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

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Abstract

Dendritic cells (DCs) initiate immunity and also antigen-specific tolerance mediated by extrathymic regulatory T (Treg) cells. Yet it remains unclear how DCs regulate induction of such tolerance. Here we report that efficient induction of Treg cells was instructed by BTLA$^+$DEC205$^+$CD8$^+$CD11c$^+$ DCs and the immunomodulatory functions of BTLA. In contrast, T cell activation in steady state by total CD11c$^+$ DCs that include a majority of DCs that do not express BTLA did not induce Treg cells and had no lasting impact on subsequent immune responses. Engagement of HVEM, a receptor of BTLA, promoted Foxp3 expression in T cells through upregulation of CD5. In contrast, T cells activated in the absence of BTLA and HVEM-mediated functions remained CD5^{lo} and therefore failed to resist the inhibition of Foxp3 expression in response to effector cell-differentiating cytokines. Thus DCs require BTLA and CD5-dependent mechanisms to actively adjust tolerizing T cell responses under steady state conditions.

Graphical abstract

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Author Contributions

A.J. designed and performed experiments, interpreted data and wrote the manuscript. J.B. performed some of the *in vitro* experiments and interpreted data, L. K. performed RT-PCR analysis and interpreted data, A.O. performed some of the chimeric antibody production, R.M.T. participated in preparation of manuscript, C.G. maintained experimental animals and participated in preparation of experiments and manuscript. D.H. conceived, designed and oversaw experiments, interpreted data and wrote the manuscript.

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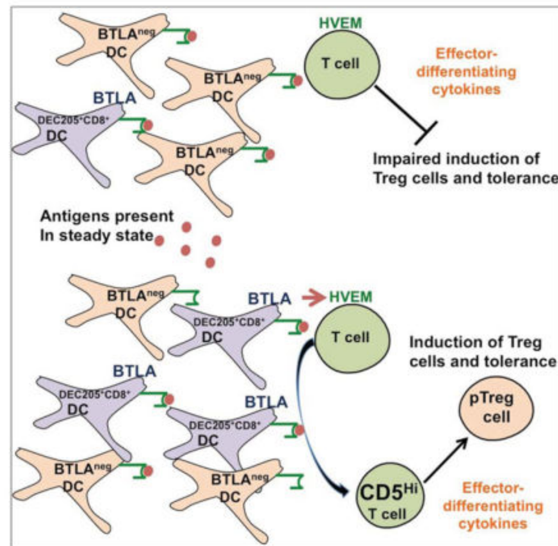

INTRODUCTION

Dendritic cells (DCs) prime and also regulate immune responses (Steinman, 2012). In the steady state, defined by the absence of pro-inflammatory stimuli, the outcome of T cell activation by DCs results in T cell tolerance (Hawiger et al., 2001; Ohnmacht et al., 2009; Probst et al., 2003; Steinman et al., 2003). Further, specialized types of DCs have tolerogenic functions (Belz et al., 2002; Coombes et al., 2007; Gottschalk et al., 2013; Idoyaga et al., 2013). However, potentially tolerogenic $CD8^+$, $CD103^+$ or $DEC205^+$ DCs constitute only relatively minor subpopulations among all $CD11c^+$ DCs in the lymphoid organs. Therefore the relevance of tolerogenic functions by such DCs remains unclear in the context of T cell responses to specific antigens that are also being presented by other DCs.

Foxp3-expressing ($Foxp3^+$) peripheral (p) regulatory T (Treg) cells converted by DCs in steady state from extrathymic T cells which induce *de novo* Foxp3 expression, prevent specific subsequent autoimmune responses (Coombes et al., 2007; Hadeiba et al., 2008; Jones et al., 2015; Josefowicz et al., 2012b; Kretschmer et al., 2005; Sun et al., 2007). Tolerogenic DCs that induce Treg cells are characterized by production of various immunomodulatory metabolites and cytokines (Coombes et al., 2007; Li and Flavell, 2008; Manicassamy et al., 2009; Mascanfroni et al., 2013; Mucida et al., 2007; Munn et al., 2002). Further, specific immunomodulatory molecules such as CTLA-4 and PD-L1 function in tolerance and Treg cell induction by DCs (Fife et al., 2009; Francisco et al., 2009; Probst et al., 2005; Wang et al., 2008; Wing et al., 2008). B and T lymphocyte associated (BTLA), an immunoglobulin domain superfamily protein, is expressed in T cells and antigen presenting cells including $CD8^+$ DCs, where it acts as a ligand for the herpesvirus entry mediator (HVEM), a tumor necrosis factor receptor superfamily member, expressed in resting and activated T cells (Cheung et al., 2005; Murphy and Murphy, 2010; Steinberg et al., 2013; Watanabe et al., 2003). The functions of HVEM and BTLA can govern T cell responses

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including their memory and regulatory functions (Flynn et al., 2013; Sharma et al., 2014; Soroosh et al., 2011).

In addition to extrinsic signals, a conversion of pTreg cells is mediated by the intrinsic specificity to self and tolerizing antigens. Self-reactive T cells are characterized by increased expression of CD5 that promotes conversion of such $CD5^{hi}$ cells into $Foxp3^+$ pTreg cells by blocking mTOR activated in response to effector cell-differentiating cytokines (Henderson et al., 2015). Therefore CD5 selectively regulates induction of Treg cells without compromising an overall high plasticity of immune responses among a total T cell repertoire. The expression of CD5 in T cells increases in response to either self-peptide(p)MHC in the thymus or tolerizing antigens presented by DCs in the periphery (Azzam et al., 1998; Hawiger et al., 2004). The expression of CD5 in T cells exiting the thymus represents a spectrum rather than discrete amounts and therefore specific upregulation of CD5 expression in T cells by peripheral DCs may be crucial for instructing the conversion of extrathymic Treg cells (Azzam et al., 2001; Hawiger et al., 2004; Henderson et al., 2015). This raises a question of how such a tolerogenic upregulation of CD5 expression in T cells is governed to ensure a specific induction of Treg cells in response to tolerizing antigens presented by DCs.

Here we show that efficient induction of Treg cells relies on a specific T cell stimulation by DCs that use BTLA and CD5-dependent mechanisms to actively adjust tolerizing T cell responses under steady state conditions. In contrast, T cells activated by DCs in the absence of BTLA and HVEM-mediated signals do not upregulate CD5 and are therefore susceptible to inhibition of Foxp3 expression by effector cell-differentiating cytokines. We propose a model whereby antigens available to most $CD11c^+$ DCs that do not express BTLA ($BTLA^{neg}$), fail to provide a BTLA-mediated signal to upregulate T cell expression of CD5 that promotes induction of pTreg cells. In contrast, only specific T cell stimulation by tolerogenic $BTLA^+$ DCs induces pTreg cells and tolerance.

