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Skin-derived TSLP stimulates skin migratory dendritic cells to promote the expansion of regulatory T cells

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Abstract

Therapeutic strategies that enhance regulatory T (Treg) cell proliferation or suppressive function hold promise for the treatment of autoimmune and inflammatory diseases. We previously reported that the topical application of the vitamin D3 analog MC903 systemically expands Treg cells by stimulating the production of thymic stromal lymphopoietin (TSLP) from the skin. Using mice lacking TSLP receptor expression by dendritic cells (DCs), we hereby show that TSLP receptor signaling in DCs is required for this Treg expansion in vivo. Topical MC903 treatment of ear skin selectively increased the number of migratory DCs in skin-draining lymph nodes (LNs) and upregulated their expression of co-stimulatory molecules. Accordingly, DCs isolated from skin-draining LNs but not mesenteric LNs or spleen of MC903-treated mice showed an enhanced ability to promote Treg proliferation, which was driven by co-stimulatory signals through CD80/CD86 and OX40 ligand. Among the DC subsets in the skin-draining LNs of MC903-treated mice, migratory XCR1^{-}CD11b^{+} type 2 and XCR1^{-}CD11b^{-} double negative conventional DCs promoted Treg expansion. Together, these data demonstrate that vitamin D3 stimulation of skin induces TSLP expression, which stimulates skin migratory DCs to expand Treg cells. Thus, topical MC903 treatment could represent a convenient strategy to treat inflammatory disorders by engaging this pathway.

Graphical Abstract

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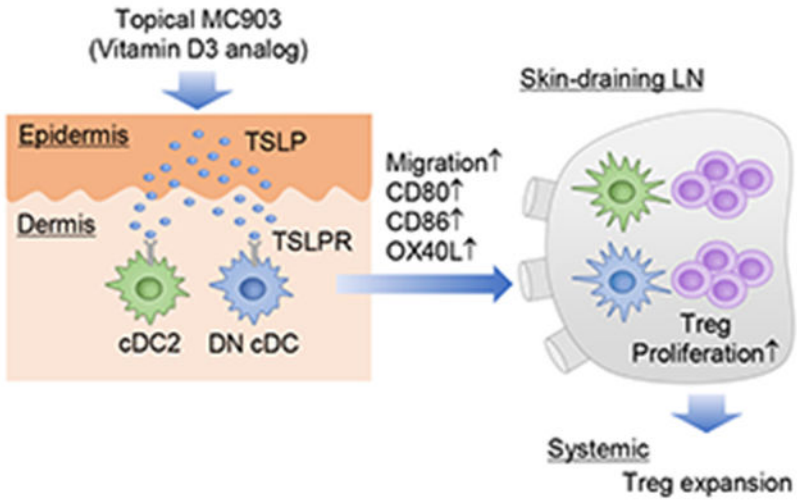

Topical application of a vitamin D3 analog MC903 stimulates skin to produce TSLP, which induces Treg expansion through TSLPR signaling in DCs. TSLP preferentially activates skin migratory cDC2s and DN cDCs to facilitate their migration and enhance their ability to promote Treg proliferation by upregulating the expression of co-stimulatory molecules.

Keywords

MC903; TSLP; dendritic cell; regulatory T cell; immune regulation

Introduction

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that play an indispensable role in the maintenance of peripheral tolerance and suppression of excessive immune responses [1, 2]. Treg cells are generated either in the thymus (tTreg, thymic Treg) or in the periphery by conversion of naïve $CD4^+$ T cells (pTreg, peripheral Treg). Treg cells are marked by the expression of the IL-2 receptor α chain CD25 and the transcription factor Foxp3, which controls the development and function of Treg cells. Loss-of-function mutations in *FOXP3/Foxp3* gene lead to deficiency of Treg cells, resulting in fatal autoimmune inflammatory disorders known as immune dysregulation polyendocrinopathy enteropathy X-linked syndrome in human and scurfy in mice [3]. An impaired number and function of Treg cells underlie many autoimmune inflammatory disorders, including type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus [4, 5]. Therefore, therapeutic strategies that expand Treg cells or enhance their suppressive function hold promise for the treatment of autoimmune and other inflammatory diseases.

The number of Treg cells is maintained in the periphery in part by their continuous proliferation at steady state [6]. Multiple signals are involved in the maintenance of Treg population. IL-2 is an essential cytokine for the maintenance and suppressive function of Treg cells [7]. Although IL-2 also activates $CD4^+$ conventional T (Tconv) cells, $CD8^+$ T cells, and NK cells, low concentrations of IL-2 selectively expand Treg cells [8]. Treg

cells do not produce IL-2 by themselves. Tconv cells produce IL-2 when they are activated by dendritic cells (DCs) through major histocompatibility complex class II (MHCII) and co-stimulatory molecules, such as CD80 and CD86. In conjugation with co-stimulatory signals provided by DCs, this paracrine IL-2 induces Treg proliferation in vitro [9]. In this process, cell-autonomous T cell receptor (TCR) signaling in Treg cells is not required as MHCII-deficient DCs can induce Treg proliferation in the presence of exogenous IL-2. This unnecessity for TCR signaling holds true for in vivo Treg proliferation when IL-2 concentrations are high or IL-2 receptor/STAT5 signaling is selectively activated in Treg cells [10]. However, at steady state IL-2 levels, Treg cells require TCR signaling for optimal proliferation [10, 11]. These and other unidentified cell-types and molecules are thought to regulate the number and function of Treg cells in the periphery to prevent autoimmunity and excessive immune responses.

Thymic stromal lymphopoietin (TSLP) is a cytokine that is mainly produced by epithelial cells at barrier sites such as skin, lung, and intestine [12–14]. TSLP has been extensively investigated as an initiator of type 2 inflammation such as atopic dermatitis and asthma. Topical application of the vitamin D3 analog MC903 stimulates keratinocytes to produce TSLP, leading to atopic dermatitis-like inflammation in mice [15, 16]. Interestingly, topical MC903 also induces systemic Treg expansion in a TSLP-dependent manner and is beneficial for the treatment of autoimmune diabetes in non-obese diabetic mice and experimental autoimmune encephalomyelitis model of multiple sclerosis [17]. Similarly, keratinocyte-derived TSLP prevents progression from local skin inflammation to systemic lethal conditions as a consequence of Mi-2β deletion in keratinocytes by expanding Treg cells [18]. Therefore, TSLP possesses therapeutic potential for autoimmune/inflammatory diseases by expanding Treg cells.

The number of Treg cells in the periphery, but not in the thymus, is reduced in the absence of DCs, indicating an important role for DCs in the maintenance of Treg cells [19–21]. Consistently, Fms-like tyrosine kinase 3 ligand treatment, which augments DC numbers, leads to an expansion of Treg cells in the periphery [9, 20]. Moreover, DCs are involved in topical MC903-induced Treg expansion [17]. DCs can be classified into conventional DCs (cDCs), plasmacytoid DCs, and non-conventional DCs, which include monocyte-derived DCs and Langerhans cells (LCs) [22, 23]. cDCs are further divided into BATF3- and IRF8-dependent type 1 cDCs (cDC1s) and IRF4-dependent type 2 cDCs (cDC2s). These DC subsets are functionally specialized. For example, cDC1s are the primary subset that cross-presents antigen to $CD8^+$ T cells, while cDC2s have been associated with a variety of $CD4^+$ Th cell responses. LCs reside in the epidermis and share functional properties with cDCs. We have previously shown that GM-CSF-stimulated splenic $CD8\alpha^-$ cDC2s, but not $CD8\alpha^+$ cDC1s, support Treg proliferation in vitro [9]. However, the responsible DC subsets for topical MC903-induced Treg expansion have not been identified.

