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Regulatory T Cells Condition Lymphatic Endothelia for Enhanced Transendothelial Migration

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SUMMARY

Regulatory T cells (Tregs) express high levels of cell surface lymphotoxin alpha beta (LT α 1 β 2) to activate the LT beta receptor (LT β R) on the lymphatic endothelial cells (LECs), modulating LEC adhesion molecules, intercellular junctions, and chemokines. We demonstrate a role for Tregs through this pathway to condition the permissiveness of lymphatic endothelia for transendothelial migration (TEM), thus gating leukocyte traffic. Human Tregs share the same property with murine Tregs. Activation of TLR2 on Tregs during inflammation specifically augments LT α 1 β 2-LT β R signaling, which further enhances the permissiveness of LECs to facilitate TEM. The conditioning of endothelia may promote the resolution of inflammation by directing leukocytes out of tissues to lymphatic vessels and draining lymph nodes (dLNs). Thus, Tregs interact with lymphatic endothelia under homeostasis and inflammation and dictate endothelial permissiveness and gating mechanisms for subsequent leukocyte migration through endothelial barriers.

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AUTHOR CONTRIBUTIONS

W.P. and J.S.B. designed the research. W.P., Y.X., L.L., V.S., K.D.S., C.P., and W.S. performed the experiments. W.P., R.A., K.L.H., B.R.B., and J.S.B. analyzed the results. W.P. and J.S.B. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.12.083>.

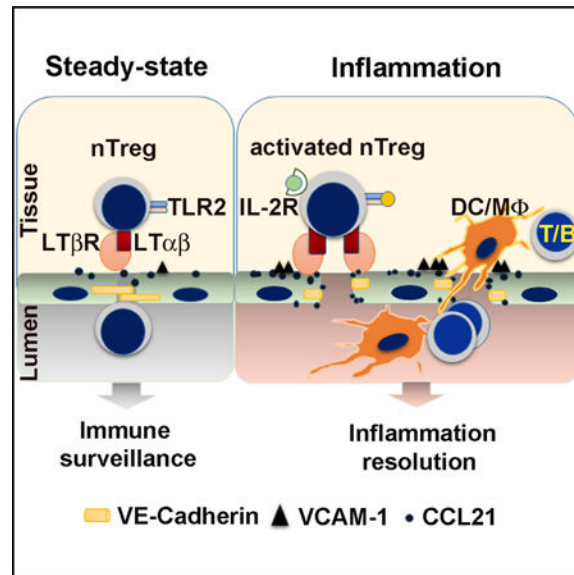
DECLARATION OF INTERESTS

The authors declare no competing interests.

In Brief

Piao et al. demonstrate that Tregs condition lymphatic endothelial cells (LECs) to be more permissive for the transendothelial migration (TEM) of other leukocytes. Activation of Toll-like receptor 2 on Tregs during inflammation specifically augments Treg $LT\alpha 1\beta 2$ expression to intensify $LT\beta R$ signalling in LECs for enhanced immune cell TEM.

Graphical Abstract:



INTRODUCTION

The lymphotoxin alpha beta ($LT\alpha 1\beta 2$) complex is located on the surface of activated lymphocytes. $LT\alpha 1\beta 2$ is well characterized and crucial for lymphatic organ development and orchestration of immune responses. As it is member of the tumor necrosis factor (TNF) superfamily, surface $LT\alpha 1\beta 2$ expression is maintained once induced by activation (Browning et al., 1997; Chiang et al., 2012; English et al., 1991). Importantly, $LT\alpha 1\beta 2$ is found to be preferentially expressed and used by regulatory T cells (Tregs) for afferent lymphatic migration (Brinkman et al., 2016). Tregs express higher levels of $LT\alpha 1\beta 2$ than naive or activated CD4 T cells and engage the LT beta receptor ($LT\beta R$) on lymphatic endothelial cells (LECs) for afferent lymphatic transendothelial migration (TEM) (Piao et al., 2018). $LT\alpha 1\beta 2$ expression on Tregs and $LT\beta R$ expression on LECs are important for the suppressive function of Tregs to migrate to draining lymph nodes (dLNs) and enhance islet allograft survival (Brinkman et al., 2016; Zhang et al., 2009).

The two subunits of $LT\alpha 1\beta 2$ form a transmembrane heterotrimer that interacts with the $LT\beta R$. Dysregulated expression or deficiency of any of the subunits has been linked to autoimmunity and inflammation. Cytokines or protein ligands that regulate $LT\alpha 1\beta 2$ expression have been described in naive T cells (Schneider et al., 2004). However, the differential expression and regulation of $LT\alpha 1\beta 2$ in Tregs or different immune cell subsets during inflammatory responses have not been well characterized. Although the induction of

LT α 1 β 2 in T cells by several distinct signals, including protein kinase C (PKC)-mediated Ets (E26 transformation-specific), nuclear factor κ B (NF- κ B) (p65/Rel), and Egr-1 (early growth response protein 1)/Sp1 (specific protein 1) promoter activation has been reported (Kuprash et al., 1996; Voon et al., 2004), functionally relevant inducers have not been clearly identified.

LT β R is widely expressed in blood and LECs, intestinal epithelial cells, dendritic cells (DCs), and lymph node (LN) stromal cells (Schneider et al., 2004). In LECs, LT β R regulates leukocyte afferent lymphatic TEM (Piao et al., 2018). Although LT β R signals to both classical and non-classical NF- κ B pathways, in LECs it predominantly signals by both a constitutive and ligand-driven non-classical NF- κ B-inducing kinase (NIK) pathway. Constitutive NIK activation in LECs is required for TEM and implicates its importance for all immune cell lymphatic recirculation. LT α 1 β 2/LT β R ligand-driven NIK signaling on LECs triggers increased expression of migration molecules and chemokines, such as CCL21 or CXCL12, resulting in enhanced leukocyte TEM across LECs. Because Tregs preferentially and directly engage LT β R-NIK signaling, we investigated the downstream influence of Treg on LECs to regulate and gate the migration of other immune cells.

Tregs constitutively express the interleukin 2 receptor α chain (IL-2R α ; CD25) and rely on IL-2 for Foxp3 induction and Treg differentiation and maintenance (Sakaguchi et al., 2008). Other signals are likely important for peripheral Treg induction, activation, and function. Of note, Toll-like receptor 2 (TLR2) signaling affects Treg expansion and function (Liu et al., 2006; Nyirenda et al., 2011; Suttmuller et al., 2006). Paired with TLR1 or TLR6 to form heterodimeric receptor complexes on the cell surface, TLR2 recognizes lipoproteins from diverse microbial sources (Akira et al., 2006) and signals through TIRAP/MyD88 (TIR domain-containing adaptor protein/myeloid differentiation primary response gene 88) to activate mitogen-activated protein kinases (MAPKs) and NF- κ B. Several endogenous TLR2 ligands have also been described, including hyaluronan (HA) (Krüger et al., 2010; Tesar et al., 2006), heat shock protein (HSP) 60, HSP Gp96 (Mkaddem et al., 2009), and high-mobility group box 1 (HMGB1) (Matsuoka et al., 2010). HA and HMGB1 are released from transplanted pancreatic islets and promote islet rejection by inducing inflammatory cytokine secretion by immune cells through TLR2 and TLR4 (Bollyky et al., 2012; Kruger et al., 2010). Tregs, conventional T cells, and activated CD4 all reportedly express functional TLR2; yet, the effects of TLR2 on T cell immunity and suppression are diverse, and many reports are even contradictory (Komai-Koma et al., 2004; Liu et al., 2006; Nyirenda et al., 2011). Thus, the precise roles and mechanisms of TLR2 ligands on different T cell subsets and early graft rejection remain incompletely defined.

Here, we observed that IL-2R signals enhanced Treg LT α 1 β 2 expression. In addition, TLR2 signaling specifically augmented IL-2-induced LT α 1 β 2 expression on activated Treg. Enhanced LT α 1 β 2 expression enabled Treg to condition the permissiveness of afferent LECs by LT β R signaling. Conditioned endothelia facilitated TEM and dLN trafficking of other leukocytes, including T cells, B cells, and myeloid cells. These interactions revealed a mechanism for Tregs to regulate endothelial gating and, thus, lymphoid trafficking from tissues to lymphatics and dLNs.

RESULTS

Tregs Express Higher LT α 1 β 2 Than Other CD4 T Cells

LT α 1 β 2 is expressed in activated T cells, although which signaling pathways lead to increased LT α 1 β 2 expression has not been reported (Brinkman et al., 2016; Browning et al., 1997). We analyzed surface LT α 1 β 2 expression on activated T cell subsets stimulated with IL-2 or anti-CD3 monoclonal antibody (mAb). IL-2 led to a dose-dependent increase of LT α 1 β 2 on *in-vitro*-stimulated induced Tregs (iTregs) (Figure 1Ai), whereas activation by anti-CD3 ligation caused a modest LT α 1 β 2 increase, which was further enhanced by anti-CD28 mAb co-stimulation or by stimulation together with IL-2 (Figure 1Aii). Notably, blocking IL-2R with anti-CD25 blocking mAb markedly diminished anti-CD3-activated LT α 1 β 2 expression (Figure 1Aiii), suggesting CD3-mediated LT α 1 β 2 is mostly and indirectly induced by CD3-triggered IL-2 secretion. Analysis of various other CD4 T cell subsets indicated that Tregs, especially iTregs, expressed the highest levels of LT α 1 β 2 (Figure 1B).

