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The transcription factor musculin promotes the unidirectional development of peripheral Treg cells by suppressing the TH2 transcriptional program

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Abstract

Although the master transcription factors (TFs) are the key to the development of specific T cell subsets, whether additional transcriptional regulators are induced by the same stimuli that dominantly repress development of other T cell lineages has not been fully elucidated. Using Transforming growth factor- β (TGF- β) induced regulatory T cell (iT_{reg}) system, we identify the TF Musculin (MSC) as critical for iT_{reg} development by repression of TH2 transcriptional program. Loss of MSC reduces Foxp3 expression and induces TH2 differentiation even under TGF- β induced iT_{reg} differentiation conditions. MSC mediates this effect by interrupting binding of GATA3 to TH2 locus and reducing intrachromosomal interactions within the Th2 locus. MSC-deficient iT_{regs} are not able to suppress TH2 responses and the *Msc*^{-/-} mice spontaneously develop gut and lung inflammation with age. Our data indicate that MSC enforces Foxp3 expression and promotes unidirectional induction of iT_{regs} by repressing development of the TH2 developmental program.

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Authors' contribution

C.W. designed, performed and wrote the manuscript. Z.C. performed experiments and wrote the manuscript. S.X., T.T., M.L., G.G., A.M., R.F.F., T.H., M.O. and V.D. performed experiments. A.M. analyzed the data. V.K.K. supervised the study and edited the manuscript.

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Introduction

Naive CD4⁺ T cells can be polarized into various T cell subsets with defined effector functions in response to differential environmental cues. Development of T cell subsets is driven by 'master' TFs that drive the genetic program leading to the promotion of core sets of signature genes that are essential for their function. However, it has been suggested that together with the master TF, other TF(s) may be induced that, repress development of alternative T cell subsets, leading to unopposed commitment to a specific T cell fate¹.

Regulatory T cells (T_{regs}) are a specialized T cell subset that plays an important role in mediating immune homeostasis and promoting peripheral tolerance^{2, 3}. Depletion of T_{regs} in mice causes a lethal aggressive autoimmune syndrome^{4, 5}. T_{regs} can be derived/differentiated in the thymus as thymic T_{reg} cells (tT_{regs}) or induced in the periphery from naive T cells as induced T_{reg} cells (iT_{regs})⁶. Although these two types of T_{regs} have complementary inhibitory functions and exhibit overlapping but different transcriptional signature gene expression patterns, both of them express the master TF Foxp3^{5, 7}. Genetic deletion of Foxp3 results in multi-organ tissue inflammation together with a loss of T_{reg} function and over-exuberant effector T cell generation, leading to premature death in both mice and human⁴.

Commitment to the T_{reg} lineage, requires prolonged and stable expression of Foxp3 in various inflammatory and tissue microenvironments³. However, even in the absence of expression of Foxp3, T_{regs} tend to express part of the T_{reg} gene program (e.g. T_{reg} 'wannabes') suggesting that there must be other TFs that are induced by environmental cues to promote T_{reg} development. The induction of Foxp3 during T_{reg} development is dependent on TCR and cytokine signaling, including signaling by TGF- β and interleukin 2 (IL-2) receptors^{8, 9}. While both tT_{regs} and iT_{regs} require IL-2 signaling for their maintenance and expansion via activation of the transcription factor signal transducer and activator of transcription 5 (STAT5), TGF- β stimulation on naive T cells results in induction of Foxp3 expression via the Smad3 signaling pathway. TGF- β signaling also suppresses differentiation of T_H1, T_H2 and T_H17 cells in a cell-intrinsic manner^{10, 11}. However, how TGF- β induces the development of iT_{regs}, while at the same time dominantly suppresses development of other effector T cell programs in a cell-intrinsic manner has not been fully characterized.