In the present study, we investigated DC subsets that are involved in topical MC903-induced Treg expansion in mice. We show that MC903-induced TSLP selectively enhances the ability of skin-draining lymph node (LN) DCs to promote Treg proliferation, which was inhibited by blockade of co-stimulatory molecules CD80/CD86 and OX40 ligand (OX40L). Among DC subsets found in skin-draining LNs, increased numbers of migratory cDC2s

and double negative (DN) cDCs with upregulated expression of co-stimulatory molecules were seen with topical MC903 treatment. Consistently, these DC subsets showed the highest ability to induce Treg proliferation. Thus, MC903-induced TSLP preferentially activates skin migratory cDC2s and DN cDCs to promote the expansion of Treg cells.

Results

Topical MC903-induced TSLP systemically expands Treg cells by acting on DCs

Topical application of MC903, a low-calcemic vitamin D3 analog, on skin stimulates keratinocytes to produce TSLP, which is detectable in circulation [15–17]. To test the effects of skin-derived TSLP on Treg frequency and number, wildtype mice were treated with vehicle (EtOH, ethanol) or MC903 once a day on both ears for 5 days (Fig. 1A). This MC903 treatment induced ear swelling as previously reported (Supporting Information Fig. 1) [24]. On day 7, the frequency of Treg cells among $CD4^+$ T cells and the absolute number of Treg cells were significantly increased in the ears and ear-draining auricular LNs after MC903 treatment (Fig. 1B and C and Supporting Information Fig. 2). Significant increases were also observed in remote sites including axillary LNs, inguinal LNs, and spleen but not in mesenteric LNs (Fig. 1B and C and Supporting Information Fig. 2). Similar to topical MC903 treatment, forced expression of TSLP in the liver using an adeno-associated virus serotype 8 (AAV8) expressing TSLP (TSLP-AAV8) [25], also increased Treg frequency and number in various secondary lymphoid organs with the exception of mesenteric LNs (Fig. 1D–F). These data suggest that TSLP is capable of inducing systemic Treg expansion irrespective of its tissue source.

We previously reported that TSLP receptor (TSLPR) was necessary for MC903-mediated Treg expansion in the spleen [17]. Moreover, using an in vitro co-culture system, we showed that TSLP-responsive DCs might be important for Treg expansion. To test the requirement of TSLPR expression by DCs in MC903-mediated Treg expansion in vivo, we generated DC-specific TSLPR conditional KO mice ($CD11c^{Cre}$ $TSLPR^{fl/fl}$ mice) and treated them with topical EtOH (vehicle) or MC903. TSLPR deletion in DCs of $CD11c^{Cre}$ $TSLPR^{fl/fl}$ mice was confirmed by flow cytometry (Supporting Information Fig. 3). While control $TSLPR^{fl/fl}$ mice showed significant increases in Treg frequency in the ears, auricular LNs, and spleen by MC903 treatment, there was no significant increase observed in $CD11c^{Cre}$ $TSLPR^{fl/fl}$ mice (Fig. 2A). This was similar to global TSLPR KO mice (Fig. 2B), which showed a similar blunted response to MC903 treatment, although there was a slight increase in Treg frequency in the ear. These data indicate that topical MC903-induced Treg expansion depends on TSLPR signaling by DCs.

Topical MC903 enhances the ability of skin-draining LN DCs to promote Treg proliferation

Given that TSLP is elevated in systemic circulation after topical MC903 treatment, it was possible that DCs from any secondary lymphoid organ could be responsible for Treg expansion. To test which DCs played a role in MC903-mediated Treg expansion, we utilized a previously established DC-Treg co-culture model to analyze the ability of DCs to promote Treg proliferation [9]. DCs were enriched from LNs from various anatomic locations and from the spleen on day 4 of EtOH or MC903 treatment and co-cultured with CellTrace

Violet (CTV)-labeled Treg cells in the presence of IL-2 for 5 days (Fig. 3A and Supporting Information Fig. 4A and B). Consistent with previous studies, DCs in LNs and spleen of EtOH-treated mice supported in vitro Treg proliferation as indicated by CTV dilution (Fig. 3B and Supporting Information Fig. 4C). Strikingly, MC903 treatment enhanced the ability of DCs in skin-draining auricular, axillary, and inguinal LNs to promote in vitro Treg proliferation (Fig. 3B). This effect was most prominent in DCs isolated from the local ear-draining auricular LNs compared with those in the remote skin-draining axillary and inguinal LNs. In contrast to skin-draining LNs, MC903 treatment did not enhance the ability of DCs in gut-draining mesenteric LNs or spleen to promote in vitro Treg proliferation (Fig. 3B). Similar results were obtained when TSLP was overexpressed in the liver of mice by TSLP-AAV8 injection (Supporting Information Fig. 4D and E). Similar to MC903 treatment, liver-derived TSLP boosted the ability of DCs from skin-draining LNs but not mesenteric LN or spleen to promote Treg expansion, although there was no bias towards auricular LN DCs. Furthermore, MC903 treatment did not affect the Treg proliferation-promoting ability of skin-draining LN DCs in TSLPR KO mice (Fig. 3C).

We have previously reported that DCs are required for Treg proliferation by providing co-stimulatory signals including those through CD80/CD86 and OX40L [9, 26]. Therefore, we examined the effects of blockade of co-stimulatory molecules by means of CTLA4-Ig, which blocks CD80 and CD86, and anti-OX40L blocking antibody on the skin-draining LN DC-Treg co-culture model. Combination of CTLA4-Ig and anti-OX40L antibody greatly suppressed Treg proliferation induced by skin-draining LN DCs from either EtOH-treated or MC903-treated mice (Fig. 3D). Together, these data suggest that topical MC903 induces TSLP, which upregulates the expression of co-stimulatory molecules on skin-draining LN DCs to enhance their ability to promote Treg proliferation.

Skin migratory DCs are responsible for Treg expansion induced by topical MC903

In LNs, DCs can be divided into lymphoid tissue-resident DCs and peripheral tissue-derived migratory DCs based on their expression of MHCII and CD11c (Supporting Information Fig. 5). To determine whether lymphoid-resident or migratory DCs were responsible for MC903-mediated Treg expansion, we examined the number of DCs and their expression of co-stimulatory molecules after topical MC903 treatment. MC903 treatment selectively increased the frequency and number of migratory DCs in auricular and inguinal LNs and upregulated their expression of CD80 and CD86 (Fig. 4A–D and Supporting Information Fig. 6). In contrast, lymphoid-resident DCs in LNs and spleen as well as migratory DCs in mesenteric LNs were almost unaffected by MC903 treatment.