Freshly isolated unstimulated-thymus-derived natural Tregs (nTregs) expressed less LT α 1 β 2 than iTregs; however, anti-CD3-activated nTreg expressed comparably high levels of CD25, Foxp3, and LT α 1 β 2 as iTreg (Figure 1C). LT α 1 β 2 was highly expressed on the CD25^{high}Foxp3^{high} iTreg fraction, at an intermediate level on CD25^{int}Foxp3^{int}, and minimally expressed on the CD25^{low}Foxp3 non-Treg portion (Figure 1D). Foxp3 expression has been linked to the T cell receptor (TCR)-phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway (Sauer et al., 2008), and inhibition of PI3K or Akt signaling increases Foxp3 expression. To determine if the LT α 1 β 2 expression was directly dependent on Foxp3 or CD25 expression, we treated iTregs with a PI3K p110 α inhibitor (PI3Ki) or pan-PI3K activator (PI3Ka). PI3Ki-treated Tregs showed enhanced Foxp3 and decreased CD25 and LT α 1 β 2. PI3Ka treatment decreased Foxp3, enhanced LT α 1 β 2, and caused no change in CD25 (Figure 1E). Together, the data suggested LT α 1 β 2 was positively regulated by PI3K/Akt signaling, and LT α 1 β 2 expression was not correlated directly with Foxp3 or CD25 expression.

IL-2R Activation Signals through NF- κ B, and MAPK Pathways Promote LT α 1 β 2 Expression in Tregs

By blocking downstream signaling molecules, we examined IL-2-mediated NF- κ B and MAPK activation pathways for effects on Treg LT α 1 β 2 expression. Blocking NF- κ B activation abolished IL-2-driven LT α 1 β 2 expression in freshly isolated nTregs (Figures 2A and 2Bi). Blocking the MAPKs c-Jun NH₂-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) inhibited LT α 1 β 2 expression to a lesser extent. Immune blot analysis of the Treg cell subsets revealed that nTreg had delayed NF- κ B and minimal JNK activation by IL-2 stimulation compared to iTregs (Figure 2C), indicating a specific role for the NF- κ B pathway in LT α 1 β 2 expression. In iTregs isolated after 5 days of stimulation culture, blocking ERK activity abolished IL-2-mediated LT α 1 β 2 increases, whereas NF- κ B blockade had a less inhibitory effect on LT α 1 β 2 expression (Figures 2A and 2Bii). Similar blocking patterns to iTregs were observed in nTregs activated by 5 days of culture with anti-CD3/28 and IL-2 (Figures 2A and 2Biii), with a stronger inhibition of expression of LT α 1 β 2

by blocking MAPK than blocking NF- κ B. NF- κ B and MAPK activations were induced by IL-2 stimulation in iTregs and activated nTregs, with a similar pattern (Figure 2C), suggesting that these cells shared IL-2-stimulated NF- κ B and ERK pathways for enhancing LT α 1 β 2 expression.

Tregs Facilitate Migration of Other Leukocytes Across LECs by LT α 1 β 2-LT β R

LT α 1 β 2 activates LT β R signaling on LECs to increase CCL21 and VCAM-1, which are crucial for Treg TEM (Piao et al., 2018). We also demonstrated that the LT β R-NIK pathway in LECs is constitutively active and that constitutive activation is required for LEC TEM of other non-Treg leukocyte subsets, such as CD4 T cells and DCs. These observations lead us to question whether Tregs, by stimulating LT β R, directly regulated the TEM of other leukocytes across LECs. LEC layers were incubated with various T cell subsets for 3, 6, and 16 h; T cells were washed away; and then a second subset of leukocytes were applied to the LEC layers and permitted to migrate toward CCL19 for 3 h. The results showed that freshly isolated wild-type (WT) nTreg but not LT α ^{-/-}nTreg or naive CD4, augmented other CD4, CD8, and immature bone marrow dendritic cell (imBMDC) migration across the LECs (Figures 3Ai–3Aiii). WT iTreg, but not LT α ^{-/-}iTreg or activated non-Treg CD4, also enhanced other CD4, CD8, and imBMDC TEM (Figures 3Bi–3Biii). WT iTreg also promoted B cell, neutrophil, and peritoneal macrophage TEM (Figures S1Ai–S1Aiii). The influence of activated nTreg was also tested. Like iTreg, activated WT nTreg but not LT α ^{-/-}nTreg enhanced other CD4 TEM (Figure 3C). This conditioning or influence of Tregs on LECs was effective as early as 6 h after the iTreg-LEC interaction (Figures S1Bi and S1Bii), with a minimum of 0.5×10^5 of iTregs added to the LEC cultures (Figures S1Ci and S1Cii). nTreg as well as naive non-Treg CD4 did not affect LEC viability, as shown by the (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide) MTT viability assay (Figures S1Di–S1Diii). Thus, migration promotion was not due to non-specific effects on LEC viability. Strikingly, the enhanced migration was dependent on nTreg or iTreg expression of LT α (Figures 3A and 3B) and LEC expression of LT β R (Figures 3D and 3E). The influence of Treg on LEC TEM regulation was further confirmed *in vivo* under both homeostatic and inflammatory conditions with the footpad migration assay. Injection of iTregs in the un-inflamed (Figure 3F) or inflamed (Figure 3G) footpads followed by the transfer of carboxyfluorescein succinimidyl ester (CFSE)-labeled non-Treg CD4 or CD8 T cells into the same footpad 1 h later promoted T cell migration into popliteal dLN. In contrast, injection of activated WT non-Treg CD4 or LT α ^{-/-}Tregs did not promote migration *in vivo* (Figures 3F and 3G). Importantly, as for mouse Tregs, only human Tregs facilitated other CD4 TEM across human LECs (Figure 3H), emphasizing the commonality of essential functions of Tregs for immune cell migration.

Treg Engage LT α 1 β 2-LT β R Pathways to Induce VCAM-1 and CCL21 on LECs and Lymphatic Vessels

Co-culture of LECs with WT iTregs, but not LT α ^{-/-}iTregs for 16 h induced strong NIK activity (Figure 4A). WT iTregs, but not LT α ^{-/-}iTregs, also increased LEC expression of VCAM-1 and CCL21 (Figure 4B) and decreased expression of tight junction proteins vascular endothelial (VE)-cadherin between LECs (Figure 4B). These data showed that Treg modulation of VCAM-1, CCL21, and VE-cadherin were LT α -dependent. Similarly, human

naive Tregs expressed higher levels of LT α 1 β 2 than effector CD4 T cells (Figure S2A) and regulated the expression of VCAM-1, CCL21, and VE-cadherin in human LECs (Figure S2B). *In vivo* Tregs induced increased CCL21 and VCAM-1 and decreased VE-cadherin expression on lymphatic vessels in an LT α -dependent manner (Figures 4C–4E). In concordance with the footpad migration assay (Figure 3F), intradermally injected WT iTregs migrated into the lymphatic vessel lumen from the tissue, whereas LT α ^{-/-}iTregs migrated much less efficiently into the lymphatics (Figure 4C). Thus, Treg LT α 1 β 2 conditioned LECs to license TEM through molecular changes of chemokine expression and tight junction structures.

TLR2 Signaling Preferentially Promotes LT α 1 β 2 Expression and iTreg Migration

TLR2 further stimulates on TCR-activated non-Treg CD4 and CD8 T cells and regulates Treg proliferation and immune suppression (Liu et al., 2006). We tested whether TLR2 stimulation of various T cell subsets influenced LT α 1 β 2 expression. iTregs, as noted above, expressed high levels of LT α 1 β 2, which were markedly increased by IL-2. Importantly, TLR2 activation by either the TLR1/TLR2 agonist Pam3Cys-SK4 (P3C) or the TLR2/TLR6 agonist Pam2CSK4 (P2C) further increased the expression of LT α 1 β 2 on iTregs (Figures 5A, S3A, and S3D). nTreg expression of LT α 1 β 2 also increased in response to IL-2; in contrast, TLR2 stimulation did not enhance LT α 1 β 2 expression (Figure 5A). To elucidate the different responses to TLR2 signaling between Treg subsets, nTregs were stimulated with anti-CD3/28 and IL-2 for 5 days followed by TLR2 stimulation, similar to how iTregs were differentiated from naive CD4. These conditions resulted in a marked increase of LT α 1 β 2 expression by activated nTregs after TLR2 stimulation. Thus, activation of both iTregs and nTregs by IL-2R and TCR determined the response to TLR2 stimulation of LT α 1 β 2 expression. Activated non-Treg CD4 did not demonstrate increased LT α 1 β 2 expression in response to either IL-2 and/or TLR2 stimulation. Naive non-Treg CD4 expressed very little LT α 1 β 2, which was also unaffected by IL-2 or TLR2 stimulation (Figure 5A). Similarly, activated human Tregs but not activated non-Treg CD4 effector T cells expressed high levels of TLR2 (Figure S3E), and TLR2 activation increased LT α 1 β 2 expression on human Tregs but not on effector T cells (Figure S3F).