Musculin (MSC), a member of the helix-loop-helix (HLH) family of TFs, is first reported in mouse skeletal muscle precursors and later on found in B cell lymphomas as a transcriptional repressor^{12, 13}. However, its role for T cell differentiation and function is still largely unclear¹⁴. In this paper, we demonstrate that MSC is induced early in iT_{regs} following TCR-stimulation of naive T cells in the presence of TGF- β . The loss of MSC results in reduced Foxp3 expression and impaired iT_{reg} suppressive function. Furthermore, loss of MSC converts iT_{regs} into T_H2 cells, enhancing expression of T_H2 cytokines even under iT_{reg} differentiation conditions. MSC mediates these effects by physically associating with GATA3 in iT_{regs} and inhibiting the DNA binding activity of GATA3 to the T_H2 locus. Our data provide a novel mechanism by which MSC induced via TGF- β -Smad3 signaling promotes iT_{reg} development by silencing the T_H2 gene program.

Results

Early induction of MSC under TGF- β stimulation

In order to identify cell-intrinsic regulators that may dominantly inhibit development of other T cell subsets during iT_{reg} differentiation, we mined published expression profiling data of iT_{regs} under conditions of TGF- β alone, TGF- β with IL-27 or TGF- β with IFN- γ for 10 and 48 hours¹⁵. We specifically searched for highly upregulated genes that encode TFs during the early phase of TGF- β stimulation, so that we could identify those that might work together with Foxp3 to promote T_{reg} development. The overlap of three different conditions revealed 4 TFs (*Foxp3*, *Batf3*, *Fosl2* and *Msc*) that were the most highly expressed in differentiating iT_{regs} in the initial stage of differentiation in the presence of TGF- β (Fig. 1a). It has been previously reported that Batf3 and Fosl2 do not have a significant impact on the differentiation of iT_{regs} (Supplementary Fig. 1a)^{16, 17}. Therefore, we focused on MSC as one TF that appears to be expressed early, and potentially functions with Foxp3 in the development of T_{regs}. We confirmed by q-PCR and Western that *Msc* was indeed highly induced during the early phase of iT_{reg} differentiation, with little or no expression observed in the other T cell subsets *in vitro* (Fig. 1b–d, Supplementary Fig. 1b). To determine whether MSC is expressed in tT_{regs} vs. iT_{regs} *in vivo*, we analyzed the expression of MSC in Neuropilin-1 (Nrp1) positive vs. negative Foxp3⁺ T_{regs} from wild-type mice^{18, 19}. Consistently, *in vivo* isolated iT_{regs} (CD4⁺Foxp3⁺Nrp1[−]) exhibited higher expression of mRNA and protein of MSC than tT_{regs} (CD4⁺Foxp3⁺Nrp1⁺) (Fig. 1e, Supplementary Fig. 1c) isolated from different compartments. These results demonstrate that MSC is induced specifically within iT_{reg} cells both *in vitro* and *in vivo*.

Smad3 regulates MSC during iT_{reg} cell differentiation

Previous studies showed that TGF- β signalling induces Foxp3 expression dependent on the Smad3 signalling pathway^{20, 21}. We therefore examined whether Smad3 can directly affect MSC expression within iT_{regs}. Indeed, we found that Smad3 bound to multiple sites across the *Msc* promoter under iT_{reg} differentiation conditions, but not under T_H0 cell conditions (Fig. 2a). Moreover, we confirmed that Smad3 transactivates the *Msc* gene in a dose-dependent manner (Fig. 2b–c). Next, we retrovirally overexpressed Smad3 in wild-type T_H0 and iT_{regs} and observed enhanced Foxp3 and MSC expression at mRNA and protein levels (Fig. 2d–e). Furthermore, the increased Foxp3 expression parallels Smad3 and MSC expression in these primary T cells (Fig. 2e). Consistent with the overexpression data, *Smad3*^{−/−} derived iT_{reg} showed both reduced Foxp3 and MSC expression (Fig. 2f–g). These results suggest that MSC expression is mediated by Smad3 that is specifically induced under TGF- β stimulation conditions.