RESULTS

$CD11c^+$ DCs fail to alter a long-term antigen-specific autoimmune response

Presentation of myelin oligodendrocyte glycoprotein (MOG) to T cells by peripheral DCs prevents subsequent experimental acute encephalomyelitis (EAE) (Hawiger et al., 2004; Idoyaga et al., 2013; Jones et al., 2015; Yogev et al., 2012). To examine the impact on such tolerance by antigens that are acquired and presented by the entire population of $CD11c^+$ DCs, we developed a recombinant chimeric antibody (denoted here as αCD11c-MOG) to deliver MOG_{35-55} peptide to all $CD11c^+$ DCs *in vivo*. CD11c mediates efficient antigen uptake for presentation to T cells (Castro et al., 2008). To help exclude a non-specific targeting by αCD11c-MOG, we used the same mutated mouse heavy and light constant regions that we previously designed for the highly specific αDEC-MOG that delivers MOG to $DEC205^+$ DCs (Hawiger et al., 2001; Hawiger et al., 2004) (Figure S1A) and **Experimental Procedures**. We confirmed targeting *in vivo* of αCD11c-MOG to subsets of $CD11c^+$DCs that either expressed or not expressed both DEC205 and CD8α ($DEC205^+CD8^+$ and $DEC205^{neg}CD8^{neg}$ DC) and verified the specific activation and proliferation of T cells (Figure S1B–D). We then pre-treated multiple groups of B6 mice

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with αCD11c-MOG and αDEC-MOG 6 weeks before we immunized such mice with MOG_{35-55} in adjuvant to induce EAE. In agreement with previously published results (Hawiger et al., 2004; Jones et al., 2015), MOG delivered to $DEC205^{+}$ DCs, prevented EAE and spinal cord T cell infiltrations. In contrast, a treatment with MOG delivered to all $CD11c^{+}$ DCs had no impact on subsequently induced EAE (Figure 1A and B). We conclude that antigens available to all $CD11c^{+}$ DCs fail to provide long-lasting specific tolerance.

The pTreg cells induced *de novo* by DCs are required for long-lasting tolerance to block responses by newly enlisted encephalitogenic T cells (Jones et al., 2015). Therefore we examined the *de novo* conversion of $Foxp3^{+}$ Treg cells from MOG-specific (2D2) Foxp3-neagtive ($Foxp3^{neg}$) T cells responding to MOG delivered to either all $CD11c^{+}$ DCs or only $DEC205^{+}$ DCs. As reported previously (Jones et al., 2015), we observed pTreg cells persisted in animals treated with αDEC-MOG. In contrast, and consistent with the absence of tolerance from EAE in mice 6 weeks after the treatment with αCD11c-MOG, we found negligible numbers of pTreg cells but similar small numbers of $Foxp3^{neg}$ T cells in all groups (Figure 1C and D). Further, we observed no transient induction of Foxp3 expression in such T cells at multiple earlier time points after a treatment with αCD11c-MOG (Figure S1E). Therefore T cell activation by all $CD11c^{+}$ DCs does not efficiently induce pTreg cells.

$CD11c^{+}$ DCs can induce specific effector cell responses (Iwasaki and Medzhitov, 2015; Palucka and Banchereau, 2013). Although effector T cells undergo skewing typically under pro-inflammatory conditions, $CD11c^{+}$ DCs could induce opposing tolerogenic and encephalitogenic T helper-1 (Th1) and Th17 effector T cell responses in steady state. However, we found only similar low expression of the corresponding transcription factors TBX21 and RORγt (Figure 1E and F). The failure of $CD11c^{+}$ DCs to differentiate T cells into either pTreg cells or effector T cells indicated anergic phenotype of such T cells consistent with a failure to expand to a re-challenge with MOG peptide (Figure S1F and G). Also, mice pre-treated with αCD11c-MOG became resistant to EAE induced 1 week later (Figure S1H). However, in the absence of the *de novo* conversion of pTreg cells, this $CD11c^{+}$ DC-induced tolerance was only transient and mice became susceptible to EAE within 6 weeks (Figure 1A and B). Overall, we conclude that T cell activation by all $CD11c^{+}$ DCs in steady state leads to a transient unresponsiveness of T cells that do not persist and fail to differentiate into pTreg cells to prevent a subsequent antigenic challenge.

To further extend our studies, we developed αCD11c-OVA (**Experimental Procedures**). We then examined cognate OVA-specific (OTII) T cell responses to OVA peptide delivered to either all $CD11c^{+}$ DCs or only $DEC205^{+}$ DCs using αDEC-OVA. As expected, we found about 21% of pTreg cells within 12 days after treatment with αDEC-OVA. In contrast, we found only negligible numbers of pTreg cells after treatment with αCD11c-OVA (Figure 1G and H), despite a similar activation of T cells in αDEC-OVA and αCD11c-OVA-treated groups (Figure S1I). We conclude that $CD11c^{+}$ DCs fail to induce pTreg cells from T cells of various TCR-specificity.

$DEC205^{+}CD8^{+}$ DCs promote induction of pTreg cells

$DEC205^{+}CD8^{neg}$DCs have been proposed to induce Treg cells (Idoyaga et al., 2013). However, other studies suggested a preferential induction of iTreg cells by $DEC205^{+}CD8^{+}$

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DCs (Wang et al., 2008; Yamazaki et al., 2008). To determine such roles of DEC205^{+}CD8^{+} DCs *in vivo*, we used *Batf3*$^{-/-}$ mice characterized by reduced numbers of these DCs (Hildner et al., 2008). 12 and 21 days after a treatment with αDEC-MOG we observed, correspondingly, only about 5–10% and 13% conversion of specific T cells into pTreg cells in the *Batf3*$^{-/-}$ mice, whereas the pTreg cell induction in *Batf3*$^{+/+}$ mice remained at about 22% and 36%, respectively (Figure 2A–D). Therefore similar to the functions in thymus (Perry et al., 2014), peripheral Batf3-dependent DEC205^{+}CD8^{+} DCs induce pTreg cells *in vivo* when antigen is delivered specifically to these DCs.