The different responses to the TLR2 ligand raised the possibility that the various CD4 subsets may express different levels of surface TLR2. iTregs and activated nTregs showed high levels of surface TLR2, whereas unstimulated nTregs, naive non-Treg CD4, and activated non-Treg CD4 had lower or minimal cell surface TLR2 (Figure 5B), suggesting that the differential responses to the TLR2 ligand among the T cell subsets was due to differences in the TLR2 expression. Notably, other TLR ligands, such as lipopolysaccharides (LPSs) (TLR4 agonist) and R848 (TLR7 agonist), had no effect on IL-2-stimulated LT α 1 β 2 expression (Figures S3A–S3D), indicating an important role for TLR2 on LT α 1 β 2 expression in Tregs.

These stimulated T cells were assessed for TEM to determine if increased LT α 1 β 2 expression functionally enhanced their migration. IL-2 activation enhanced WT but not LT α ^{-/-}iTreg TEM, and WT but not LT α ^{-/-}iTreg migration was further enhanced by TLR2 co-stimulation (Figure 5Ci). Correlated with LT α 1 β 2 expression, IL-2 increased resting nTreg

migration, whereas TLR2 co-stimulation did not (Figure 5Cii), and TLR2 co-stimulation further increased activated nTreg migration (Figure 5Ciii). Activated non-Treg CD4 migration was not influenced by IL-2 or TLR2 co-stimulation (Figure 5Ciiii). For TLR2^{-/-}iTregs, IL-2 promoted both TLR2^{-/-} and WT TEM; however, TLR2 co-stimulation failed to increase TEM by TLR2^{-/-}iTregs (Figure 5D).

We investigated which signaling pathways TLR2 engaged in T cell subsets stimulated with IL-2 by immunoblotting for classical NF- κ B, MAPK, and PI3K-Akt activities. Classical NF- κ B (p65) activation and ERK (p44/p42) phosphorylation were induced by IL-2 in all T cell subsets and were further enhanced by TLR2 co-stimulation in iTregs (Figure 5E) and activated nTregs but not in resting nTregs (Figure S3A), which was likely due to the higher levels of surface TLR2 expression in iTregs and activated nTregs (Figure 5B). IL-2 also induced JNK MAPK signaling in all T cell subsets except nTreg (Figures 2C and S4B), whereas TLR2 co-stimulation failed to promote JNK (p54/p46) phosphorylation in any of the T cell subsets. PI3K-Akt (Thr308) signaling was also induced by IL-2, but only in iTregs and activated nTregs (Figures S4A–S4C). Notably, Akt (Thr308) was constitutively activated in activated non-Treg CD4 (Sauer et al., 2008) and was weakly or not activated in nTreg or naive non-Treg CD4. TLR2 co-stimulation increased Akt (Thr308) in both activated nTregs and iTregs (Figures S4A–S4C). Because IL-2R-mediated LT α 1 β 2 expression is controlled by NF- κ B and MAPK signaling (Figure 2), together the results indicated that TLR2 augmented classical NF- κ B, ERK, and PI3K-Akt (Thr308), but not JNK signaling, by IL-2R for increasing LT α 1 β 2 expression in the Treg subsets.

Pancreatic Islets Mobilize iTregs by Enhancing LT expression

Pancreatic islets express endogenous TLR2 ligands, such as HMGB1 and HA (Matsuoka et al., 2010; Tesar et al., 2006). We hypothesize that transplanted pancreatic islets may trigger TLR2 signaling on local or patrolling Tregs to promote LT α 1 β 2 expression and, thus, mobilize Treg into the dLNs and protect the graft (Zhang et al., 2009). To test this, we challenged iTregs with various doses of allogenic pancreatic islets for 16 h *in vitro* and found that LT α 1 β 2 expression was dramatically increased (Figure 6A). Blocking with TLR2 inhibitor (2R9) (Piao et al., 2015) abolished the increase. In contrast, for TLR2^{-/-}iTregs, the islets and TLR2 agonists failed to increase LT α 1 β 2 expression (Figure 6B). WT iTreg, but not TLR2^{-/-} iTreg, pre-treated with islets showed a significant increase in TEM *in vitro* (Figure 6C). To show modulation of LT α 1 β 2 *in vivo* by islet-derived TLR2 ligands, we co-transferred WT or TLR2^{-/-}nTregs with islets underneath the kidney capsule, and 2 days later, the nTregs within the islets were harvested and analyzed for LT α 1 β 2 expression. Like the *in vitro* assay, higher LT α 1 β 2 expression was observed on WT nTregs than on TLR2^{-/-}nTregs (Figure 6D). Consistent with the need for increased expression of LT α 1 β 2 to promote migration (Brinkman et al., 2016), WT but not TLR2^{-/-}Tregs prolonged islet graft survival (median survival time: 34 versus 15 days, $p < 0.05$) (Figure 6E). Furthermore, TLR2^{-/-}Treg migration into dLNs (Figure 6F) was also impaired. To test whether TLR2^{-/-}iTregs had impaired suppressive capability, we analyzed *in vitro* immune suppression. Consistent with a previous report (Sutmuller et al., 2006), TLR2^{-/-} iTregs retained *in vitro* immune suppressive function (Figure S5). Thus, islet-derived TLR2 ligands

triggered TLR2 signaling on iTregs to enhance LT α 1 β 2 expression, promote migration from the graft to the dLN, and prolong graft survival.

DISCUSSION

Here, we showed that iTregs and activated nTregs each directly regulated the responses of LECs, thereby modifying the TEM of other leukocyte subsets. This activity was shared by human Tregs and LECs. With high levels of LT α 1 β 2, transmigrating Tregs engaged LT β R on LECs to modulate endothelial cell responses and structures. The Treg-LEC interaction rapidly induced the cell adhesion molecule VCAM-1, downregulated the cell junction protein VE-cadherin, and promoted the TEM of Tregs and other immune cells, including non-Treg CD4, CD8, B cells, neutrophils, macrophages and immature DCs. Importantly, iTregs and activated nTregs expressed the highest level of TLR2, which shared NF- κ B, ERK, MAPK, and PI3K-Akt pathways with IL-2R, and activation of which enhanced LT α 1 β 2 expression, thus efficiently mobilizing immune cell trafficking during inflammation.

Treg trafficking between inflamed tissues and dLNs is essential for optimal immune suppression (Rudensky and Campbell, 2006; Zhang et al., 2009). Tregs use multiple mechanisms to curb autoimmunity and inflammation by suppressing differentiation, activation, proliferation, and effector function of multiple immune cell types, including non-Treg CD4 and CD8 T cells, B cells, and DCs (Fontenot et al., 2005; Rudensky and Campbell, 2006; Sakaguchi et al., 2008). However, it is uncertain how multiple suppressive mechanisms can be coordinated and deployed to restrict such a large variety of cell types and responses. Tregs are also required for the active regulation of appropriately timed effector cell migration and immune responses (Lund et al., 2008; Rogers et al., 2018). Thus, Tregs have dual roles in suppressing and ensuring appropriate immune responses. We recently demonstrated that Tregs use LT α 1 β 2 to trigger LT β R-NIK signaling in LECs for Treg lymphatic TEM (Brinkman et al., 2016; Piao et al., 2018) and that LT β R-NIK signaling is constitutively active *in vivo*. The results here suggest that in homeostatic conditions, nTregs equipped with high levels of LT α 1 β 2 patrol tissues and tissue lymphatic vessels and may maintain LEC LT β R constitutive activation. This activity may permit regulatory or effector T cell homing and recirculation to maintain immune surveillance of tissue-derived endogenous or exogenous antigens for suppression of immune responses to the former and induction of immune responses to the latter. During acute inflammation, activated Tregs expressing the highest levels of LT α 1 β 2 are rapidly mobilized and regulate LECs for TEM of a variety of leukocyte subsets. Enhancing TEM may allow rapid antigen presentation between effector T cells and DCs in the dLN. Enhanced TEM of effector cells out of inflamed site may also resolve inflammation and decreased local tissue destruction (Figure S5). Although the fate or stability of Tregs during infection has been challenged because exposure to pro-inflammatory cytokines may convert Tregs into effector T cells (Zhou et al., 2009a, 2009b), their stability under physiologic and inflammatory conditions has been confirmed (Rubtsov et al., 2010) and the Tregs further restrain immunity (Koch et al., 2009). Together, these observations suggest Treg conditioning of endothelial TEM gating might be required at multiple levels of the immune response to regulate systemic trafficking in and out of tissues and lymphoid organs. This also suggests that this single Treg function might be

able to regulate a multitude of cell types and functions. These relationships remain to be proven and may require the development of an $LT\alpha$ -flox strain, which does not currently exist.

