultured *in vitro* with $0.2x10^6$/well T cells as indicated in RPMI-1640 media containing 10% FBS, Penicillin-Streptomycin, L-Glutamine, b-Mercaptoethanol (Gibco), Sodium pyruvate (Gibco), and in some experiments also synthetic MOG_{35-55} (60 µg/ml) recombinant IL-2 (200 units /ml), and TGF-β (2 ng/ml) were added or T cells were labeled with 3 µM CFSE (Sigma) in 5% FCS RPMI at 37°C for 20 min.

Immunoblot and Real-time RT- PCR analysis

Naïve sorted $CD4^+$ T cells were stimulated with anti-CD3, anti-CD28, anti-HVEM or isotype control as described above for times as indicated in the text and then collected and lysed in RIPA buffer (Cell Signalling) with Protease/Phosphatase inhibitor cocktail (Cell Signalling) and phenylmethylsulfonyl fluoride (Sigma) 1 mM. Antibodies used:anti-ETS-1 (D8O8A), anti-MEK (D1A5), anti-Phospho-MEK (Ser217/221) (41G9), anti-STAT3 (79D7), anti-Phophso-STAT3 (Tyr705) (D3A7), anti-NF-kB2 p100/p52, anti-β-actin (D6A8), anti-Phospho-NF-kB p65 (Ser536) (93H1), anti-NF-kB p65 (D14E12) (all from Cell Signaling).

RNA was isolated from cultured T cells using TRIZOL Reagent (Invitrogen) and Qiagen mRNAEasy kit (Qiagen). Total RNA was reverse transcribed and the cDNA was subsequently used for real-time PCR on an ABI Prism instrument using commercial primer-probe sets (Applied Biosystems): *Cd5*: Mm00432417_m1, *Ets-1*: Mm01175819_m1, *E47* (Tcf3): Mm01175588_m1, Hypoxanthineguanine phosphoribosyltransferase (*Hprt*): Mm03024075_m1. The results of Q-PCR were standardized to the HPRT expression and analyzed by the dd CT method.

Statistical analysis

Mice of particular genotypes were randomly assigned to individual experimental groups. All experimental groups and individual mice were included in statistical analysis. The numbers of groups and mice in each group were determined to achieve statistical significance based on commonly used statistical techniques, two-way and one-way ANOVA and the Student's t test. In all experiments data was pooled from two to four

independent experiments and individual P values were calculated using Student's t-test with Welch's correction, one-way ANOVA or two-way ANOVA.