CCR7 is important for migration of peripheral tissue DCs to the draining LNs [27]. To test the role of CCR7 in MC903-mediated migration of DCs to the LNs, we treated CCR7 KO mice with MC903. CCR7 deficiency greatly reduced the baseline frequency and number of migratory, but not resident, DCs in auricular LNs (Supporting Information Fig. 7). Moreover, only a slight increase in migratory DCs was observed upon MC903 treatment of CCR7 KO compared to wildtype mice. Thus, topical MC903 promotes the CCR7-dependent migration of skin DCs to the draining LNs and upregulates their expression of co-stimulatory molecules. These data are consistent with results from the

DC-Treg co-culture model (Fig. 3B), which show that only skin-draining LN DCs increase their ability to promote Treg proliferation after MC903 treatment.

The preferential effect of topical MC903 on skin migratory DCs raised the possibility that TSLPR expression could be restricted to the migratory DC subset. Thus, we examined the expression of TSLPR on lymphoid-resident and migratory DCs in LNs and on lymphoid-resident DCs in spleen. TSLPR was uniformly expressed on all DC subsets in all secondary lymphoid organs tested, although the expression level was higher on lymphoid-resident DCs (Fig. 4E and Supporting Information Fig. 8). Intriguingly, MC903 treatment reduced the expression of TSLPR by migratory DCs in the auricular and inguinal LNs but not in mesenteric LNs, which was most likely caused by TSLPR internalization caused by TSLPR signaling in these DCs [28]. This implies that topical MC903-derived TSLP more selectively signals in migratory DCs of skin-draining LNs.

We next attempted to identify the DC subset(s) that are critical for Treg expansion by topical MC903 treatment. Skin-draining LN DCs can be classified into lymphoid-resident cDC1s and cDC2s and migratory LCs, cDC1s, cDC2s, and DN cDCs (Supporting Information Fig. 9) [29]. LCs were only found in the migratory DC compartment and defined as Ep-CAM^{high}XCR1^{-}. cDC1s, cDC2s, and DN cDCs were defined as XCR1^{+}CD11b^{-}, XCR1^{-}CD11b^{+}, and XCR1^{-}CD11b^{-}, respectively. The frequency and number of skin-draining LN DC subsets and their expression of CD80 and CD86 were largely comparable between control TSLPR$^{fl/fl}$ and CD11c^{Cre} TSLPR$^{fl/fl}$ mice, indicating that TSLPR signaling is not required for the development or maintenance of these DCs (Supporting Information Fig. 10). MC903 treatment selectively increased the frequency and number of migratory cDC2s and DN cDCs in auricular LNs of wildtype mice (Fig. 5A and B). The expression of CD80 and CD86 by all four migratory DC subsets, especially cDC2s and DN cDCs, were upregulated by MC903 treatment (Fig. 5C and D and Supporting Information Fig. 11A). The expression of TSLPR was downregulated and the mRNA expression of *Tnfsf4*, which encodes OX40L, was upregulated by MC903 treatment in migratory cDC2s and DN cDCs, but not in other DC subsets in skin-draining LNs (Fig. 5E and F and Supporting Information Fig. 11B). Consistent with the expression pattern of co-stimulatory molecules, migratory cDC2s and DN cDCs purified from skin-draining LNs of MC903-treated mice showed the highest ability to promote in vitro Treg proliferation (Fig. 5G). These data suggest that skin migratory cDC2s and DN cDCs are responsible for Treg expansion by topical MC903 in skin-draining LNs.

Discussion

Topical application of MC903 is a convenient way to increase systemic TSLP levels in mice [15–17]. We have previously reported that topical MC903 expands Treg cells in a TSLP-dependent manner and proposed its use for the treatment of autoimmune diseases [17]. In the present study, we demonstrated that forced expression of TSLP by means of TSLP-AAV8 also expanded Treg cells. Moreover, we generated DC-specific TSLPR conditional KO mice and showed that TSLPR signaling in DCs is required for topical MC903-induced Treg expansion in vivo. Finally, we found that MC903 treatment selectively

activates skin-draining LN migratory cDC2s and DN cDCs to enhance their ability to induce Treg proliferation.

At least three factors are known to be involved in Treg proliferation: IL-2, TCR, and co-stimulatory molecules [30]. In this study, we utilized a previously established DC-Treg co-culture model with exogenous IL-2 to test the ability of DCs to induce Treg proliferation [9]. In this setting, co-stimulatory, but not TCR, signals are required for Treg proliferation [9, 26]. Stimuli that upregulate the expression of co-stimulatory molecules on DCs, such as GM-CSF, lipopolysaccharide, and CpG DNA, can enhance the ability of DCs to induce Treg proliferation [9, 31]. In line with these previous results, DCs purified from skin-draining LNs of MC903-treated mice showed enhanced ability to induce Treg proliferation, which was attributed to their increased expression of CD80, CD86, and OX40L. These co-stimulatory signals contribute to Treg homeostasis and function, although the precise mechanisms are not fully understood [32, 33]. The OX40-OX40L axis can be either a positive or negative regulator of Treg cells, presumably in a context- and timing-dependent manner [31, 32, 34]. Combined blockade of CD80/CD86 and OX40L did not completely inhibit Treg proliferation in our DC-Treg co-culture model, suggesting a role for other signals provided by DCs, such as ICOS ligand and a Notch ligand Jagged1 [35, 36]. In the absence of exogenous IL-2, Tconv cells activated by DCs through MHCII and co-stimulatory molecules can be the source of IL-2 to induce in vitro Treg proliferation [9]. Thus, we predict that upregulated expression of co-stimulatory molecules on skin migratory DCs by MC903 treatment contributes to in vivo Treg expansion by providing co-stimulatory signals both directly to Treg cells and indirectly to Tconv cells, leading to increased production of paracrine IL-2. Further studies are needed to clarify the precise role of co-stimulatory molecules in MC903/TSLP-induced Treg expansion and immune suppression.

The in vivo relevance of our in vitro Treg proliferation model is supported by recent histological studies showing that in LNs, self-activated Tconv cells produce IL-2, which triggers paracrine signaling in Treg cells, leading to their local proliferation and thus resulting in the formation of a negative feedback loop to constrain self-activated Tconv cells [37, 38]. Interestingly, migratory DCs, especially with the $CD11b^{+}$ cDC2 phenotype, are associated with Treg clusters in LNs [37]. Our current study shows that topical MC903 increases the number of migratory cDC2s and DN cDCs in skin-draining LNs. Therefore, it is possible that MC903 treatment increases the number of Treg clusters in skin-draining LNs, which may also contribute to in vivo Treg expansion.