Loss of MSC disrupts iT_{reg} generation

Next, we wanted to study whether loss of MSC *in vivo* has any impact on development of tissue inflammation or homeostasis. We observed wild-type and MSC deficient mice over time for the development of any overt autoimmunity. While the young mice (6–8 weeks old) exhibited comparable T cell composition in the peripheral lymph nodes (LN), we observed increased proportions of activated CD4⁺ (CD62L^{lo}CD44⁺) T cells in 40 week old *Msc*^{−/−} mice (Fig. 3a–b). Further examination of non-lymphoid tissues from the 40 week old mice

revealed immune cell infiltration, particularly in the small intestine and lung, indicating disruption of immune homeostasis at the mucosal surface (Fig. 3c–d).

Increased tissue inflammation at mucosal sites in MSC-deficient mice led us to hypothesize that there might be a defect in T_{reg} generation or function following loss of MSC. We therefore analysed the $Foxp3^+ T_{reg}$ compartment in 8 week old mice. Although no dramatic differences were found in different peripheral lymphoid organs (Supplementary Fig. 1d–e), we observed a reduction of T_{regs} in the gut lamina propria (LP) but not in the lung from $Msc^{-/-}$ mice compared with wild-type littermates (Fig. 3e–f, Supplementary Fig. 2a–b). The gut is known as the major site for iT_{reg} generation and accumulation^{22, 23}. Analysis of T_{regs} isolated from the lamina propria using Nrp1 staining showed fewer $Foxp3^+Nrp1^+ iT_{regs}$ in the $Msc^{-/-}$ mice whereas $Foxp3^+Nrp1^+ iT_{regs}$ remained unchanged (Supplementary Fig. 2c–d). Given the distribution of MSC in both lymphocytes (hematopoietic) and muscle cells (non-hematopoietic)^{12, 13}, we generated chimeras transferring wild-type or $Msc^{-/-}$ bone marrow into $Rag2^{-/-}$ recipients. The bone marrow chimeras that carried $Msc^{-/-}$ lymphocytes exhibited fewer iT_{regs} within the gut LP compared to wild-type controls (Supplementary Fig. 2e–h), indicating that T cell intrinsic MSC is required for the development of $Foxp3^+ iT_{regs}$ *in vivo*.

During *in vitro* differentiation, we observed no defect in the differentiation of T_H1 , T_H2 and T_H17 cells from $Msc^{-/-}$ mice as compared with wild-type mice (Supplementary Fig. 2i). However, naive $Msc^{-/-}$ T cells exhibited reduced $Foxp3$ expression when differentiated in the presence of TGF- β , and the addition of exogenous IL-2 did not rescue this defect (Fig. 3g). We next asked whether MSC functions in a cell intrinsic or extrinsic manner for iT_{reg} cell differentiation. We polarized CD45.1⁺ wild-type and CD45.2⁺ $Msc^{-/-}$ T cells in the presence of TGF- β either separately or together. Reduced $Foxp3$ expression in $Msc^{-/-}$ T cells as compared with wild-type T cells was still observed, even when $Msc^{-/-}$ and wild-type T cells were cultured together (Supplementary Fig. 2j–k). A cell intrinsic effect of MSC was also observed *in vivo* by developing a bone-marrow chimera. Ten weeks after reconstitution of mixed 1:1 congenic CD45.2⁺ $Msc^{-/-}$ and CD45.1⁺ wild-type bone marrow within irradiated $Rag2^{-/-}$ host mice, we observed that $Msc^{-/-}$ T_{regs} had lower $Foxp3$ expression than wild-type T_{regs} in LP (Supplementary Fig. 2l). Therefore, MSC affects iT_{reg} cell differentiation in a cell intrinsic manner.