The failure to induce pTreg cells by CD11c^{+} DCs that include DEC205^{+}CD8^{+} DCs (Figure 1) could result from targeting through the CD11c integrin receptor. Alternatively, induction of pTreg cells may be inefficient in the presence of the majority of non-DEC205^{+}CD8^{+} DCs presenting the same antigen. Therefore we used a *Itgax*^{cre}*Irf4*$^{fl/fl}$ (*Irf4*$^{-/-}$) genetic mouse model characterized by a proportional increase of DEC205^{+}CD8^{+} DCs (Figure S2A) due to a reduction of numbers of DEC205^{neg}CD8^{neg} DCs (Persson et al., 2013; Schlitzer et al., 2013). We found that in *Irf4*$^{-/-}$ mice treated with αCD11c-MOG the pTreg cell conversion rate increased about 3–6 fold compared to the corresponding pTreg cell induction in *Irf4*$^{+/+}$ mice, despite similar T cell activation in both types of recipients (Figure 2E and F and Figure S2B). This indicates that pTreg cell conversion depends on the proportion of the DEC205^{+}CD8^{+} DCs among all CD11c^{+} DCs that can present the same antigen. We collectively conclude that T cells stimulated predominantly by DEC205^{+}CD8^{+} DCs are converted to pTreg cells *in vivo*.

Tolerogenic induction of pTreg cells depends on BTLA

The immunemodulatory molecules such as PD-L1 expressed by CD8^{+} DCs help mediate the induction of Treg cells by these DCs (Shortman and Heath, 2010; Wang et al., 2008). However, it remains unclear how a conversion of pTreg cells is specifically determined by tolerogenic DCs *in vivo*. CD8^{+} DCs express BTLA (Flynn et al., 2013) and we confirmed BTLA to be specifically expressed in DEC205^{+}CD8^{+} DCs from multiple lymphoid organs (Figure 3A and S3A). We therefore hypothesized that these DCs may require BTLA to induce pTreg cells.

To test such a role for BTLA, we tested an impact on pTreg cell conversion of blocking BTLA by αBTLA (6A6) (Hurchla et al., 2005) that prevents BTLA binding to its ligand HVEM. In mice treated with αBTLA we found a consistent about 70% decrease in conversion of Foxp3^{+} pTreg cells (Figure 3B and C). In contrast, blocking of BTLA did not alter expression of effector transcription factors TBX21 and RORγt in the remaining T cells or initial T cell activation following αDEC-MOG treatment (Figure S3B and C).

A treatment with αBTLA could also impact functions of BTLA expressed in T cells (Albring et al., 2010). Therefore we used *Btla*$^{-/-}$ *and Btla*$^{+/+}$ mice as 2D2 T cell recipients. We found that the specific pTreg cell conversion in *Btla*$^{-/-}$ recipient mice treated with αDEC-MOG decreased by about 50–60% compared to *Btla*$^{+/+}$ recipients (Figure 3D and E) despite similar specific T cell activation and similar numbers of DEC205^{+}CD8^{+} DCs in *Btla*$^{-/-}$ and *Btla*$^{+/+}$ recipient mice (Figure S3D and E). We also observed an increased induction of iTreg cells by *Btla*$^{+/+}$ DEC205^{+}CD8^{+} DCs as compared to DEC205^{neg}CD8^{neg}

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DCs and *Btla*$^{-/-}$ DEC205^{+}CD8^{+} DCs (Figure S3F and G). This was accompanied in cultured DCs by a partial, non-specific down-regulation of BTLA expression consistent with its moderate impact on *in vitro* iTreg cell induction (Figure S3H). In contrast to specific functions of BTLA in DCs, we observed a similar conversion of pTreg cells from *Btla*$^{-/-}$ or *Btla*$^{+/+}$ T cells *in vivo* (Figure S3I and J). We collectively conclude that BTLA promotes an efficient *de novo* conversion of Treg cells by DCs.

A genetic deletion of BTLA increases severity of EAE (Watanabe et al., 2003). Therefore to examine the role of BTLA in specific anti-EAE tolerance, we pre-treated B6 mice with αDEC-MOG followed by a treatment with αBTLA. After 6 weeks we then immunized such mice to induce EAE. We observed a similar EAE induction in groups pre-treated with either 6A6 αBTLA antibody or an isotype control in the absence of MOG targeting to DCs, consistent with a lack of an impact of a transient blockade of BTLA on EAE induced 6 weeks later. In contrast, in mice treated with αDEC-MOG, despite an initial resistance to EAE, a concomitant blocking of BTLA abolished the DC-induced anti-EAE tolerance in agreement with the role of BTLA in induction by DCs of pTreg cells to efficiently inhibit EAE (Figure 3F and G).

BTLA and HVEM function to upregulate CD5 expression in peripheral T cells

2D2 T cells in mice treated with αDEC-MOG respond with an increased expression of CD5 that precedes a *de novo* induction of Foxp3 expression (Henderson et al., 2015). In contrast, we found an unaltered expression of CD5 in T cells from mice treated with either PBS or αCD11c-MOG (Figure S4A). Therefore consistent with the inefficient conversion of pTreg cells in response to stimulation by all CD11c^{+} DCs, these DCs also fail to increase expression of CD5 in T cells. However, T cells upregulated CD5 expression in response to MOG delivered by αCD11c-MOG in *Irf4*$^{-/-}$ mice that have an increased proportion of BTLA^{+}DEC205^{+}CD8^{+} DC, consistent with the role of these DCs in mediating pTreg cell induction (Figure 4A). To determine if BTLA functions are required for this specific increase of CD5 expression, we examined adoptively transferred 2D2 T cells responding to MOG delivered to DEC205^{+}CD8^{+} DCs in *Btla*$^{-/-}$ *and Btla*$^{+/+}$ recipient mice. As expected in *Btla*$^{+/+}$ mice Foxp3^{neg} T cells increased expression of CD5. However, expression of CD5 remained low in T cells recovered from *Btla*$^{-/-}$ mice (Figure 4B-**upper panel**). Further, T cells stimulated *in vitro* by *Btla*$^{+/+}$ DEC205^{+}CD8^{+} DCs had higher expression of CD5 than T cells responding to DEC205^{neg}CD8^{neg} DCs or *Btla*$^{-/-}$ DEC205^{+}CD8^{+} DCs, further consistent with the specific functions of BTLA to mediate CD5 induction (Figure S4B). In contrast, we observed a similar activation and CD5 expression in *Btla*$^{+/+}$ and *Btla*$^{-/-}$ T cells *in vivo* (Figure S4C and Figure 4B-lower panel). We collectively conclude that BTLA is required for the DEC205^{+}CD8^{+} DC-mediated upregulation of CD5 expression in T cells.