DC subsets in skin-draining LNs are not altered in naïve TSLPR KO mice compared with wildtype mice, suggesting that TSLP is not involved in skin DC homeostasis at steady state [39]. We obtained similar results using DC-specific TSLPR conditional KO mice. However, when TSLP expression is induced by MC903, we find that skin migratory cDC2s and DN cDCs are increased in skin-draining LNs and are the key DC subsets responsible for MC903-induced Treg expansion. TSLP-TSLPR signaling in these DC subsets is supported by the reduced expression of surface TSLPR seen after MC903 treatment, which implies receptor internalization following ligand activation [28]. Intriguingly, the same DC subsets that expand Treg cells also drive Th2 differentiation during TSLP-dependent contact

sensitization [39, 40]. Moreover, topical MC903 induces Th2 cell differentiation in draining LNs in a TSLP-dependent manner [41, 42]. Even in the absence of allergens, high levels of TSLP induced by topical MC903 can trigger skin inflammation characterized by a Th2 cytokine milieu provided by type 2 innate lymphoid cells [24, 43, 44]. One molecule that could be important for simultaneously driving Treg expansion and Th2 differentiation is OX40L. Our data shows that upregulation of *Tnfsf4* (encodes OX40L) expression by topical MC903 is limited to migratory cDC2s and DN cDCs among DC subsets in skin-draining LNs. OX40L is a critical co-stimulatory molecule for the priming of Th2 cells [45]. Blockade of OX40L during MC903 treatment inhibits the development of Th2 cells in the draining LNs [41]. Therefore, skin migratory cDC2s and DN cDCs may have dual Treg-expanding and Th2-inducing function in the context of MC903/TSLP-induced skin inflammation.

Topical MC903 triggers skin inflammation despite an increase in the frequency and number of local Treg cells. Although Treg cells are insufficient to completely quell the local skin inflammation, they are likely involved in its attenuation. This notion is supported by a recent report showing that RORα-expressing skin Treg cells restrain local inflammation induced by topical MC903 and allergen application to tape-stripped mouse skin [46]. The latter model involves a low level of TSLP production by keratinocytes and depends on allergen-specific Th2 responses [43]. Thus, while TSLP-stimulated DCs can promote type 2 inflammation, a concurrent feedback mechanism involving Treg expansion likely counterbalances excessive inflammation induced by TSLP. In addition to their expansion, topical MC903 treatment upregulates the expression of functional markers including CTLA4, GITR, and ICOS on Treg cells, although their suppressive activity on in vitro Tconv proliferation is not significantly enhanced [17].

In the current study, topical MC903 or TSLP-AAV8 injection had no effects on the ability of mesenteric LN DCs to induce Treg proliferation, suggesting that intestinal DCs are unresponsive to TSLP. Intestinal DCs transport food antigens to mesenteric LNs, where they induce pTreg cells to maintain peripheral tolerance [47]. Importantly, oral tolerance to food antigens depends on pTreg cells [48]. If intestinal DCs responded to TSLP in the same way as skin DCs, elevated TSLP in circulation might lead to the induction of Th2 cells instead of pTreg cells to food antigens, resulting in the development of food allergy. We previously showed that topical MC903 augments the proliferation of pre-existing tTreg cells, but not the generation of pTreg cells [17]. Indeed, although co-stimulatory signals via CD28 is required for pTreg generation, strong CD28 co-stimulation prevents it [33, 49]. Thus, upregulation of co-stimulatory molecules on DCs in response to TSLP is thought to be undesirable for the pro-tolerogenic property of mesenteric LNs, if it occurs. Unresponsiveness of intestinal DCs to TSLP may partly contribute to the prevention of food allergy. This hypothesis is consistent with a current view that food allergy is caused by allergen exposure through barrier-disrupted skin rather than intestinal mucosa [50].

Human TSLP has short and long isoforms whereas mouse TSLP has only long isoform [51, 52]. The receptor for short-form TSLP is not known. Short-form TSLP is constitutively expressed in barrier surfaces under steady-state conditions and is anti-inflammatory, whereas long-form TSLP is induced by inflammatory stimuli and is pro-inflammatory. Based on

the present and previous studies using mice [17, 18], we expect that long-form TSLP has the potential to expand Treg cells besides its well-known function to initiate type 2 inflammation. Indeed, human DCs upregulate co-stimulatory molecules, including CD80, CD86, and OX40L, in response to long-form TSLP [45]. Thus, it is possible that long-form TSLP enhances the ability of human DCs to promote Treg proliferation. However, some inflammatory cytokines and toll-like receptor ligands, but not vitamin D3 or MC903, induce the production of long-form TSLP by human keratinocytes and skin biopsies [53, 54]. Thus, there is a need for novel approaches other than topical application of MC903 to induce long-form TSLP in human. Also, it is of interest to assess the role of short-form TSLP in Treg homeostasis and function.

In conclusion, we herein revealed that TSLP systemically expands Treg cells by activating skin migratory cDC2s and DN cDCs, which have been previously reported as TSLP-responsive DCs that drive Th2 differentiation. Thus, we identified a novel regulatory role for these skin migratory DCs. This study provides a rationale for targeting skin migratory DCs to expand Treg cells for the treatment of autoimmune/inflammatory diseases.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories or Japan SLC. B6 TSLPR KO mice were kindly provided from Dr. W.J. Leonard (National Institutes of Health) [55]. B6 TSLPR$^{fl/fl}$ mice were described previously [25]. B6 CD11c^{Cre} mice were purchased from Jackson Laboratories (strain # 008068) and crossed with TSLPR$^{fl/fl}$ mice to generate DC-specific TSLPR conditional KO mice. B6 Foxp3^{EGFP} and CCR7$^{GFP/GFP}$ (CCR7 KO) mice were purchased from Jackson Laboratories (strain # 006772 and 027913, respectively). TSLPR KO, TSLPR$^{fl/fl}$, CD11c^{Cre}, Foxp3^{EGFP}, and CCR7 KO mice were bred and maintained at the University of Pennsylvania. All mice were housed in specific pathogen-free conditions and used at 7 to 12 weeks of age. All experiments were performed with age- and gender-matched mice.

Treatment of mice

Mice were treated on both ears with 10 μL of 200 μM MC903 (Tocris Bioscience) dissolved in EtOH or EtOH vehicle for up to 5 consecutive days. To evaluate skin inflammation, thickness of both ears was measured using a dial thickness gauge and averaged. Ear swelling was expressed as % change in ear thickness from baseline (day 0). Alternatively, mice were injected intravenously with 5×10^{10} genome copies of control AAV8 or TSLP-AAV8 as described previously [25]. On the indicated days post treatment, mice were sacrificed and tissues were collected for analysis.

Isolation of cells

For the analysis of Treg cells, LNs and spleen were mashed and filtered through a 70-μm nylon mesh. Splenocytes were hemolyzed with 140 mM ammonium chloride in 17 mM Tris-HCl (pH 7.5). Ears were split into dorsal and ventral halves and digested with 250 μg/mL Liberase TL (Roche) and 50 μg/mL DNase I (Sigma-Aldrich) in RPMI 1640 medium

containing 10% fetal bovine serum (FBS) at 37°C for 90 min with gentle shaking. After digestion, the ear cells were filtered through a 70-μm cell strainer. For the analysis of DCs, LNs and spleen were cut into small pieces and digested with 1 mg/mL collagenase D (Roche) and 50 μg/mL DNase I (Sigma-Aldrich) in RPMI 1640 medium containing 10% FBS at 37°C for 30 min with gentle shaking. Then, the cells were filtered through a 70-μm nylon mesh. In some experiments, DCs were enriched using EasySep mouse pan-DC enrichment kit (StemCell Technologies). In other experiments, DC subsets were purified by cell sorting without a prior enrichment step.