MSC-deficient iT_{regs} exhibit enhanced T_H2 responses

Smad3 is known to function as one critical TF for *Foxp3* gene induction. We then examined whether MSC deficiency affects Smad3 activity, resulting in reduced $Foxp3$ expression. Immunoblotting showed that phosphorylation of Smad3 following exposure to TGF- β was intact in MSC deficient T cells, excluding the possibility that MSC directly interferes with Smad3 activation (Supplementary Fig. 3a). It has also been reported that MSC possesses a transcriptional repression domain within the bHLH region, forming heterodimers with E proteins and therefore attenuating E protein-mediated gene activation²⁴. We therefore asked whether the absence of MSC could induce compensatory expression of E proteins, but we found no difference in expression of E47 (encoded by *Tcf3*) and HEB (encoded by *Tcf12*) in

Msc^{-/-} iT_{regs} (Supplementary Fig. 3b), excluding a possible compensatory effect from these E proteins.

In order to gain more insight into MSC regulation of Foxp3, we performed mRNA sequencing (mRNA-seq) on both wild-type and *Msc*^{-/-} iT_{regs} generated *in vitro* in the presence of TGF-β. In addition to the downregulation of *Foxp3*, we noted that *Msc*^{-/-} iT_{regs} appeared to attain a T_H2 phenotype with increased expression of T_H2 associated genes (*Il4*, *Il5*, *Il13* and *Gata3*) even under iT_{reg} differentiation conditions (Fig. 4a, Supplementary Fig. 3c). This increased expression of T_H2 genes was further confirmed by qPCR (Fig. 4b) and at the protein level (Fig. 4c, Supplementary Fig. 4a). We thus hypothesized that MSC induced by TGF-β signaling must promote iT_{reg} differentiation by repressing T_H2 development. Indeed, we observed uncontrolled T_H2 responses in MSC deficient mice, which were mainly observed in the gut, but not in the peripheral lymphoid organs (Fig. 4d–e, Supplementary Fig. 4b–c). IL-4 signaling has been reported to suppress Foxp3 expression under TGF-β stimulation^{25, 26}. Given that *Msc*^{-/-} iT_{regs} produced large amounts of T_H2 cytokines and were defective in iT_{reg} differentiation, we assessed IL-4 production from *Msc*^{-/-} iT_{regs} *in vivo* employing a model of ovalbumin (OVA)-induced generation of iT_{regs}²³. We crossed *Msc*^{-/-} mice with *Rag2*^{-/-} OT-II mice to generate *Msc*^{-/-} OVA specific T cells. Indeed, adoptive transfer of OVA-specific *Msc*^{-/-} T cells into *Rag2*^{-/-} mice resulted in fewer iT_{regs} but the transferred cells produced more IL-4 than wild-type T cells upon exposure to OVA *in vivo* (Supplementary Fig. 4d–f). Altogether, these data indicate that the loss of MSC induces uncontrolled T_H2 responses during iT_{reg} cell differentiation, repressing the expression of Foxp3.

MSC suppresses T_H2 response to promote Foxp3 expression

In order to address the primary cause for the Foxp3 reduction in *Msc*^{-/-} iT_{regs}, we examined the kinetics of *Foxp3* expression and T_H2-specific gene expression during iT_{reg} differentiation. *Msc*^{-/-} T cells showed reduced *Foxp3* but elevated *Gata3* expression at 24 hours after TGF-β stimulation compared to wild-type T cells (Fig. 5a). This suggested that there might be other elements prior to GATA3 induction that repress Foxp3 expression in *Msc*^{-/-} T cells. The expression of T_H2 cytokine genes in *Msc*^{-/-} iT_{regs}, including *Il4*, *Il5* and *Il13*, all started to elevate as early as 12 hours after T cell activation while *Il4* transcription was observed even at 4 hours *Msc*^{-/-} T cells (Fig. 5a). Based on these data, we hypothesized that enhanced expression of T_H2 cytokines could be one of the reasons for inhibition of Foxp3 expression in *Msc*^{-/-} iT_{regs}.