To investigate if engagement of HVEM, a receptor for BTLA, upregulates expression of CD5, we cross-linked HVEM in naïve sorted Foxp3^{neg}CD25^{neg} 2D2 and polyclonal CD4^{+} T cells also activated through TCR stimulation. We found that an additional engagement of HVEM upregulated surface expression of CD5 and increased *Cd5* gene expression (Figure S4D and Figure 4C and D). HVEM can govern multiple signaling pathways in immune and epithelial cells (Shui et al., 2012; Shui et al., 2011; Steinberg et al., 2011). In developing

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$CD5^{hi}$ T cells a specific engagement of HVEM had no impact on STAT3 phosphorylation and a minimal impact on TCR-induced NFkB activation (Figure S4E-G). In contrast, HVEM engagement increased phosphorylation of mitogen-activated protein kinase (MAPK) kinase (MEK) (Figure 4E). MEK activation can increase expression of E26 avian leukemia oncogene 1 (ETS1) as well as decrease an expression of transcription factor 3 (TCF-3 also known as E2A or E47), correspondingly, positive and negative regulators of *Cd5* expression (Khanna et al., 2011; Page et al., 2004; Paumelle et al., 2002; Tung et al., 2001; Yang et al., 2004). We found a decreased expression of E2A (E47) and an increased expression and abundance of ETS1 in developing $CD5^{hi}$ T cells, consistent with their specific functions in transcriptional regulation of *Cd5* expression (Figure 4F–H). We conclude that HVEM governs in developing $CD5^{hi}$ T cells pathways regulating expression of CD5.

HVEM-mediated upregulation of CD5 relieves inhibition of Treg cell induction by effector cell-differentiating cytokines

The $CD5^{hi}$ T cells, but not $CD5^{lo}$ or $Cd5^{-/-}$ T cells, can efficiently convert into Treg cells in the presence of effector cell-differentiating cytokines (Henderson et al., 2015). We engaged HVEM in naïve $Cd5^{+/+}$ and $Cd5^{-/-}$ 2D2 $CD4^+$ T cells cultured under Treg cell-differentiating conditions and in the absence or presence of IL-4 or IL-6 (Figure 5A–D). We found a similar high rate of Treg cell induction in $Cd5^{+/+}$ and $Cd5^{-/-}$ T cells in the absence of these cytokines. Without an engagement of HVEM, IL-4 or IL-6 decreased such Treg cell induction by about 60–80% both in $Cd5^{+/+}$ and $Cd5^{-/-}$ T cells. However, consistent with its role to increase expression of CD5, an engagement of HVEM overcame an IL-4 and IL-6-mediated inhibition of Treg cell induction and over 70% and 50% of $Cd5^{+/+}$ T cells converted to Treg cells despite the presence of IL-4 and IL-6, respectively (Figure 5A–D). In contrast, a conversion of $Cd5^{-/-}$ T cells into Treg cells remained low at about 37% and 21% in the presence of IL-4 and IL-6, respectively, despite an engagement of HVEM (Figure 5A–D), In contrast, an engagement of HVEM had no effect on the induction of iTreg cells in the absence of IL-4 or IL-6. We also observed a similar CD5-dependent promotion of Treg cell induction upon engagement of HVEM in polyclonal T cells (Figure S5A and B). Thus an engagement of HVEM in T cells leads to the upregulation of CD5 expression to promote induction of Treg cells.

DISCUSSION

In this study we have identified a crucial mechanism governing induction of extrathymic Treg cells and tolerance by DCs. BTLA and HVEM-mediated functions increase in T cells the expression of CD5 to instruct Treg cell differentiation. Thus, only T cells specifically activated by $BTLA^+DEC205^+CD8^+$ DCs efficiently convert into tolerogenic pTreg cells. In contrast, Treg cell conversion is impaired in T cells that respond to antigens presented by the majority of $CD11c^+$ DCs that are $BTLA^{neg}$.

$CD11c^+$ DCs in lymphoid tissues constitute phenotypically heterogeneous populations whose development is dependent on distinct transcription factors (Satpathy et al., 2012). This diversity matches the versatile T cell responses induced by such DCs (Guilliams et al., 2014; Mellman, 2013; Steinman, 2012). In the pro-inflammatory environment, priming of T

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cells by DCs leads to immune responses. In contrast, the presentation of antigens by DCs in steady state can result in multiple mechanisms of T cell tolerance (Hawiger et al., 2001; Ohnmacht et al., 2009; Probst et al., 2003; Steinman et al., 2003). However, a broad tolerogenic outcome of antigenic presentation by DCs in steady state could hamper subsequent protective immune responses against cross-reactive antigens. Therefore induction of tolerance by DCs may rather be mediated by specialized “tolerogenic” DCs to enhance the specificity of such immune regulation (Coquerelle and Moser, 2010; Merad et al., 2013). Yet, despite the proposed tolerance-inducing functions of some DCs, a precise functional definition of tolerogenic DCs remains elusive. The proposed populations of tolerogenic DCs expressing CD8, DEC205 or CD103, include the “lymphoid resident” DCs and migratory DCs that transfer peripheral antigens to the lymphoid tissues (Coquerelle and Moser, 2010; Lukacs-Kornek and Turley, 2011; Merad et al., 2013; Randolph et al., 2008; Shortman and Heath, 2010). However, it remains unknown if the induction of tolerance requires T cell activation by antigens whose presentation is restricted to tolerogenic DCs. Our findings that only antigens presented specifically by BTLA$^+$DEC205$^+$CD8$^+$ DCs induce T cell tolerance, support a model of tolerance that relies on a compartmentalization of antigenic presentation by various DC types. Our results further indicate that by governing the crucial tolerogenic functions of DCs, BTLA helps to further shape a plasticity of DC-mediated T cell responses determined by various pathways (Iwasaki and Medzhitov, 2015; Kumamoto et al., 2013; Sancho and Reis e Sousa, 2012; Segura and Amigorena, 2013; Steinman, 2012). In contrast, the roles in specific immune responses of BTLA expressed solely in various types of immune cells require further investigation.