DC-Treg co-culture

$CD4^+$ T cells were isolated from LNs and spleen of untreated $Foxp3^{EGFP}$ mice using EasySep mouse $CD4^+$ T cell isolation kit (StemCell Technologies). Then, Treg cells ($CD90.2^+CD8a^-Foxp3\text{-}EGFP^+$) were sorted and labeled with 2.5 μM CTV (Thermo Fisher Scientific). To assess the ability of DCs to promote Treg proliferation, 2×10^4 DCs were co-cultured with 2×10^4 CTV-labeled Treg cells in 200 μL of T cell media (MEM-α containing 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 50 μM 2-mercaptoethanol) in the presence of 10 ng/mL human IL-2 (PeproTech) in 96-well flat-bottomed plates. To block co-stimulatory signals, CTLA4-Ig (20 μg/mL; BioXcell) and anti-OX40L (20 μg/mL; clone RM134L; BioLegend) were added to culture media. After a 5-day culture, the cells were analyzed by flow cytometry and Treg proliferation was evaluated by CTV dilution.

Flow cytometry

Dead cells were stained with either Live/Dead Near-IR or Aqua (Thermo Fisher Scientific) or DAPI (Dojindo). Anti-CD16/32 (2.4G2; BD Biosciences) was used to block Fc receptors. Intracellular staining of Foxp3 was performed using a Foxp3 staining buffer set (Thermo Fisher Scientific). Flow cytometric antibodies used in this study were shown in Supporting Information Table 1. Data were acquired on an LSR II or an LSRFortessa (BD Biosciences), and analyzed using FlowJo software (Tree Star). Cell sorting was performed with a FACSAria cell sorter (BD Biosciences). Flow cytometry was performed at the Flow Cytometry Core Facility of the University of Pennsylvania and the Biomedical Research Core of Tohoku University Graduate School of Medicine.

Quantitative RT-PCR

Sorted DC subsets ($\geq 3\times10^4$) were lysed in Isogen (Nippon Gene), and total RNA was extracted following the manufacturer's instructions. cDNA was generated using a ReverTra Ace qPCR RT kit (Toyobo). Quantitative RT-PCR was performed using a Thunderbird SYBR qPCR Mix (Toyobo) and a CFX96 cycler (Bio-Rad). The primers used for PCR were as follows: *Tnfsf4*, forward 5'-GGGATGCTTCTGTGCTTCATCT-3' and reverse 5'-TTTGGATTGGAGGGTCCTTTG-3'; *Gapdh*, forward 5'-ATGTGTCCGTCGTGGATCTG-3' and reverse 5'-GGTGGAAGAGTGGGAGTTGC-3' (Nihon Gene Research Laboratories). The PCR conditions were 40 cycles for *Tnfsf4* or 35 cycles for *Gapdh* of 95°C for 10 s, 64°C for 10 s, and 72°C for 30 s. The expression levels of *Tnfsf4* were normalized to *Gapdh* and expressed as relative units.

Statistics

Statistical analysis was performed by Welch's *t*-test or two-way ANOVA as described in the Figure legends using Prism 9 software (GraphPad). *P* values less than 0.05 were considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations

AAV8	adeno-associated virus serotype 8
B6	C57BL/6
cDC	conventional DC
cDC1	type 1 cDC
cDC2	type 2 cDC
CTV	CellTrace Violet
DC	dendritic cell
DN	double negative
EtOH	ethanol
FBS	fetal bovine serum
LC	Langerhans cell
LN	lymph node
MHCII	major histocompatibility complex class II
OX40L	OX40 ligand
pTreg	peripheral Treg
Tconv	conventional T

TCR	T cell receptor
Treg	regulatory T
TSLP	thymic stromal lymphopoietin
TSLPR	TSLP receptor
tTreg	thymic Treg

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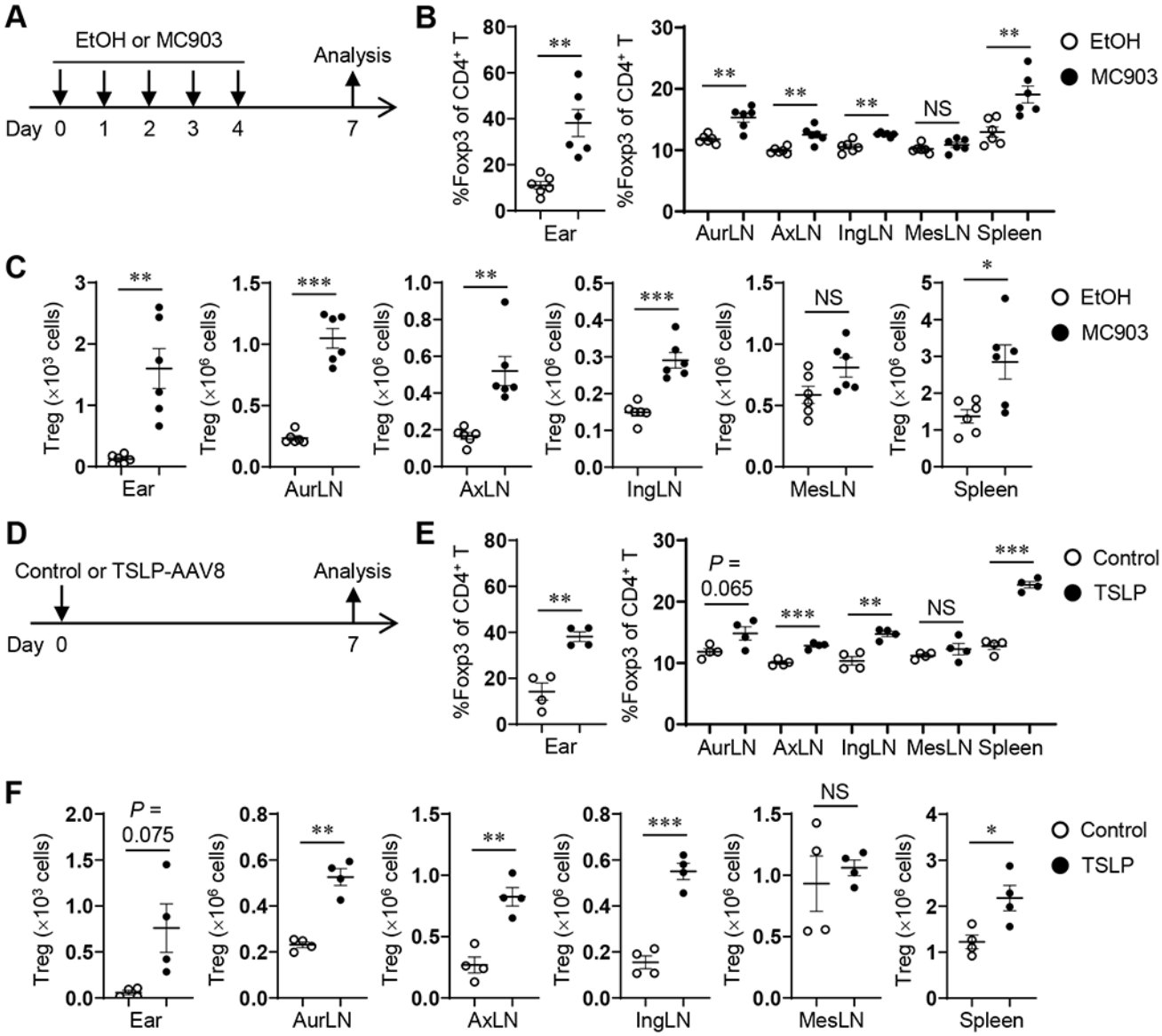

Figure 1.