The transcription factor Foxp3 is the signature marker of immune suppressive Treg (Fontenot et al., 2005), which includes thymic-derived nTregs and peripherally generated iTregs. nTregs and iTregs have been compared, and several differences in gene expression or signal pathways have been described. nTregs are demethylated at Treg-specific promoter and enhancer regions, whereas iTregs are predominately methylated in these regions (Kim et al., 2012), and demethylation correlates with the stability of Foxp3 gene expression (Polansky et al., 2010). We observed that activated nTregs resemble iTregs with respect to Foxp3 and CD25 expression; increased $LT\alpha 1\beta 2$ expression after IL-2, CD3/CD28, and/or TLR2 stimulation; and use of NF- κ B-, ERK-, and PI3K-Akt-related intracellular signaling pathways. IL-2/IL-2R induced the greatest increases in $LT\alpha 1\beta 2$ expression, and we clarified that $LT\alpha 1\beta 2$ is mainly induced by IL-2R signals after CD3/CD28 activation. Despite an equal expression of high-affinity IL-2R (Busse et al., 2010; Kim et al., 2001; Sakaguchi et al., 1995), iTregs and activated nTregs had distinct IL-2R signaling patterns compared to activated non-Treg CD4 cells. IL-2 induced transient classical NF- κ B and ERK activation in iTregs or activated nTregs but persistent activation in activated non-Treg CD4 (Figure 5E); PI3K-Akt (Thr308) was inducible in iTregs and activated nTregs but was constitutively activated in activated non-Treg CD4; and iTregs favored p54 JNK activation, whereas activated non-Treg CD4 had preferential p46 JNK activation (Figure S4B). Compared to activated T cells, resting naive non-Treg CD4 had weak responses to IL-2, likely due to lower IL-2R expression (Busse et al., 2010; Kim et al., 2001; Sakaguchi et al., 1995). Blocking assays showed that $LT\alpha 1\beta 2$ expression in resting nTregs was controlled by IL-2-activated NF- κ B-p65 signaling and in iTregs or activated nTregs by MAPK signaling.

iTregs and nTregs that had been activated *in vitro* expressed the highest levels of $LT\alpha 1\beta 2$, which engaged $LT\beta R$ on LECs to then modulate LEC function and structure and promote Treg TEM. We tested the hypothesis that the Treg-LEC interaction and changes in LECs may facilitate the TEM of other immune cells. The results showed that Tregs conditioned LECs so that subsequent migration by non-Treg CD4, CD8, and DC were enhanced, which promoted effective egress out of inflamed tissues. Treg conditioning also mobilized B cell, neutrophil, and macrophage migration, suggesting that Tregs may control leukocyte lymphatic migration during many different inflammatory and immune responses. Treg conditioning of LECs is specific and LT-dependent because $LT\alpha^{-/-}$ Treg failed to provide this activity. $LT\alpha 1\beta 2$ triggered $LT\beta R$ signaling on LEC-induced VCAM-1 and select chemokines, such as CCL21, CXCL12, and CCL2 (Piao et al., 2018), and decreased the intercellular tight junction protein VE-cadherin between LECs. These observations suggested that Tregs use $LT\alpha 1\beta 2$ to stimulate the chemokine repertoire (including CCL19, CCL21, CXCL12, and CCL2) of LECs, and thus, cells expressing CCR7, CXCR4, or CCR2/4 can be recruited to the proximal lymphatic vessels and transmigrate across the conditioned LECs under the influence of Tregs. Activated Tregs may upregulate other receptors and ligands important for TEM that we have not yet investigated. An analysis of TLR2 surface expression showed that activated Tregs expressed the highest levels of TLR2, and TLR2 activation of Treg, but not activated or naive non-Treg CD4, dramatically

increased $LT\alpha.1\beta2$ expression and further enhanced the ability of Tregs to condition LECs for increased leukocyte TEM. Activation of other TLRs such as TLR4 or TLR7 had no such effect, showing the specific role for TLR2 signaling on immune cell migration.

TLR2 reportedly has diverse effects on T cells, including immune suppression, survival, and proliferation (Chen et al., 2009; Liu et al., 2006; Suttmuller et al., 2006). However, TLR2 signaling influence on Treg migration has not yet been reported. Here, we found increased TLR2 expression in activated Tregs, which are likely present during inflammatory events. Increased TLR2 expression correlated with the ability of TLR2 ligands plus IL-2 to induce NF- κ B-p65, ERK (p44/42), and PI3K-Akt to increase $LT\alpha.1\beta2$ expression and regulate migration. Because Treg migration from islets to dLNs is important for allograft protection and survival (Zhang et al., 2009), we tested the role of TLR2 in vivo and proved it was important for mobilizing trafficking and maintaining suppressive function. Islet-derived endogenous ligands triggered TLR2 to increase $LT\alpha.1\beta2$ expression in vitro and in vivo. TLR2 activated Tregs, then conditioned LECs by $LT\beta R$, and licensed them for enhanced TEM of other leukocytes. TLR2-deficient Tregs failed to upregulate $LT\alpha.1\beta2$, enhance LEC TEM properties, migrate from islets to dLNs, or prolong islet graft survival, suggesting that TLR2 agonists may be tools for regulating Tregs and other immune functions in tissues for protection of the allograft. On the other hand, TLR2 on myeloid cells has also been targeted for anti-inflammatory effects in innate immunity (Meng et al., 2004; Piao et al., 2015). Activation of TLR2 on macrophages or DCs by TLR2 agonists may exaggerate innate immune responses and be directly detrimental to islet integrity so that manipulation of this complex system may be difficult (Leventhal and Schröppel, 2012; Krüger et al., 2010). Nevertheless, the data provide insight into the immune regulatory function of TLR2 agonists for transplantation tolerance.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jonathan S. Bromberg (jbromberg@som.umaryland.edu). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal strains—Female C57BL/6J (WT, $LT\beta R^{-/-}$, $LT\alpha^{-/-}$) (7–10 weeks), were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3GFP mice on a C57BL/6 background were from Dr. A. Rudensky (Memorial Sloan Kettering Cancer Center) (Fontenot et al., 2005). All animal experiments were performed in accordance with Institutional Animal Care and Use Committee approved protocols.

Antibodies and Reagents—Antibodies against phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, phospho-JNK (Thr183/Tyr185), phospho-p65 (Ser536), phospho-Akt (Thr308), NF κ B-p100/p52, and GAPDH were obtained from Cell Signaling (San Diego, CA). Anti-CD25 (IL-2R α , clone MAB702) was purchased from R&D Systems (Minneapolis, MN). Lipopolysaccharides (LPS, *E. coli* O55:B5, L4524), NF κ B-I κ B α inhibitor BAY11-7082

and ERK inhibitor U0126 were purchased from Sigma-Aldrich (St. Louis, MO); INK inhibitor JNK-IN-8 from Calbiochem (San Diego, CA); PI3K p110 α inhibitor PIK2 and activator 740Y-P from Echelon Biosciences Inc. (Salt Lake City, UT) and from Cayman (Ann Arbor, MI), respectively; Pam3CSK4, Pam2CSK4, and R848 (Resiquimod) from InvivoGen (San Diego, CA).

Cells—C57BL/6 mouse (C57–6064L) or human (H-6064L) primary dermal LECs were from Cell Biologics, Inc. (Chicago, IL), and were cultured according to the manufacturer's instructions in manufacturer-provided mouse endothelial cell medium supplemented with 5% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, vascular endothelial growth factor, endothelial cell growth supplement, heparin, epidermal growth factor, and hydrocortisone.

METHOD DETAILS

T Cell Subset, B cell, Neutrophil, Peritoneal Macrophage, and Bone Marrow Derived Dendritic Cell Generation—cCD4, cCD8, and B cells from mouse LNs and spleens were isolated using mouse CD4⁺, CD8⁺, and B cell negative selection kits (StemCell Technologies, Inc., Cambridge, MA), and were cultured as previously described (1). Briefly CD4⁺CD25⁻Foxp3-GFP-WT or LT α ^{-/-}naive CD4 cells with > 98% purity were sorted using a FACS Aria II (BD Biosciences, San Jose, CA). The sorted Foxp3-GFP⁻ naive CD4 T cells (5×10^4) were then cultured in flat-bottom 96-well plates for 5 days at 37 °C in 5% CO₂, with IL-2 (20 μ g/ml, eBioscience, San Diego, CA), plate-bound anti-CD3 ϵ mAb (1 μ g/mL, clone 145–2C11, eBioscience), and anti-CD28 mAb (1 μ g/mL, clone 37.52, eBioscience) for activated CD4 T cells; and human TGF β 1 (10 μ g/mL, eBioscience) and anti-IL-4 (10 ng/mL, clone 11B11, eBioscience) for induced regulatory T cells (iTreg). At the end of the 5-day culture, the CD4⁺CD25⁺Foxp3-GFP⁺ iTregs and CD4⁺CD25⁺Foxp3-GFP⁻ activated CD4 cells were FACS-sorted to > 99% purity. Cells were cultured in complete RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μ g/mL streptomycin, non-essential amino acids and 2×10^5 M 2-ME (Sigma-Aldrich). Neutrophils were positively selected with Gr-1FITC (clone RB6–8C5) followed by FITC-selection kit (StemCell Technologies, Inc.) from mouse peripheral blood. Peritoneal macrophages were kind gift of Dr. Stefani Vogel, University of Maryland School of Medicine, Baltimore, MD (Dobrovolskaia et al., 2003). In brief, 3 mL of 3% sterile fluid thioglycollate (Remel, San Diego, CA) was injected i.p. into wild-type C57/BL6J mice and four days later macrophages were harvested by peritoneal lavage with sterile saline. BMDCs were generated as described (Lutz et al., 1999). Briefly, bone marrow (BM) cells of wild-type mice were treated with 10 ng/ml GM-CSF (R&D Systems) for 10 days in Petri dishes, and the loosely attached cells were collected as immature BMDC (imBMDC). 100 ng/mL LPS was added for 48 hours to induce mature BMDC (mBMDC). CD11c⁺ DC were purified by CD11c positive selection kit (StemCell Technologies, Inc.).