To further explore the molecular mechanism of MSC regulation of T_H2 responses in iT_{regs}, we performed formaldehyde-assisted isolation of regulatory elements (FAIRE) to assess the chromatin accessibility of the T_H2 cytokine locus. Compared to wild-type iT_{regs}, we found enhanced open chromatin regions throughout the T_H2 cytokine locus in *Msc*^{-/-} iT_{regs} as observed at 12 hours post-differentiation, while the *Foxp3* promoter (Foxp3 Pro) was less open in *Msc*^{-/-} iT_{regs} at 48 hours post differentiation (Fig. 5b). However, we did not observe such differences in chromatin marks at either time point during differentiation of the other effector T cell subsets (**data not shown**). Chromatin accessibility is known to be associated with histone modifications²⁷. Consistent with the FAIRE data, the absence of MSC led to

enhanced acetylated histone H4 (AcH4) and acetylated histone H3 at Lys9 (H3K9Ac), and of histone H3 trimethylated at Lys4 (H3K4me3) modifications on the T_H2 cytokine locus at 12 hours but not on the *Foxp3* locus in iT_{regs} (Supplementary Fig. 5a–c). Meanwhile, H3K27me3 modifications were elevated at the wild-type T_H2 cytokine locus at 12 hours as compared with *Msc*^{-/-} iT_{regs} (Supplementary Fig. 5d). Thus, in *Msc*^{-/-} iT_{regs}, the T_H2 cytokine locus exhibited epigenetic modifications associated with active gene expression, potentially resulting in higher T_H2 cytokine production.

As IL-4 is known to suppress the generation of iT_{regs} by TGF-β²⁸, we analyzed whether the reduction of Foxp3 in *Msc*^{-/-} iT_{regs} is due to increased early IL-4 production. Addition of a neutralizing antibody for IL-4 during iT_{reg} polarization resulted in full restoration of Foxp3 levels in *Msc*^{-/-} iT_{regs} (Supplementary Fig. 6a–b). Using *Msc*^{-/-} *Il4*^{-/-} mice, we found that Foxp3 expression was restored in iT_{regs} compared with iT_{regs} from *Msc*^{-/-} *Il4*^{+/-} littermates (Fig. 5c). Further analysis of the Foxp3⁺ T_{regs} in draining LN and LP also suggested that depletion of IL-4 rescued Foxp3 expression from MSC deficient iT_{regs} *in vivo* (Fig. 5d, Supplementary Fig. 6c–f). Thus, these data suggest that T_H2 cytokines, particularly the over-induction of IL-4 within the *Msc*^{-/-} iT_{regs}, are fully responsible for the reduction of Foxp3 expression.

MSC suppresses GATA3 activity within iTreg cells

As a master TF for T_H2 cells, GATA3 has been reported to be critical for the initiation and regulation of the T_H2 cytokine locus²⁹. As MSC was known as a transcriptional repressor³⁰, we suspected there might be a potential interaction between MSC and GATA3, which could lead to suppression of T_H2 responses. In differentiated iT_{regs}, we observed that GATA3 and MSC formed a protein complex by co-immunoprecipitation (Co-IP) (Fig. 6a), which was confirmed by *in situ* proximity ligation assay (PLA) (Fig. 6b, Supplementary Fig. 7a). Treatment with ethidium bromide did not interfere with the GATA3-MSC interaction, suggesting that this interaction does not involve DNA (Supplementary Fig. 7b–c).

It has been established that T_H2 specific genes are regulated by the T_H2 cytokine locus (Fig. 6c). We prepared iT_{regs} and analyzed the T_H2 locus by ChIP of MSC. We observed MSC binding signals at the CGRE (*Il13* distal promoter), *Il13* promoter, *Il4* intronic enhancer (HS2), *Il4* promoter, Rad50 DNase I-hypersensitive sites 6 (RHS6), RHS7, *Il5* promoter, and CNS-2 enhancer (Fig. 6d). Given the physical interaction of GATA3 and MSC, we performed ChIP for GATA3 followed by re-ChIP for MSC (ChIP-re-ChIP) to evaluate whether GATA3 and MSC occupied the same binding sites. We observed that during iT_{reg} differentiation, GATA3 and MSC indeed co-occupy multiple DNA sites within the T_H2 locus at the same time (Fig. 6e). Since *Il4*, *Il5*, *Il13* are known to be regulated by GATA3^{31, 32}, we also tested the binding of GATA3 on the T_H2 locus within iT_{regs}. Enhanced GATA3 binding to the *Il4* and *Il13* locus was detected already at 12 hours after TGF-β stimulation in *Msc*^{-/-} iT_{regs}, despite its identical mRNA expression as in the wild-type T cells at this time point (Fig. 6f, Fig. 5a). These results indicate that MSC primarily inhibits GATA3 DNA binding activity to suppress the T_H2 response during iT_{reg} differentiation.