Systemic Treg expansion induced by topical MC903 and exogenous TSLP. (A-C) Both ears of B6 mice were treated with vehicle (EtOH) or MC903 (2 nmol/ear) once daily for 5 days. On day 7, mice were sacrificed and analyzed by flow cytometry. The gating strategy is shown in Supporting Information Fig. 2. (D–F) B6 mice were injected intravenously with control AAV8 or TSLP-AAV8 (5×10^{10} genome copies) and analyzed on day 7. (B and E) The frequencies of $Foxp3^+$ Treg cells among $CD4^+$ T cells in indicated tissues are plotted as mean ± SEM. (C and F) The absolute numbers of $Foxp3^+$ Treg cells in indicated tissues are plotted as mean ± SEM. (B and C) Data are pooled from 3 independent experiments (n = 6 mice/group). (E and F) Data are pooled from 2 independent experiments (n = 4 mice/group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired two-tailed Welch's *t*-test. AurLN, auricular LN; AxLN, axillary LN; IngLN, inguinal LN; MesLN, mesenteric LN; NS, not significant.

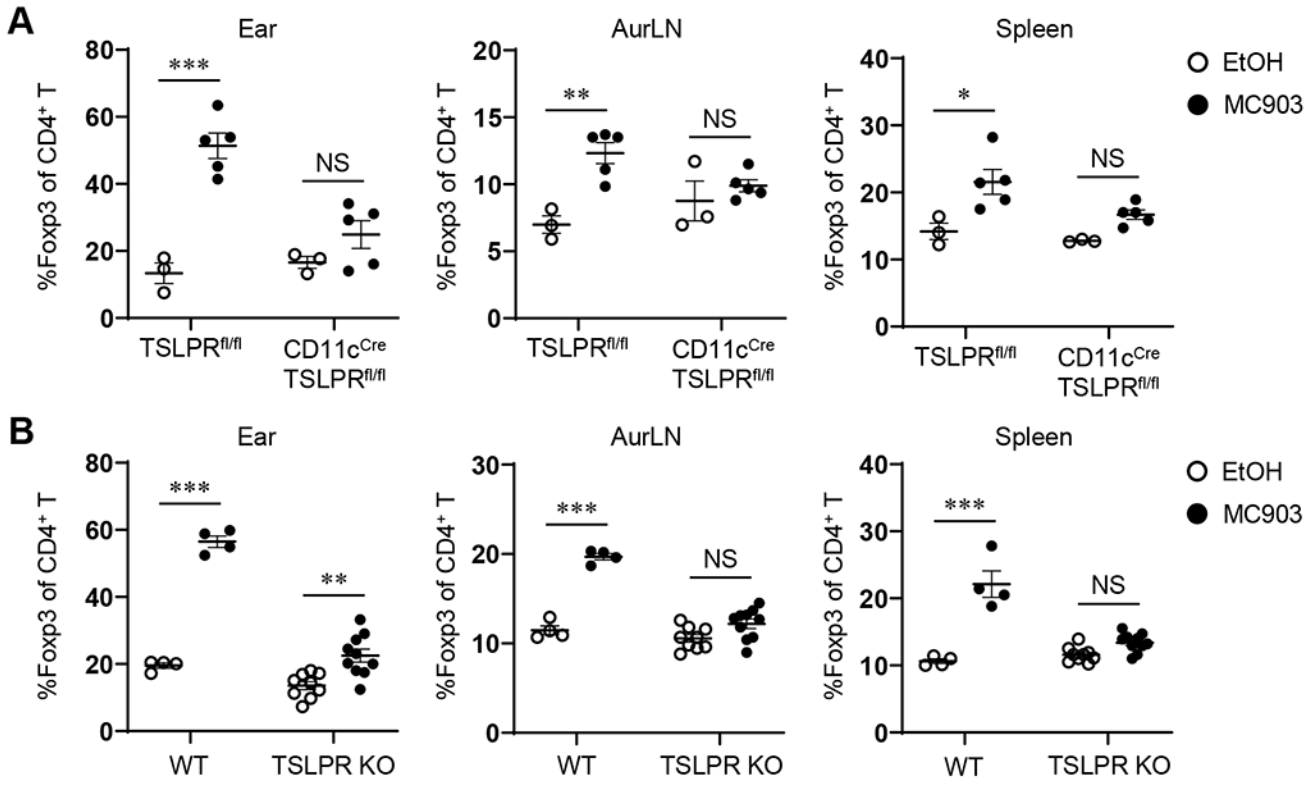

Figure 2.

TSLPR signaling in DCs is required for MC903-induced Treg expansion. Mice were topically treated with vehicle (EtOH) or MC903 and analyzed as in Fig. 1A. The frequencies of $Foxp3^+$ Treg cells among $CD4^+$ T cells in indicated tissues are plotted as mean ± SEM. (A) $TSLPR^{fl/fl}$ control and $CD11c^{Cre}$ $TSLPR^{fl/fl}$ mice were examined. Data are pooled from 2 independent experiments (n = 3–5 mice/group). (B) Wildtype control and TSLPR KO mice were examined. Data are pooled from 2 independent experiments (n = 4–10 mice/group). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by 2-way ANOVA with Tukey's *post hoc* test. AurLN, auricular LN; NS, not significant; WT, wildtype.