A conversion of Foxp3$^+$ pTreg cells by DCs mediates tolerance and induction of pTreg cells by DCs is independent of other potential antigen presenting cells (Esterhazy et al., 2016; Hawiger et al., 2010; Jones et al., 2015; Kretschmer et al., 2005). Several mechanisms proposed to mediate the induction of Treg cells by DCs rely on specific cytokines and metabolites as well as signaling by PD-1 and CTLA-4 (Coombes et al., 2007; Francisco et al., 2009; Li and Flavell, 2008; Manicassamy et al., 2009; Mascanfroni et al., 2013; Mucida et al., 2007; Probst et al., 2005; Sun et al., 2007; Wang et al., 2008; Yogev et al., 2012). Although the relevant mechanisms by which these immunomodulatory pathways induce Foxp3$^+$ Treg cells remain incompletely understood, such pathways may directly affect the cell-intrinsic mechanisms that induce Foxp3 expression (Benoist and Mathis, 2012; Josefowicz et al., 2012a).

However, the regulation of immune tolerance also depends on the specificity of T cells to self and tolerizing antigens as reflected by expression of CD5 (Azzam et al., 1998; Klein et al., 2014). CD5 is a surface protein that recruits multiple positive and negative regulators of T cell signaling (Soldevila et al., 2011). CD5 expression parallels T cell receptor (TCR) signal strength during thymic T cell development leading to an increased CD5 expression in developing CD4$^+$ T cells bearing high affinity TCR for self-antigens (Azzam et al., 1998). Despite the functions of CD5 as a negative regulator of TCR signaling in thymus, the peripheral CD5^{hi} T cells remain responsive to antigenic stimulation and can form cross-reactive effector T cells thereby risking the development of autoimmune responses (Klein et al., 2014; Mandl et al., 2013; Persaud et al., 2014). Recently, we established that CD5 instructs the conversion of such self-reactive and potentially autoagressive extrathymic

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$CD5^{hi}$ T cells into pTreg cells by modulating their responsiveness to effector cell-differentiating cytokines (Henderson et al., 2015). In addition to thymic development, CD5 expression can also increase in response to peripheral tolerogenic signals (Hawiger et al., 2004). Therefore an upregulation of CD5 expression is a common mechanism for both thymic and peripheral processes to instruct subsequent induction of pTreg cells (Henderson et al., 2015).

However, mechanisms governing in peripheral T cells the expression of CD5 and the impact of such mechanisms on tolerance remained unknown. Our results now indicate that only $BTLA^{+}DEC205^{+}CD8^{+}$ DCs specifically mediate a tolerizing upregulation of CD5 expression in T cells. Therefore, the antigens acquired and presented predominantly by these tolerogenic DCs may be perceived as "tolerizing" by T cells. In contrast, antigens that are available to all $CD11c^{+}$ DCs and presented by a majority of non-tolerogenic $BTLA^{neg}$ DCs fail to provide a BTLA-mediated signal to upregulate CD5 expression in T cells and do not alter the subsequent immune responses in steady state. Our results also reveal that HVEM, a receptor of BTLA, expressed in T cells, governs expression of transcription factors ETS1 and E2A (E47) that regulate expression of CD5. Therefore functions of BTLA and HVEM enable DCs to link the T cell responsiveness to specific tolerogenic antigens with a CD5-dependent process governing pTreg cell induction to maintain the *de novo*-induced peripheral T cell tolerance. In the absence of these specific functions of BTLA and CD5, the steady state conditions alone may not facilitate a sufficient induction of tolerance by DCs. Therefore, we propose that through the combined functions of BTLA and CD5 tolerogenic DCs can actively adjust T cell responses by favoring a differentiation of $CD5^{hi}$ T cells that can more readily become tolerizing pTreg cells.

The differences in cytokine milieu between *in vivo* and *ex vivo* environments as well as phenotypic alterations of DCs *in vitro* may contribute to the specific variations of especially iTreg cell induction, also possibly regulated by multiple mechanisms (Wang et al., 2008; Yamazaki et al., 2008). However, the functions of BTLA and HVEM are both necessary and sufficient to promote a differentiation of Treg cells that are crucial to govern T cell tolerance and prevention of autoimmunity by DCs *in vivo.*

In conclusion, by showing that induction of pTreg cells relies on specific DCs that use BTLA and CD5-dependent mechanisms to actively adjust T cell responses, our findings help to further define and extend a concept of tolerogenic DCs. In contrast, a majority of $CD11c^{+}$ DCs are $BTLA^{neg}$ and therefore are not tolerogenic even in the steady state. Thus a selective induction of pTreg cells by specialized tolerogenic functions of $BTLA^{+}$ DCs may promote tolerance while helping to maintain an overall high plasticity of immune responses among the total repertoire of T cells that respond to antigens presented by all DCs.

EXPERIMENTAL PROCEDURES

For complete experimental procedures see supplemental information.

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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

Chimeric antibodies were produced as previously described (Hawiger et al., 2001; Hawiger et al., 2004). 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

See supplemental experimental procedures.

Adoptive transfers

Lymph nodes and spleen cells 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). 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 100μg synthetic Myelin Oligodendrocyte Glycoprotein peptide (MOG$_{35\text{-}55}$, Yale Keck Protein Synthesis Facility) in Complete Freund's Adjuvant subcutaneously in each flank. *Pertussis Toxin* (List Biological Laboratories Inc.) was injected 200ng per mouse in PBS intraperitoneally on days 0 and 2 after MOG$_{35\text{-}55}$ injections. Clinical score of EAE was graded on a scale of 1–4.

Cell cultures

Lymph node and spleen cells were pooled and CD4^{+} T cells were enriched using magnetic microbeads (Miltenyi) and then sorted on ARIA III (BD). 0.25x10^{6}/well T cells were cultured 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, β-Mercaptoethanol, Sodium pyruvate (Gibco), recombinant IL-2 (200 units/ml), and in some

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experiments TGF-β (1–4 ng/ml) (BioLegend). Anti-CD3 was cross-linked by addition of anti-Armenian Hamster IgG (15 μg/ml) after 20 min incubation at 37°C. In some experiments recombinant IL-4 (2ng/ml) or IL-6 (8ng/ml) were additionally added. 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 and were cross-linked by addition of anti-Armenian Hamster IgG (15 μg/ml) after 20 min incubation at 37°C. 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). 0.35–0.7x10^5/well DCs and $CD11c^{neg}$ cells were cultured with 0.2x10^6/well T cells in RPMI-1640 media containing 10% FBS, Penicillin-Streptomycin, L-Glutamine, β-Mercaptoethanol (Gibco), and Sodium pyruvate (Gibco). In some experiments synthetic MOG_{35-55} (60 μg/ml), recombinant IL-2 (200 units/ml), and TGF-β (2 ng/ml) were also 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

See supplemental experimental procedures.