Primary Murine Skin LEC Purification and Culture—Ears from 5–6-week-old wild-type and LT β R^{-/-}C57BL/6J mice were collected and digested in 4 mg/ml collagenase D (Roche, Indianapolis, IN) at 37°C for 1 hour. The dissociated cells were washed and re-suspended in mouse endothelial cell medium (Cell Biologics, Inc) and plated in 6-well tissue culture plates for 1 weeks until the adherent cells reached confluency. The cells were

dissociated with 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA) and the LEC were purified with Lyve-1-FITC (Novus Biologicals, Littleton, CO) using anti-FITC positive selection kit (StemCell Technologies, Inc.).

Human T Cell Purification and Culture—Human Treg were enriched from peripheral blood mononuclear cells with anti-CD25 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and were sorted via FACS Aria as nTreg ($CD4^+CD25^{\text{high}}CD127^-CD45RA^+$) and naive CD4 T cells ($CD4^+CD25^-CD127^+CD45RA^+$). The cells were expanded as previously described (Hippen et al., 2011). Briefly, purified nTreg were stimulated with irradiated KT64/86 cells cultured in XVivo-15 (BioWhittaker, Walkersville, MD) media containing 10% human AB serum (Valley Biomedical, Winchester, VA), Pen/Strep (Invitrogen, Carlsbad, CA), N-acetyl cysteine (USP), and recombinant IL-2 (300 IU/mL; Chiron, Emeryville, CA) for 14 days and frozen. When needed, frozen nTreg and naive CD4 were thawed and re-stimulated with anti-CD3/CD28 mAb-Dynabeads (Life Technologies, Carlsbad, CA) at 1:3 (cell to bead) plus recombinant IL-2 (300 U/ml) for 10 days before assay.

MTT Viability Assay—LEC were plated into 24-well tissue culture plates, incubated overnight, and treated with iTreg or naive non-Treg CD4 for 16 hours. The cells were washed and followed by 3-hour incubation with 0.5 mg/mL MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide) (Sigma-Aldrich). 50 μ L DMSO was added to cells before reading OD at 540 nm.

In Vitro Suppression Assays— $CD4^+CD25^+$ iTreg and $CD4^+CD25^-$ non-Treg T cells were FACS-sorted from the indicated strains. Treg were labeled with eFluor 670 (Molecular Probes, Eugene, OR). CFSE (Life Technologies)-labeled responder $CD4^+CD25^-$ T cells (5×10^4 cells per well) were cultured with or without Treg at responder:Treg ratios of 1:0, 1:1, 2:1, 4:1 and 8:1 with irradiated (800 rad) syngeneic T cell-depleted splenocytes (5×10^4 cells per well) in 96-well U-bottom plates (Corning) and anti-CD3 ϵ mAb (1 μ g /mL, clone 145-2C11, eBioscience) for 3 days. Cells were collected, and cell division was measured by assessing relative CFSE dilution with an LSR Fortessa cytometer (Beckton Dickinson).

Flow Cytometry—For detection of LT α 1 β 2, T cells were incubated with MOPC21 (BioExCel, West Lebanon, NH) or mouse LT β RIg (recognizing both human and mouse LT α 1 β 2, gift from Biogen Idec, Cambridge, MA) at 2 μ g /mL for 60 minutes at 37 °C in HBSS with 2% FBS. Cells were washed, and then stained for 45 minutes at 4 °C with anti-CD16/32 (clone 93, eBioscience), followed with BV421-rat anti-mouse IgG1 (Biolegend) APC-eFlour780-anti-mouse CD4 (GK1.5, eBioscience), and PE-anti-mouse CD25 (PC61.5, eBioscience); cells were then washed and fixed with 4% paraformaldehyde and run on an LSR Fortessa flow cytometer (BD Biosciences). Surface TLR2 staining used anti-mouse or human TLR2-APC (clone T2.5, BioLegend). Results were analyzed with FlowJo 8.7 (Treestar).

Immunoblotting—Cells were lysed in buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM NaF, 2 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche). Protein in the cell

extract was quantified using protein quantification kit (Bio-Rad, Philadelphia, PA) and 10 μ g total protein was run on Novex WedgeWell 4%–20% Tris-Glycine Mini Gels (Invitrogen) and transferred to an Immobilon-P membrane (Bio-Rad). Membranes were probed with indicated antibodies. Quantification of blots was performed with ImageJ from the National Institutes for Health. Relative intensity of blots was normalized to housekeeping gene GAPDH and presented as fold induction to non-stimulation.

Lymphatic Transendothelial Migration—Transmigrations across endothelial cells were described previously (Brinkman et al., 2016; Xiong et al., 2017). Briefly the inverted transwell insert (24-well, Corning International) with 5 μ m pore size was coated with 0.2% (w/v) gelatin (Bio-Rad) for 1 hour at 37 °C before loading with 1.0×10^5 primary skin LEC in 100 μ L LEC medium. After 2 days, the LEC cell layers were treated with various conditions as noted in the text and figure legends prior to adding 2×10^5 T cells or DCs migrating cells in 100 μ L to the upper chamber of transwell plate while the lower chamber contained mouse or human CCL19 or CCL21 (50 ng/mL or 200 ng/mL respectively, R&D systems). All cells or reagents were prepared in IMDM containing transferrin and 0.5% (w/v) fatty acid-free BSA (Gemini, West Sacramento, CA). T cells that migrated to the lower chamber after 3 hours at 37 °C were counted.

Immunohistochemistry—Murine or human LEC monolayers were stained for surface VCAM-1, VE-Cadherin or Lyve-1 with rat anti-mouse VCAM-1 (Clone 429, eBioscience) or mouse anti-human VCAM-1 (Clone STA, Biolegend), rat anti-mouse VE-Cadherin (Clone 11D4.1, BD Biosciences) or mouse anti-human VE-Cadherin (Clone BV9, Biolegend), goat-anti-mouse or anti-human CCL21 polyclonal antibody (R&D System), or rabbit anti-mouse Lyve-1 (70R-LR003, Fitzgerald, Acton, MA), then were fixed for 20 min at 4 °C with 4% (w/v) paraformaldehyde (Affymetrix, Santa Clara, CA), Cells were permeablized with PBS 0.2% (v/v) Triton X-100 (Sigma-Aldrich), and treated with 5% donkey serum for 30 min then incubated with listed primary antibodies for overnight at 4 °C. Samples were incubated with secondary antibodies conjugated with Alexa Fluor 448, 546 or 647 (1:400, Jackson ImmunoResearch, West Grove, PA) for 1 hour at 4 °C and mounted in Prolong Gold with DAPI (Thermo Fisher, P36931). Transwell membranes were transferred onto glass slides and visualized by fluorescent microscopy (Zeiss LSM 510 Meta and LSM5 Duo) with a 60 \times or 20 \times objective. Images were analyzed with Volocity version 6.3 software. Fluorescence intensity was measured as Mean Fluorescence Intensity (MFI) over the entire field.

Footpad and Whole-Mount Ear Migration Assays—Mice were anaesthetized and 1×10^6 Foxp3-GFP⁺CD25⁺ Tregs or activated Foxp3-GFP⁻CD25⁺ no-Tregs were injected intradermally into the footpads or ear pinnae in 20 μ L PBS as we previously described. For footpad assays, draining popliteal LNs were collected 12 hours post injection and processed for flow cytometry. For ear pinnae assays, whole-mount ears were collected 16 hours post injection. Sample were fixed for 10 minutes at room temperature with 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100 for 30 min at 4°C; Samples were blocked for 1 hour in 5% donkey serum in PBS containing 0.1% Triton X-100 and incubated with listed antibodies overnight at 4 °C. Anti-Lyve-1 (1:100, Fitzgerald 70R-

LR003), anti-CCL21 (1:100, R&D Systems), or anti-VCAM-1 (1:100, eBioscience 429). secondary antibodies were conjugated to Alexa Fluor 488, 546 or 647 (1:400, Jackson ImmunoResearch) for 1 hour at 4 °C, mounted in Prolong Gold with DAPI (Thermo Fisher, P36931). Distance of T cells from lymphatic vessels calculated with minimum distance program in Volocity 6.3.