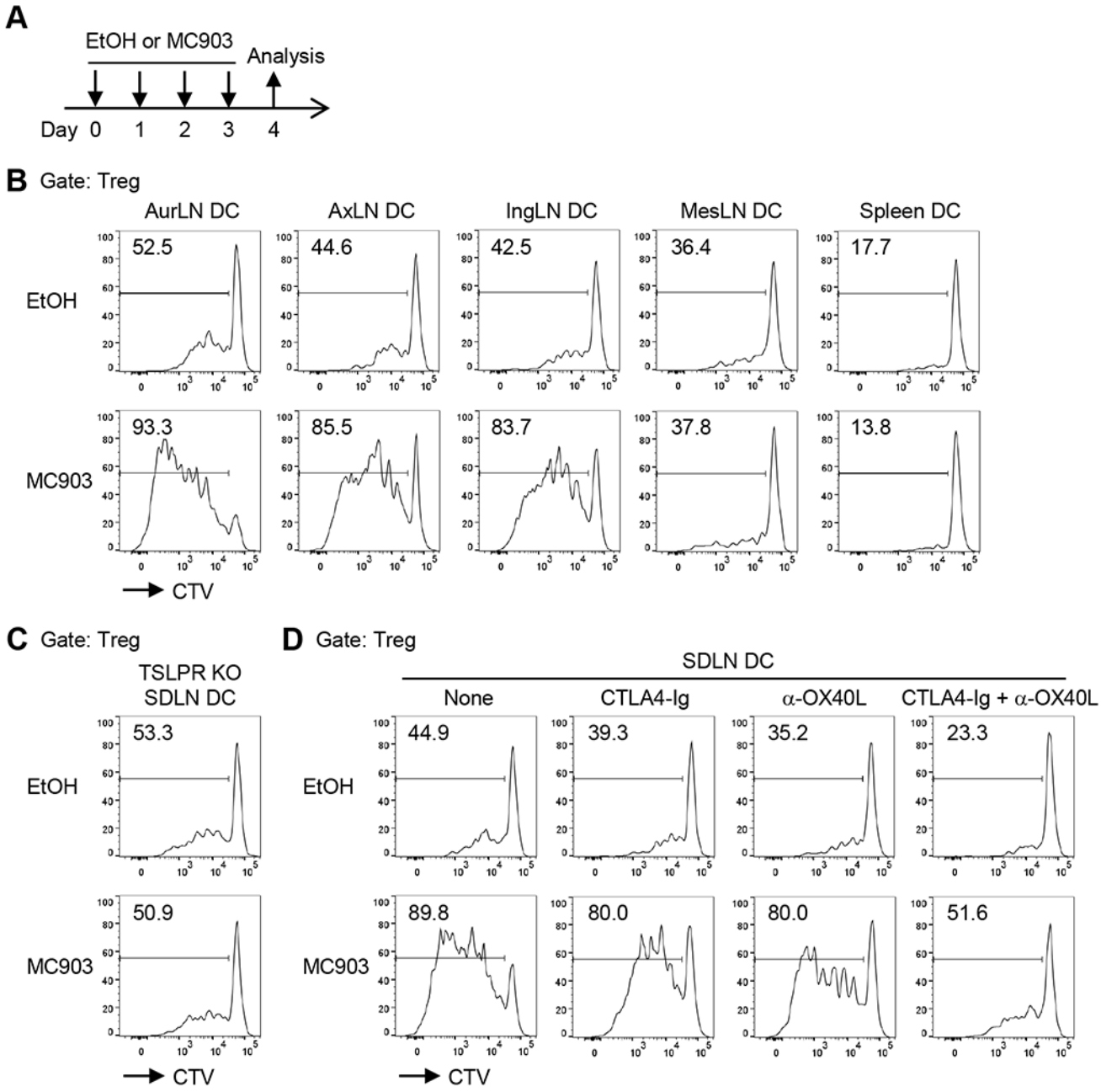

Figure 3.

Topical MC903 enhances the ability of skin-draining LN DCs to promote in vitro Treg proliferation, which depends on co-stimulatory signals. (A–D) Both ears of mice were treated with vehicle (EtOH) or MC903 (2 nmol/ear) once daily for 4 days. On day 4, mice were sacrificed and tissues were collected for the analysis of DCs. (B–D) DCs were enriched from indicated tissues of B6 mice (B) or skin-draining auricular, axillary, and inguinal LNs of TSLPR KO (C) or B6 mice (D). DCs were co-cultured with CTV-labeled Treg cells, sorted as shown in Supporting Information Fig. 4A and B, in the presence of IL-2. After 5 days of culture, Treg cells were gated as in Supporting Information Fig. 4C and analyzed for

CTV dilution by flow cytometry. CTLA4-Ig and/or anti-OX40L were added to culture media as indicated (D). Representative histograms from 2 independent experiments are shown. AurLN, auricular LN; AxLN, axillary LN; IngLN, inguinal LN; MesLN, mesenteric LN; SDLN, skin-draining LN.

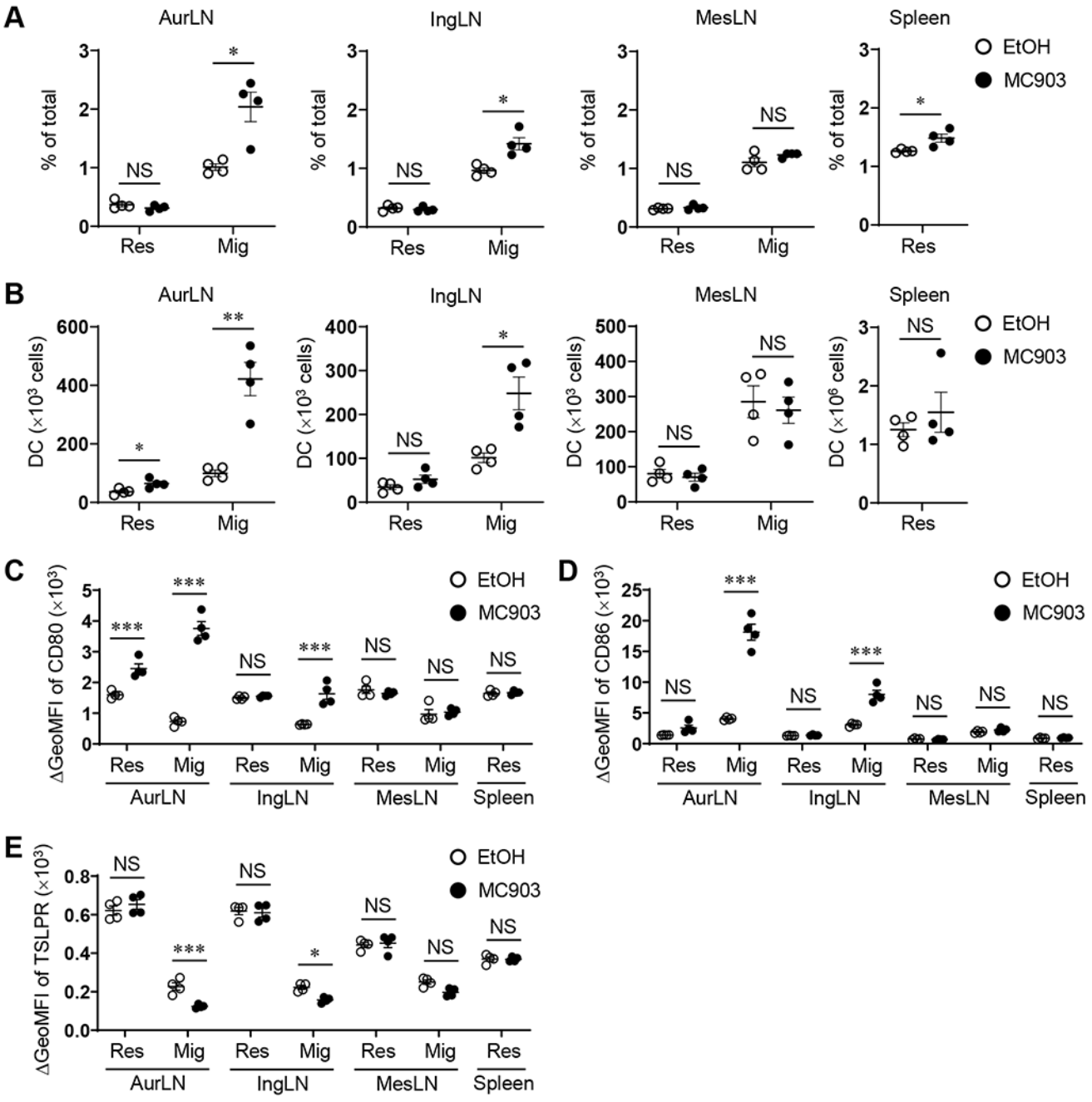

Figure 4.