Statistical analysis

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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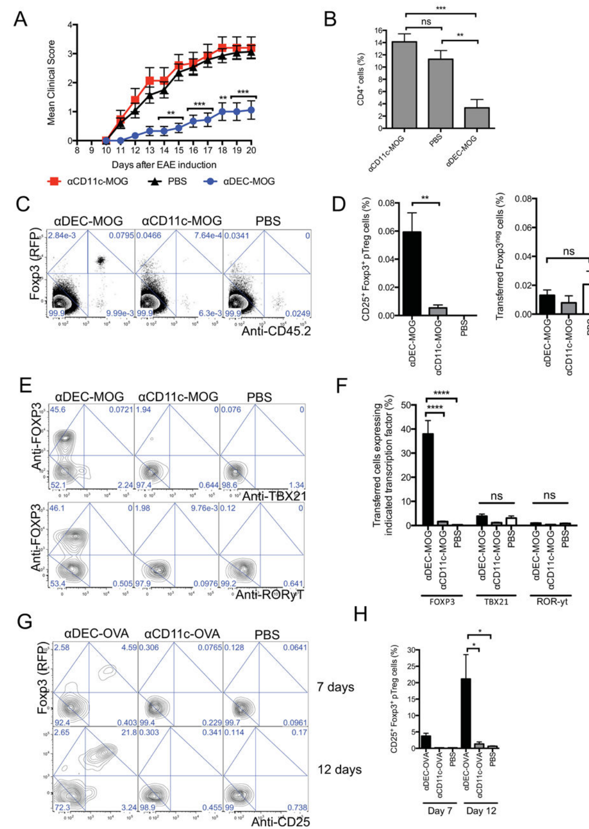

Figure 1. Activation of T cells by CD11c+ DCs in steady state has no lasting impact on autoimmune responses (A and B)

Targeting of MOG to all CD11c+ DCs fails to prevent subsequently induced EAE. Mice were treated with chimeric antibodies as indicated 6 weeks before induction of EAE. **(A)** Graphs show mean EAE disease scores (n=10–20). **(B)** Results show mean percentages of CD4+ T cells in spinal cords 19 days after EAE induction (n=3–4). (**C** and **D**) CD11c+ DCs fail to induce pTreg cells in steady state. Sorted 2D2 Foxp3negCD25neg T cells were adoptively transferred and analyzed by flow cytometry 6 weeks after treatment of recipients with chimeric antibodies as indicated. **(C)** Plots show Foxp3 (RFP) expression and anti-CD45.2 staining intensity among total CD4+ T cells from splenocytes. **(D)** Graphs show percentages of remaining Foxp3negCD25neg T cells and converted Foxp3+CD25+ pTreg cells among total CD4+ splenocytes (n=5–7). (**E** and **F**) Expression of transcription factors. Mice treated as in (C and D) and analyzed after 2 weeks**. (E)** Plots show anti-FOXP3 and anti-TBX21 **(upper panel)** or anti-RORyt **(lower panel)** intracellular staining intensity in transferred CD4+ T cells among splenocytes. **(F)** Graphs show percentages of cells that were positively stained for FOXP3, TBX21 and RORyt (n=4). (**G** and **H**) CD11c+ DCs fail to induce pTreg cells from T cells of various TCR-specificity. Sorted OTII Foxp3negCD25neg T cells were transferred into recipient mice and analyzed by flow cytometry at indicated days after indicated treatments. **(G)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in transferred CD4+ T cells from splenocytes. **(H)** Graphs show percentages of pTreg cells (n=3). **(C, E and G)** Numbers in quadrants indicate corresponding percentages. Results represent one of two to four similar experiments. **(A, B, D, F and H)** Graphs show

mean +/− standard error of mean (SEM), * P< 0.05, ** P< 0.01 ***, P< 0.001 and **** P< 0.0001 determined by one-way or two-way ANOVA, n = number of mice per group from two to four independent experiments. Please see also Figure S1.

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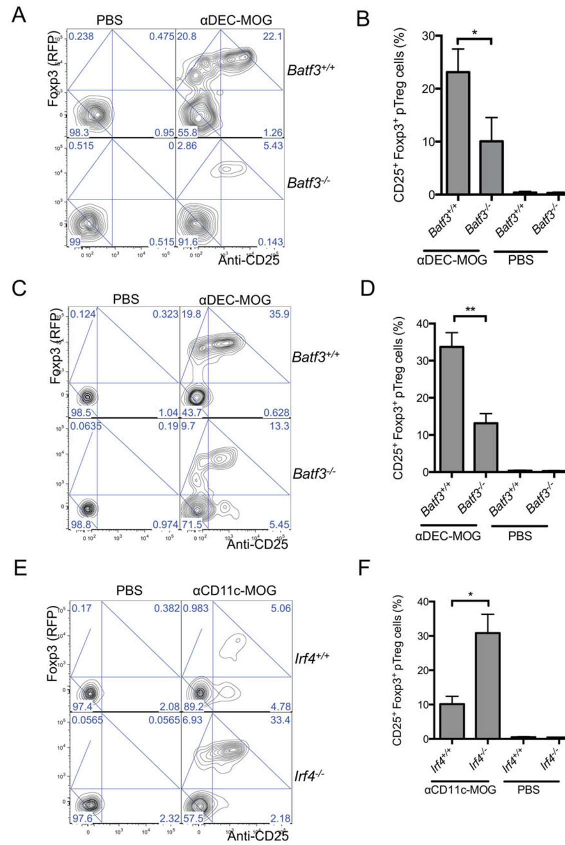

Figure 2. DEC205$^+$CD8$^+$ DCs mediate induction of pTreg cells

(A–D) Batf3-dependent DCs are required for pTreg cell induction. Sorted 2D2 Foxp3^{neg}CD25^{neg} T cells were adoptively transferred into *Batf3*$^{+/+}$ and *Batf3*$^{-/-}$ recipient mice that were treated as indicated. Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in transferred CD4$^+$ T cells from splenocytes analyzed by flow cytometry after 12 days **(A)** or 21 days **(C)**. Graphs show percentages of pTreg cells (n=4–5) after 12 days **(B)** or 21 days **(D)**. **(E and F)** Conversion of pTreg cells depends on the proportion of the DEC205$^+$CD8$^+$ DCs among CD11c$^+$ DCs. Cells as in (A) were transferred into *Irf4*$^{-/-}$ and *Irf4*$^{+/+}$ recipient mice treated as indicated. **(E)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in transferred CD4$^+$ T cells from splenocytes analyzed by flow cytometry after 21 days. **(F)** Graphs show percentages of pTreg cells (n=9–11). **(A, C and E)** Numbers in quadrants indicate corresponding percentages. Results represent one of three to four similar experiments. (**B, D and F**) graphs show mean +/− SEM, * $P< 0.05$ and ** $P< 0.01$ determined by one-way ANOVA, n = number of mice per group from three to four independent experiments. Please see also Figure S2.