Islet Isolation and Transplantation—BALB/c islets were isolated as previously described (Zhang et al., 2009). Briefly, pancreata were perfused with collagenase P (Roche), harvested, digested, and purified over a discontinuous Ficoll (Sigma-Aldrich) gradient. 400 freshly isolated islets were handpicked and used for T cell stimulation or transplanted beneath the renal capsule of C57BL/6J recipients made diabetic (blood glucose > 300 mg/dl) by intraperitoneal injection of 200 mg/kg streptozotocin (Sigma-Aldrich) at least 1 week before transplant. Blood glucose < 150 mg/dl after transplantation was considered engraftment, and > 300 mg/dl was considered diabetic or graft rejection as appropriate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Numerical data are presented as mean \pm SEM. Asterisks mark data statistically different from the controls, with p values noted in the figure legends. A p value of < 0.05 was considered significant for one-way ANOVA and Student's t tests using Prism 5 (La Jolla, CA). The number of replicates is noted in the figure legends.

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Tregs modulate lymphatic endothelial cells (LECs) for transmigration of other cells
- IL-2R stimulates NF κ B and MAPK pathways to promote lymphotoxin expression on Tregs
- Tregs stimulate LEC LT β R to increase VCAM-1 and CCL21 and decrease VE-cadherin
- TLR2 signaling on activated Tregs promotes lymphotoxin expression and Treg migration

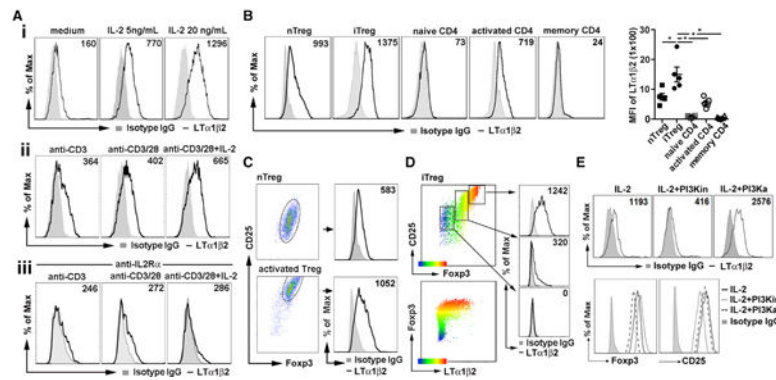


Figure 1. LT α 1 β 2 Is Induced by IL-2R and TCR Signaling in Tregs.

(A) Flow cytometry analysis of LT α 1 β 2 expression on fluorescence-activated cell sorting (FACS)-sorted Foxp3-GFP⁺CD4⁺ iTregs treated with indicated dose of IL-2 (i); anti-CD3 (5 μ g/mL) alone, together with anti-CD28 (1 μ g/mL), or IL-2 (10 ng/mL) (ii); and 2 μ g/mL anti-CD25 pretreatment prior to TCR activation as in (ii), for 16 h (iii).

(B) LT α 1 β 2 expression of FACS-sorted Foxp3-GFP⁺CD4⁺ iTregs, Foxp3-GFP⁺CD44^{low}CD4⁺ natural Tregs (nTregs), Foxp3-GFP⁻CD44^{low}CD4⁺ naive CD4, Foxp3-GFP⁻CD44^{high}CD4⁺ memory CD4 T cells, or IL-2 + anti-CD3/28-stimulated Foxp3-GFP⁻CD4⁺-activated CD4 from Foxp3-GFP reporter mice. Quantitative difference expressed as geometric mean fluorescence intensity (MFI) \pm SEM for specific staining minus isotype immunoglobulin G (IgG) MFI.

(C) LT α 1 β 2 expression of Foxp3-GFP⁺ CD4⁺nTregs sorted from Foxp3-GFP reporter mice, before and after stimulation with plate-bound anti-CD3 (5 μ g/mL), anti-CD28 (1 μ g/mL), and IL-2 (20 ng/mL) for 16 h.

(D) LT α 1 β 2 expressed in iTreg subsets stratified by CD25 and Foxp3 expression.

(E) LT α 1 β 2, CD25, and Foxp3 expression in iTregs treated with 500 nM PI3K inhibitor (PI3Ki) or 500 nM PI3K activator (PI3Ka) for 16 h.

Data are representative of 3 (A, C, and D) and 5 (B) independent experiments. * $p < 0.05$ by one-way ANOVA (B).

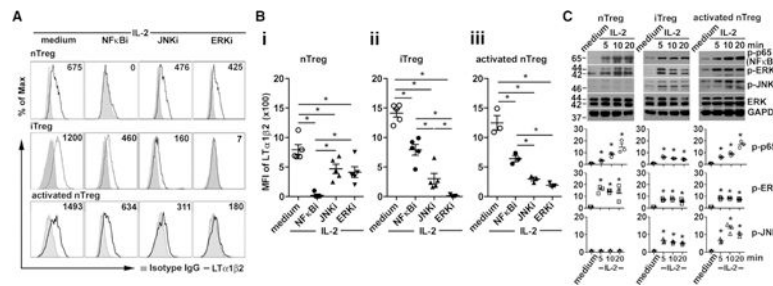


Figure 2. IL-2 and TCR Signaling through NF- κ B and MAPK Promote LT α 1 β 2 Expression in T Cell Subsets.

(A) Flow cytometry analysis of LT α 1 β 2 expression on Foxp3-GFP⁺CD25⁺CD4⁺ nTregs, iTregs, or activated nTregs treated with IL-2 (20 ng/mL) together with NF- κ B inhibitor (NF κ Bi; 5 μ M), JNK inhibitor (JNKi; 2 μ M), or ERK inhibitor (ERKi; 5 μ M) at 37°C for 16 h.

(B) Δ MFI fold changes of LT α 1 β 2 of nTregs (i), iTregs (ii), and activated nTregs (iii). Five (i and ii) or 3 (iii) independent experiments are shown. * $p < 0.05$ by one-way ANOVA.

(C) Immunoblots for NF κ B and MAPKs activation in FACS-sorted nTregs, iTregs, and activated nTregs stimulated with IL-2 (20 ng/mL) as indicated. Representative blot shown. The relative band intensities (normalized to GAPDH, mean \pm SEM) from three independent experiments shown under the panels. * $p < 0.05$ by one-way ANOVA.

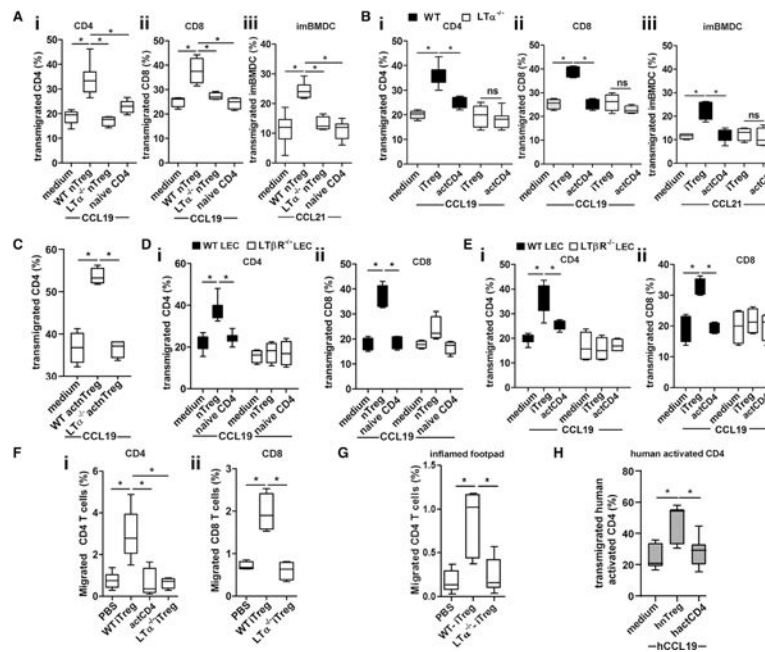


Figure 3. Tregs Promote CD4, CD8, and DC TEM by $LT\alpha 1\beta 2$ - $LT\beta R$.

(A) A total of 1×10^5 wild-type (WT) or $LT\alpha^{-/-}$ nTregs (Foxp3-GFP⁺CD25⁺CD4⁺) or naive CD4 (Foxp3-GFP⁻CD25⁻CD4⁺) were incubated with LEC layers in Boyden chambers for 16 h, T cells were removed by washing, and chambers were then loaded with 2×10^5 naive CD4 (i) or CD8 (ii) T cells or 1×10^5 BMDCs (iii) and migrated toward 50 ng/mL CCL19 (CD4 and CD8) or 200 ng/mL CCL21 (BMDC) for 3 h.

(B) WT or $LT\alpha^{-/-}$ iTregs or activated non-Treg CD4 (actCD4) cells were incubated with LECs and washed, and then chambers were loaded for CD4 (i), CD8 (ii), and imBMDc (ii) TEM as in (A).