MSC regulates T_H2 chromatin conformation within iT_{reg} cells

It is known that the coordinate expression of T_H2 cytokines is largely dependent on the chromatin conformation of the T_H2 cytokine locus. GATA3 has been shown to fully participate in establishing such long-range chromatin interactions³³. As can be seen from the spatial organization of the T_H2 cytokine locus (Fig. 7a), we performed chromosome conformation capture (3C) assay. We chose the *Il4* proximal promoter region (HS1) and RHS7 as the anchor points to evaluate this intrachromosomal interaction³³. We first tested the interaction of the fragments within different T cell subsets. As expected, the T_H2 locus exhibited greater interaction in T_H2 cells than in either iT_{reg} or naive T cells (Fig. 7b). We next examined T_H2 locus fragment interactions in *Msc*^{-/-} and wild-type T cells under iT_{reg} differentiation conditions. We observed enhanced interactions within the T_H2 locus in *Msc*^{-/-} iT_{regs} (Fig. 7c).

It has been previously reported that CTCF and cohesion (Rad21) are important for long-range chromosomal associations³⁴. As the CTCF binding sites (CBS) within the T_H2 locus have been identified³⁵, we examined the binding profiles of both CTCF and Rad21 to the T_H2 locus within the iT_{reg} setting (Fig. 7d). We found that CTCF binding was enriched at CBS1, CBS3, CBS5 and CBS7 in the absence of MSC as compared with wild-type iT_{regs} (Fig. 7e). Furthermore, greater binding of Rad21 was found in the *Msc*^{-/-} iT_{regs} in most of the CBS sites except for CBS2 (Fig. 7f). Overall, these results indicate that MSC plays a critical role in silencing the T_H2 response during iT_{reg} differentiation via a chromatin looping mechanism.

MSC promotes the suppressive activity of iT_{reg} cell

iT_{regs} are known to be responsible for immune self-tolerance and control of inflammation *in vivo*⁷. Considering the impaired Foxp3 expression in MSC-deficient T_{regs}, we next assessed the function of these iT_{regs}. We first performed an *in vitro* suppression assay. Compared with wild-type iT_{regs}, *Msc*^{-/-} Foxp3⁺ iT_{regs} showed a reduced ability to inhibit the TCR-stimulated proliferation and cell division of wild-type naive CD4⁺ T cells (Fig. 8a). The signature markers and co-stimulatory molecules of T_{regs} were shown to be decreased in Foxp3⁺ iT_{regs} in the absence of MSC. Interestingly, the co-inhibitory molecule TIGIT which has been shown to be expressed on T_{regs} and able to differentially inhibit T_H1/T_H17 responses but promote T_H2 responses, was found to be elevated in *Msc*^{-/-} iT_{regs}³⁶ (Supplementary Fig. 8a, Fig. 8b). Due to the high level of TIGIT, we suspected that besides their general reduced suppressive ability, *Msc*^{-/-} iT_{regs} might be defective in suppressing T_H2 responses. Indeed, both wild-type and *Msc*^{-/-} iT_{regs} inhibited expression of IL-17 and IFN- γ produced by T_H17 and T_H1 cells, respectively. However, *Msc*^{-/-} iT_{regs} did not suppress differentiation of T_H2 cells, but enhanced IL-4 production compared with controls, similar to what we have previously observed in TIGIT⁺ T_{regs} (Supplementary Fig. 8b, Fig. 8c).