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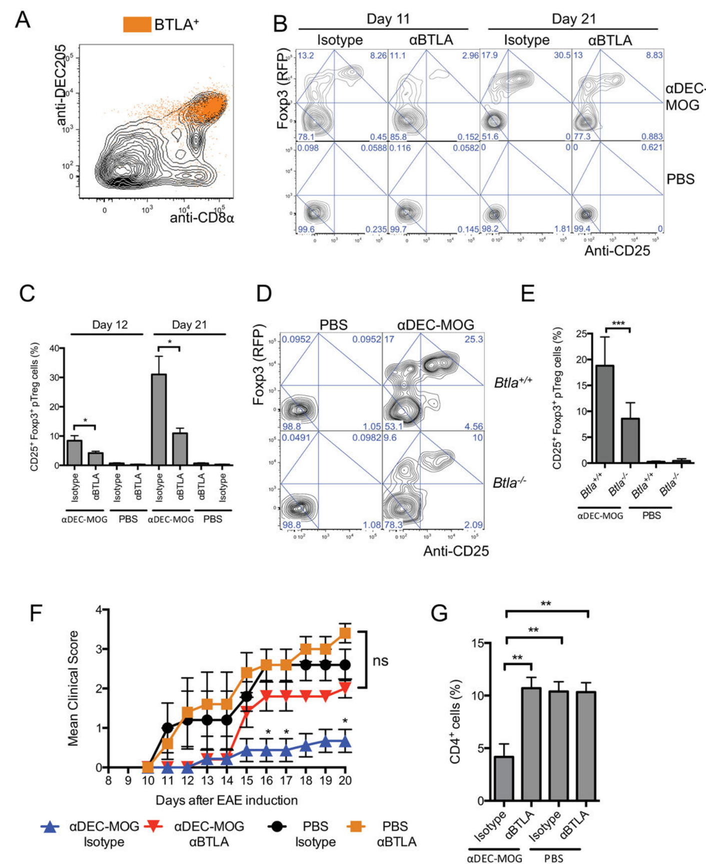

Figure 3. BTLA is required for induction of tolerogenic pTreg cells

(A) BTLA is specifically expressed in DEC205+CD8+ DCs. The plot shows anti-DEC205 and anti-CD8α staining intensities analyzed by flow cytometry in splenic CD11c+MHCII+ DCs with superimposed distribution of BTLA+ events. **(B and C)** Blocking of BTLA reduces DC-mediated pTreg cell conversion. 2D2 Foxp3negCD25neg T cells were transferred into recipient mice treated with chimeric antibodies and αBTLA or isotype control as indicated. **(B)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in transferred CD4+ T cells from splenocytes analyzed by flow cytometry after 11 and 21 days. **(C)** Graphs show percentages Foxp3+CD25+ pTreg cells (n=4–5). **(D and E)** BTLA is required for DC-mediated pTreg cell induction. 2D2 Foxp3negCD25neg T cells were adoptively transferred into *Btla*+/+ and *Btla*−/− recipient mice that were treated as indicated. **(D)** Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity in transferred CD4+ T cells from splenocytes analyzed by flow cytometry after 9 days. **(E)** Graphs show percentages of pTreg cells (n=4–6). **(F and G)** Blocking of BTLA abolishes DC-induced tolerance against EAE. Mice were treated as indicated and subsequently treated with αBTLA or isotype control 6 weeks before induction of EAE. **(F)** Graphs show mean EAE disease scores (n=10–20). **(G)** Results show mean percentages of CD4+ T cells in spinal cords 19 days after EAE induction (n=3–4). **(B and D)** Numbers in quadrants indicate corresponding percentages. **(A, B and D)** Results represent one of three similar experiments. **(C, E, F and G)** Graphs show mean +/– SEM, * $P< 0.05$, ** $P< 0.01$ and *** $P< 0.001$

determined by one-way or two-way ANOVA, n = number of mice per group from two to three independent experiments. Please see also Figure S3.

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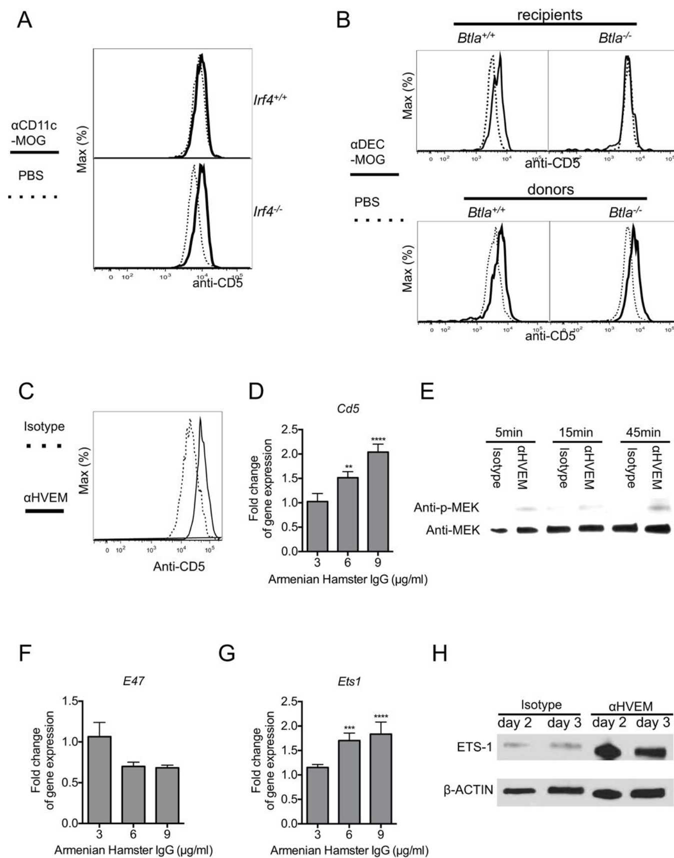