(C) WT or $LT\alpha^{-/-}$ nTregs (Foxp3-GFP⁺CD25⁺CD4⁺) were stimulated *in vitro* with plate-bound anti-CD3 (5 μ g/ml), anti-CD28 (1 μ g/mL), IL-2 (20 ng/mL), and TGF β 1 (10 ng/mL) for 3 days and harvested, and 1×10^5 Tregs were added to LEC layers in Boyden chambers. After 16 h, Tregs were removed by washing, and chambers then loaded with 2×10^5 purified non-Treg CD4 T cells, which were migrated toward CCL19 for 3 h.

(D and E) WT or $LT\beta R^{-/-}$ LECs were incubated with 1×10^5 nTregs (D) or iTregs (E) for 16 h and washed, and then chambers loaded for TEM with non-Treg CD4 (i) or CD8 (ii) as in (A).

(F) A total of 1×10^6 iTregs or activated non-Treg CD4 T cells injected into hind footpads of WT C57BL/6J mice 1 h before injection of 2×10^6 CFSE-labeled non-Treg CD4 (i) or CD8 (ii) T cells. After 16 h, popliteal dLNs were analyzed for migrated cells by flow cytometry.

(G) Hind footpads of WT C57BL/6J mice intradermally injected (i.d.) with LPS (1 μ g/10 μ L PBS/footpad i.d.). After 3 h, 5×10^5 WT or $LT\alpha^{-/-}$ iTregs was transferred to the footpads. After another 1 h, 2×10^6 CFSE-labeled non-Treg CD4 T cells was transferred to the same footpads. Sixteen hours later, CD4 T cell migration to the draining popliteal dLN was assessed.

(H) Activated human naive Tregs (hnTreg) or activated non-Treg CD4 (hactCD4) T cells were incubated with human dermal LEC layers in Boyden chambers for 16 h; T cells were

removed by washing, and chambers were then loaded with 3×10^5 human activated non-Treg CD4 for 3-h TEM as in (A). (A–H) Representative of 3 independent experiments; quadruplicate wells for each condition. Data are presented as mean \pm SEM, * $p < 0.05$ by one-way ANOVA.

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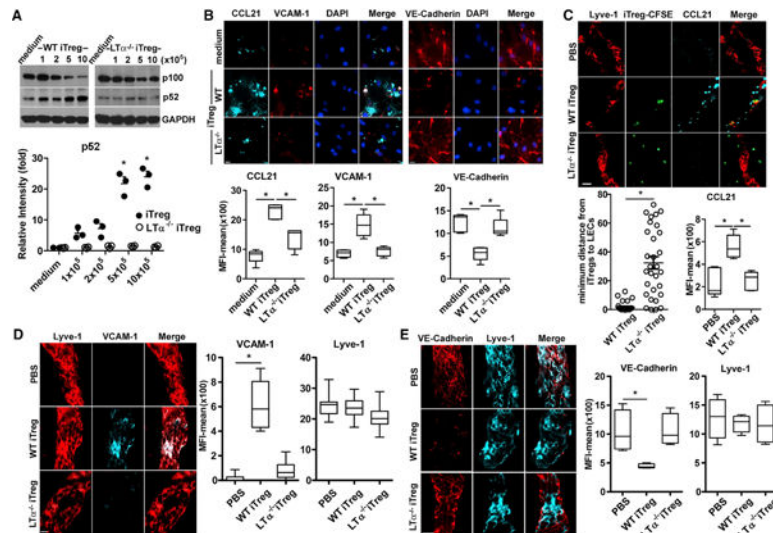


Figure 4. Treg $LT\alpha 1\beta 2$ Activates LEC $LT\beta R$ by the NIK Pathway to Induce VCAM-1, VE-cadherin, and CCL21.

(A) Immunoblot for NIK activation and p100 conversion to p52. Primary LECs incubated with variable numbers of WT or $LT\alpha^{-/-}$ iTregs. After 16 h, cells were lysed for immune blot. Relative band intensities (mean \pm SEM) from three independent experiments are shown.

(B) Immunohistochemistry for CCL21, VCAM-1, and VE-cadherin expression in primary LECs incubated with WT or $LT\alpha^{-/-}$ iTreg for 16 h. MFIs of 5 fields are shown as mean \pm SEM, * $p < 0.05$ by one-way ANOVA.

(C–E) Ear whole-mount staining. Pinnae injected with 1×10^6 WT or $LT\alpha^{-/-}$ CSFE-iTregs; after 16 h, ears were collected and stained for Lyve-1 and CCL21 (C), VCAM-1 (D), or VE-cadherin (E). Magnification is 60 \times (B, D, and E) or 20 \times (C); scale bar: 14 μ m (B, D, and E) or 20 μ m (C). Single lymphatic vessels, which are Lyve-1⁺ (C and E). MFIs of 5 Lyve-1⁺ vessels are shown as mean \pm SEM, * $p < 0.05$ by one-way ANOVA. Representative of 3 independent experiments (A–E). Four ears/two mice in each experiment (C–E).

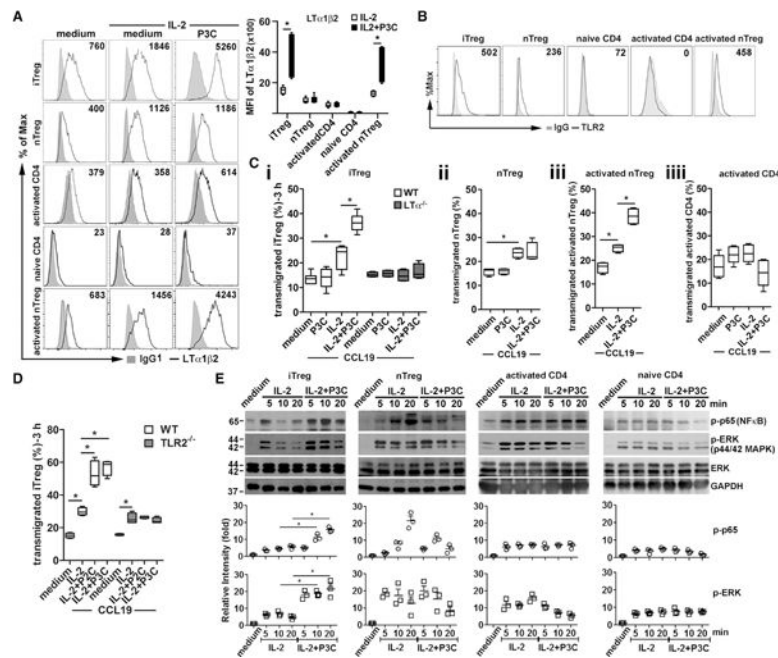


Figure 5. TLR2 Signaling Promotes iTreg $LT\alpha 1\beta 2$ Expression and TEM.

(A) iTregs, nTregs, activated non-Treg CD4, naive non-Treg CD4, and activated nTreg stimulated with IL-2 (20 ng/ml) and/or P3C (500 ng/ml) as indicated. After 16 h, cells were analyzed for $LT\alpha\beta$ expression by flow cytometry (left panel). $LT\alpha 1\beta 2$ MFI (right panel) of 5 independent experiments is shown. Mean \pm SEM. * $p < 0.05$ by one-way ANOVA.

(B) Surface TLR2 expression of various T cell subsets analyzed by flow cytometry.

(C) WT or $LT\alpha^{-/-}$ cells with the same stimulation as in (A) assessed for iTreg (i), nTreg (ii), activated nTreg (iii), and activated non-Treg CD4 (iiii) TEM to CCL19 (50 ng/mL).

(D) WT or $TLR2^{-/-}$ iTregs treated and assayed as in (A). Representative of 3 independent experiments with triplicate wells (C and D). * $p < 0.05$ by one-way ANOVA.

(E) iTregs, nTregs, activated non-Treg CD4, or naive non-Treg CD4 stimulated with IL-2 with or without P3C. After indicated times, cells were lysed and immunoblotted for NF- κ B and MAPK activation. Representative blot shown. The relative band intensities (normalized to GAPDH, mean \pm SEM) of three independent experiments shown under the panels. IL2 + P3C co-stimulation compared to IL-2 stimulation. * $p < 0.05$ by one-way ANOVA.

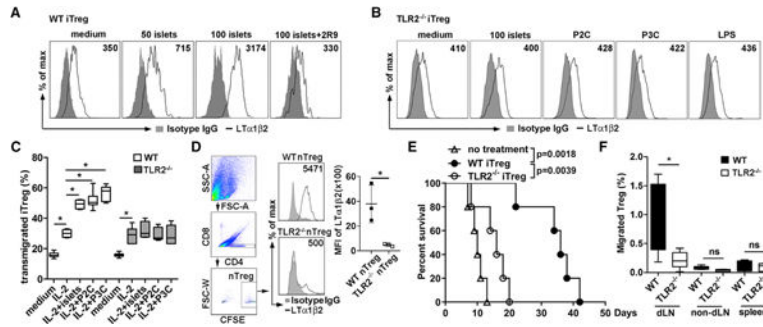


Figure 6. Pancreatic Islets Stimulate Treg LTα1β2 Expression and Migration by TLR2 Activation

(A) A total of 5×10^5 iTregs from WT C57/BL6J mice were co-cultured with various doses of BALB/c pancreatic islets for 16 h. A total of 20 μ M TLR2 inhibitory peptide (2R9) was used as control. Tregs were assessed for LTα1β2 expression by flow cytometry.