Topical MC903 increases the number of migratory DCs in skin-draining LNs and upregulates their expression of co-stimulatory molecules. B6 mice were topically treated with vehicle (EtOH) or MC903 and analyzed by flow cytometry as in Fig. 3A. Gating strategy is shown in Supporting Information Fig. 5. (A and B) The frequencies (A) and absolute numbers (B) of resident and migratory DCs in indicated tissues are plotted as mean ± SEM. (C–E) The Δ geometric mean fluorescence intensities of CD80 (C), CD86 (D), and TSLPR (E) by indicated DCs are plotted as mean ± SEM. Representative plots and

histograms are shown in Supporting Information Fig. 6 and 8. Representative data from 2 independent experiments are shown (n = 4 mice/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Welch's *t*-test (A and B) and 2-way ANOVA with Bonferroni's *post hoc* test (C–E). AurLN, auricular LN; GeoMFI, geometric mean fluorescence intensity; IngLN, inguinal LN; MesLN, mesenteric LN; Mig, migratory; NS, not significant; Res, resident.

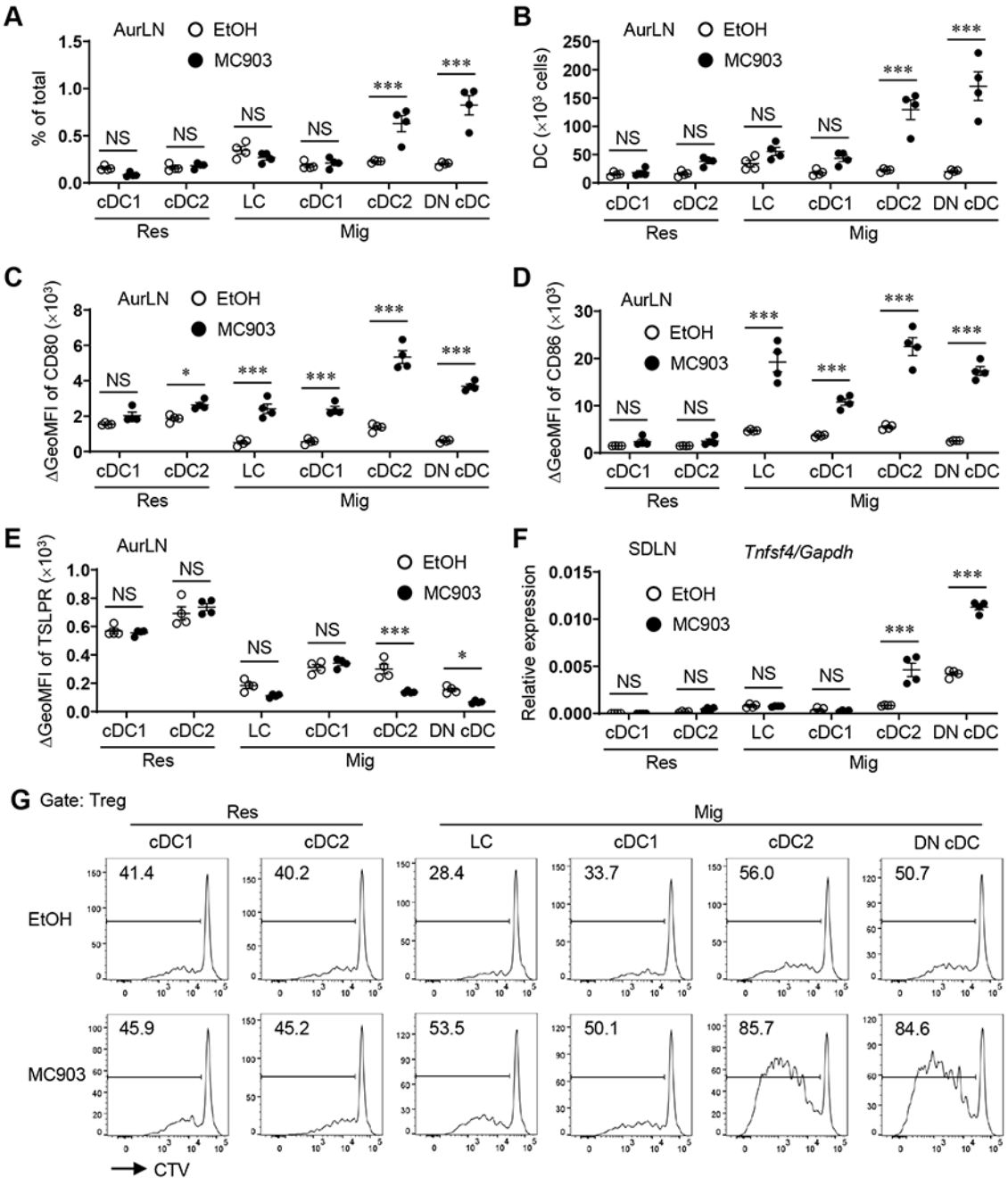

Figure 5.

Skin migratory cDC2s and DN cDCs are the most potent DC subsets to induce Treg expansion in response to topical MC903. B6 mice were topically treated with vehicle (EtOH) or MC903 and analyzed as in Fig. 3A. (A and B) The frequencies (A) and absolute numbers (B) of indicated DC subsets in auricular LNs are plotted as mean ± SEM. Gating strategy is shown in Supporting Information Fig. 9. (C–E) The Δ geometric mean fluorescence intensities of CD80 (C), CD86 (D), and TSLPR (E) by auricular LN DC subsets are plotted as mean ± SEM. Representative plots and histograms are

shown in Supporting Information Fig. 11. (A–E) Representative data from 2 independent experiments are shown (n = 4 mice/group). (F) The skin-draining LNs were pooled from 2–4 mice/sample and DC subsets were sorted. The mRNA levels of *Tnfsf4* were determined by quantitative RT-PCR and normalized to *Gapdh*. Data are plotted as mean ± SEM (n = 4 samples/group). (A–F) $*P < 0.05$, $***P < 0.001$ by 2-way ANOVA with Bonferroni's *post hoc* test. (G) The skin-draining LN DC subsets were sorted and co-cultured with CTV-labeled Treg cells in the presence of IL-2. After 5 days of culture, Treg cells were analyzed for CTV dilution by flow cytometry. Representative histograms from 2 independent experiments are shown. AurLN, auricular LN; GeoMFI, geometric mean fluorescence intensity; Mig, migratory; NS, not significant; Res, resident; SDLN, skin-draining LN.