Figure 4. Upregulation of CD5 in T cells depends on BTLA and HVEM functions

(A) $BTLA^+DEC205^+CD8^+CD11c^+$ DCs increase CD5 expression in T cells *in vivo.* 2D2 $Foxp3^{neg}CD25^{neg}$ T cells were adoptively transferred into *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ recipient mice treated as indicated. Overlaid histograms show staining intensity with anti-CD5 in transferred $Foxp3^{neg}$ T cells among splenocytes analyzed by flow cytometry after 9 days. **(B)** BTLA is required for DC-mediated upregulation of CD5 in T cells. Upper panel - 2D2 $Foxp3^{neg}CD25^{neg}$ T cells were transferred into *Btla*$^{+/+}$ and *Btla*$^{-/-}$ recipient mice that were treated as indicated. Lower panel - *Btla*$^{+/+}$ and *Btla*$^{-/-}$ 2D2 $Foxp3^{neg}CD25^{neg}$ T cells were transferred into recipient mice that were treated as indicated. Overlaid histograms show staining intensity with anti-CD5 in transferred $Foxp3^{neg}$ $CD4^+$ T cells among splenocytes analyzed by flow cytometry after 9 days. **(C)** Engagement of HVEM in T cells induces CD5 upregulation. Naïve 2D2 $Foxp3^{neg}CD25^{neg}$ $CD4^+$ T cells were stimulated *in vitro* for 3 days with αCD3 and αCD28 and in the presence of either αHVEM or isotype control followed by cross-linking with a secondary reagent. Overlaid histograms show CD5 expression in $Foxp3^{neg}$ $CD4^+$ T cells as indicated. **(D)** Engagement of HVEM in T cells induces *Cd5* gene expression. Naïve 2D2 $CD4^+Foxp3^{neg}CD25^{neg}$ T cells were stimulated for 2 days *in vitro* in the presence of either αHVEM or isotype control at indicated concentrations followed by cross-linking with a secondary reagent. Gene expression was analyzed by quantitative real-time RT-PCR, normalized for expression of HPRT and calculated using CT method. Graph shows a fold difference in αHVEM over isotype treated with an arbitrary value indicating no change set at 1. **(E)** Engagement of HVEM in T cells activates MEK

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phosphorylation. Immunoblot analysis in lysates of T cells stimulated as in **(D)** and for the indicated times. **(F and G)** Engagement of HVEM in T cells reduces *E47* gene expression **(F)** and induces *Ets1* gene expression **(G)**. T cells were stimulated and gene expression analyzed as in **(D). (H)** Engagement of HVEM in T cells increases ETS1 protein expression. Immunoblot analysis in lysates of T cells stimulated as in **(D)** and for the indicated times. **(A, B, C, E, and H)** Results represent one of two to three similar experiments. **(D, F, and G)** Results (n=4) from two independent experiments represent mean +/− SEM. ** p 0.01 *** p 0.001 **** p 0.0001, analyzed by two-way ANOVA. Please see also Figure S4.

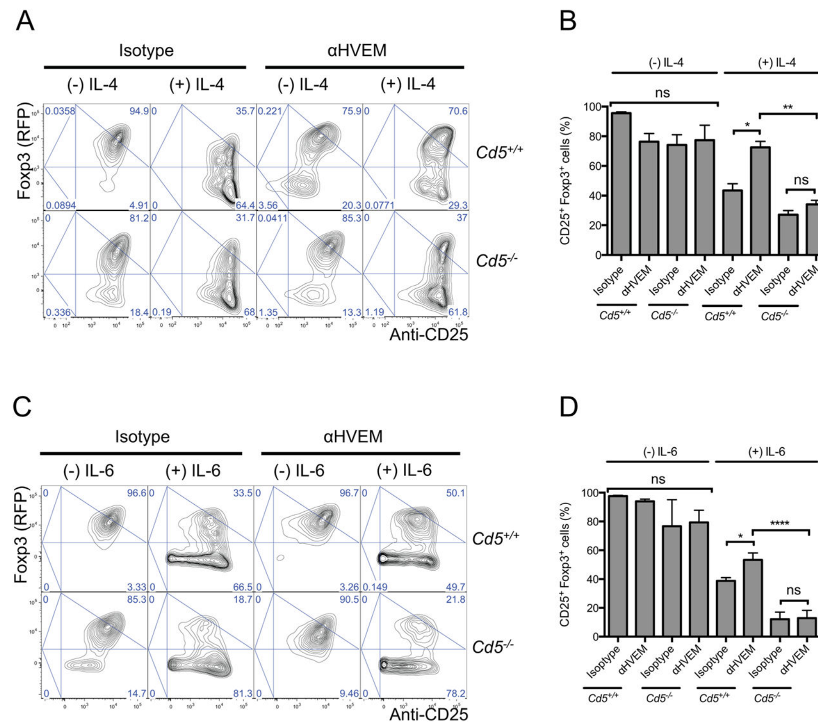

Figure 5. Engagement of HVEM in T cells induces CD5-dependent Treg cell induction

(A and B) HVEM-mediated upregulation of CD5 relieves inhibition of Treg cell induction by IL-4. **(A)** Naïve *Cd5*$^{+/+}$ and *Cd5*$^{-/-}$ 2D2 Foxp3^{neg}CD25^{neg} CD4^{+} T cells were stimulated for 5 days *in vitro* with αCD3 and αCD28 in the presence of TGF-β and αHVEM or isotype control followed by cross-linking with a secondary reagent and also in the presence or absence of IL-4. Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity. **(B)** Graphs show percentages of Foxp3^{+}CD25^{+} cells (n=3–4). **(C and D)** HVEM-mediated upregulation of CD5 relieves inhibition of Treg cell induction by IL-6. **(C)** T cells were cultured as in (A) but in the presence or absence of IL-6. Plots show Foxp3 (RFP) expression and anti-CD25 staining intensity. **(D)** Graphs show percentages of Foxp3^{+}CD25^{+} cells in the indicated groups (n=3–5). **(A and C)** Numbers in quadrants indicate corresponding percentages. Results represent on of two to three similar experiments. **(B and D)** Results represent mean +/− SEM, * P< 0.05, ** p 0.01 and **** p 0.0001 determined by one-way ANOVA, Results in each group are from two to three independent experiments. Please see also Figure S5.

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