(B) A total of 5×10^5 TLR2^{-/-}iTregs stimulated with 100 BALB/c islets, 50 ng/mL P2C, 500 ng/mL P3C, or 100 ng/mL LPS for 16 h prior to LTα1β2 staining.

(C) A total of 5×10^5 WT and TLR2^{-/-}iTregs co-cultured with 100 BALB/c islets for 16 h, and then 2×10^5 of each iTreg assessed for TEM to 50 ng/mL CCL19 for 3 h.

(D) Flow cytometry analysis of LTα1β2 expression on CFSE-labeled WT or TLR2^{-/-}nTregs co-transferred with islets underneath the kidney capsule. Two days after transplantation, the nTregs within the islets were harvested. n = 3 mice per group. Gating strategy and MFI of LTα1β2 expression shown.

(E) Islet survival after co-transfer of WT or TLR2^{-/-}iTregs with transplanted islets. n = 5 mice/group.

(F) Migration of Tregs into the dLNs. After co-transfer of CFSE-labeled WT or TLR2^{-/-}Tregs with transplanted islets, non-dLNs (inguinal LNs), dLNs (peri-renal), and spleen were harvested at day 4 for analysis of percentage of CFSE-Tregs among all CD4 T cells. n = 4 mice/group. Data representative of three independent experiments (A–D). *p < 0.05 by one-way ANOVA (C and F) or by Kaplan-Meier method using the log rank test (E).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit-anti-NFκB122 p100/p52	Cell Signaling Technology	Cat# 4882P; RRID:AB_10828354
anti-phospho-NF-κB p65 (Ser536) (93H1)	Cell Signaling Technology	Cat#3033; RRID:AB_331284
Rabbit-anti-GAPDH (clone 14C10)	Cell Signaling Technology	Cat# 2118; RRID:AB_561053
Anti-phospho-JNK (Thr183/Tyr185) (81E11)	Cell Signaling Technology	Cat#4668; RRID:AB_2307320
Anti-phospho-ERK (D13.14.4E)	Cell Signaling Technology	Cat#4370; RRID:AB_2315112
Rabbit-anti-p44/42 MAPK (D137F5)	Cell Signaling Technology	Cat#4695; RRID:AB_390779
Rabbit-anti-phospho-Akt (Thr308) (244F9)	Cell Signaling Technology	Cat#4056; RRID:AB_331163
Anti-CD3e (145-2C11)	eBioscience	Cat# 14-0031-86; RRID:AB_467051
Anti-CD28 (CD28.2)	eBioscience	Cat# 16-0289-81; RRID:AB_468926
Rat-anti-mouse LTβR (eBio38)	eBioscience	Cat# 16-5671-82; RRID:AB_763451
Anti-rat IgG1 (RG11/39.4)	BioXCell	Cat# BE-0250; RRID: NA
Rat-anti-mouse VCAM-1 (429)	eBioscience	Cat# 553329; RRID:AB_394785
Mouse-anti-human VCAM-1 (Sta)	Biolegend	Cat# 305802; RRID:AB_314558
Rat-anti-mouse VE-Cadherin (BV13)	Biolegend	Cat# 138001; RRID:AB_10570497
Mouse anti-human VE-Cadherin (BV9)	Biolegend	Cat# 348501; RRID:AB_10574464
Anti-Rabbit IgG Alexa Flour 647	Invitrogen	Cat# 1013785; RRID: NA
Anti-Rabbit IgG Alexa Flour 546	Jackson ImmunoResearch	Cat# 705-546-147; RRID:AB_2340430
Anti-Rabbit IgG Alexa Flour 488	Jackson ImmunoResearch	Cat# 711-545-152; RRID:AB_2313584
Anti-Rat IgG Alexa Flour 647	Jackson ImmunoResearch	Cat# 712-605-153; RRID:AB_2340694
Anti-Rat IgG CyTM3	Jackson ImmunoResearch	Cat# 712-165-153; RRID:AB_2340667
Anti-Goat IgG CyTM3	Jackson ImmunoResearch	Cat# 705-165-003; RRID:AB_2340411
Anti-Goat IgG Alexa Flour 647	Jackson ImmunoResearch	Cat# 705-605-003; RRID:AB_2340436
Anti-Mouse IgG Alexa Flour 647	Jackson ImmunoResearch	Cat# 715-605-150; RRID:AB_2340862
Anti- CXCL12	eBioscience	Cat# 14-7992-81; RRID:AB_468513
Anti-CD3e	Abcam	Cat#ab49943; RRID:AB_868900
Anti-CD11c (HL3)	BD Bioscience	Cat# 553863; RRID:AB_395099
Anti-mouse CCL21 polyclonal Goat IgG	R&D Systems	Cat# AF457; RRID:AB_207208
Anti-human CCL21 polyclonal Goat IgG	R&D Systems	Cat# AF366; RRID:AB_355327
Rabbit-anti-Lyve-1	Fitzgerald	Cat# 70R-LR003; RRID:AB_1287923
Anti-CD16/32 (93)	eBioscience	Cat# 14-0161-82; RRID:AB_467133
Anti-CD4-APC-eflour780 (RM4-5)	eBioscience	Cat# 47-0042-82; RRID:AB_1272183
Rat-anti-CD44-APC (IM7)	eBioscience	Cat# 17-0441-82; RRID:AB_469390
Rat-anti-CD25-PE (PC61.5)	eBioscience	Cat# 12-0251-83; RRID:AB_465608
Rat-anti-VCAM-1-Alexa Flour 647(429)	eBioscience	Cat# 14-1061-82; RRID:AB_467419
Anti-mouse IgG1-BV421 (RMG1-1)	Biolegend	Cat# 406616; RRID:AB_2562234
Anti-human or mouse TLR2 (T2.5)	Abcam	Cat# ab16894; RRID: AB_443530
Rat-anti-LTβR-PE (3C8)	eBioscience	Cat# 12-5671-82; RRID: NA
Rat-anti-mouse Gr-1-FITC (RB6-8C5)	Biolegend	Cat#108406; RRID: AB_313371

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
LT β RIg	Biogen Idec	gift
4H-isoquinoline-1,3-dione (NIKi)	Enamine LLC	Cat# EN300-15352
BAY-11-7082	Sigma-Aldrich	Cat# B5556-10MG
U0126	Sigma-Aldrich	Cat# 662005-1MG
JNK-IN-8	Calbiochem	Cat# 1410880-22-6
PI3K p110 α inhibitor (PIK2)	Echelon Biosciences Inc.	Cat#B-0304
PI3K activator (740Y-P)	Cayman	Cat# 22598
Lipopolysaccharides (LPS, E. coli O55:B5)	Sigma-Aldrich	Cat# L4524-5MG
Pam3CSK4	InvivoGen	Cat # tlr1-pms
Pam2CSK4	InvivoGen	Cat # tlr1-pm2s-1
Resiquimod (R848)	InvivoGen	Cat# tlr1-r848-5
Recombinant mouse M-CSF	R&D Systems	Cat# 416-ML-050
Recombinant CCL19	R&D Systems	Cat# 440-M3-025
Recombinant CCL21	R&D Systems	Cat# 457-6C-025
human TGF- β 1	eBioscience	Cat# 14-8348-62
Recombinant mouse IL-2	eBioscience	Cat# 14-8021-64
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich	Cat# M2128-250MG
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen	Cat# C34570
Rat Ig G1 (MOPC21)	BioXCell	Cat# BE0083
Collagenase D	Roche	Cat# 11088866001
Collagenase P	Roche	Cat# 11213857001
Critical Commercial Assays		
mouse CD11c positive selection kit	StemCell Technologies, Inc.	Cat# 18758
mouse FITC positive selection Kit	StemCell Technologies, Inc.	Cat# 17682
mouseCD4+ T cell isolation kit	StemCell Technologies, Inc.	Cat# 19852
mouse CD8+ T cell isolation kit	StemCell Technologies, Inc.	Cat# 19853
mouse B cell isolation kit	StemCell Technologies, Inc.	Cat# 19854
Experimental Models: Cell Lines		
Primary mouse dermal LEC	Cell Biologics, Inc.	Cat# C57-6064 L
Primary human dermal LEC	Cell Biologics, Inc.	Cat# H-6064 L
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J WT	The Jackson Laboratory	JAX: 000664
Mouse: B6.129S2-Ltatm1Dch/J	The Jackson Laboratory	JAX: 002258
Mouse: C57BL/6J Foxp3GFP	(Fontenot et al., 2005)	N/A
Software and Algorithms		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Velocity 6.3	Quorum Technologies	http://quorumtechnologies.com/
FlowJo 8.7	FlowJO, LLC	https://www.flowjo.com
Prism 5	GraphPad Software, Inc	https://www.graphpad.com

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