To evaluate whether the MSC expression in iT_{regs} also impacts *in vivo* immune-regulation, and given that *Msc*^{-/-} iT_{regs} do not suppress T_H2 responses *in vitro*, we used a model of house dust mite (HDM)-induced asthma in the lung in which T_H2 cytokines play a dominant role in tissue inflammation³⁷. Ten days prior to challenging CD45.1 congenic mice with

HDM, we transferred either CD45.2 wild-type or *Msc*^{-/-} Foxp3⁺ iT_{regs} to the CD45.1 recipient mice. Consistent with previous data⁴⁸, wild-type iT_{regs} suppressed the mononuclear cell infiltration in the lung following HDM challenge³⁸. However, MSC-deficient iT_{regs} were not able to prevent lung inflammation induced by HDM (Fig. 8d). The bronchioalveolar lavage (BAL) fluid from the recipients that received *Msc*^{-/-} iT_{regs} exhibited more inflammatory leukocytes, including eosinophils and lymphocytes (Fig. 8e). *Msc*^{-/-} iT_{regs} also induced higher immunoglobulin E (IgE) and IL-4 in the BAL fluid than did wild-type iT_{regs} (Fig. 8f). Consistent with the *in vitro* data, transfer of *Msc*^{-/-} iT_{regs} enhanced the T_H2 response, but not the T_H1 or T_H17 cell response in the lung (Fig. 8g, Supplementary Fig. 8c). These results suggest that MSC potentially enhances the suppressive function of iT_{regs} by selectively inhibiting the T_H2 gene program during iT_{reg} differentiation and promotes mucosal immune-homeostasis.

Discussion

iT_{regs} are generated in the periphery upon stimulation with TGF- β in specific tissue microenvironments such as tumors and mucosal sites^{39, 40}. The activation and development of iT_{regs} require a series of transcriptional regulators that orchestrate functional development of iT_{regs} from naive T cells. It has been shown that polarization of T cell subsets into a defined fate requires not only induction of subset specific genes, but also silencing of transcriptional programs for other T cell subsets to ensure unidirectional development of a specific T cell subset. In this study, we demonstrate that a key TF MSC, plays an essential role within iT_{regs} to enforce the expression of Foxp3 while restraining development of the T_H2 gene program.

iT_{regs} developing at mucosal sites play a critical role in maintaining immune homeostasis and tolerance. Loss of the genomic CNS1 element in the *Foxp3* locus results in a selective defect in the generation of iT_{regs}, and CNS1 deficient mice develop spontaneous T_H2 inflammation as the mice age^{41, 42}. Consistent with this observation, aged *Msc*^{-/-} mice, which have a defect in the development of iT_{regs}, also develop spontaneous inflammation at mucosal sites, both in the gut and lungs. However, these mice do not exhibit spontaneous systemic, multi-organ inflammation as observed with other T_{reg}-deficient mice at a young age^{4, 26}. Therefore, the loss of MSC has selective effects on iT_{regs}, limiting the pathological effects of MSC-dependent T_{reg} deficiency. Although the generation of peripheral iT_{regs} is compromised in *Msc*^{-/-} mice, the thymus derived tT_{regs} from *Msc*^{-/-} mice are still sufficient to maintain immune homeostasis and control systemic tissue inflammation. Although there is weak expression of MSC protein in T cells following activation, MSC protein expression is suppressed in other T cell subsets (T_H1, T_H2 and T_H17 cells) and over-expressed under iT_{reg} conditions, supporting the idea that MSC plays an important role in the differentiation of iT_{regs} but not in other T cell subsets.

MSC can function as a transcriptional repressor by binding to E2A proteins, resulting in the formation of inactive heterodimers^{24, 30}. Repression of E2A protein binding to the *Foxp3* locus leads to inhibition of Foxp3 expression⁴³. However, the impaired iT_{reg} differentiation we observed in *Msc*^{-/-} T cells is inconsistent with a model in which MSC directly represses E2A binding to the *Foxp3* promoter. Functional redundancies have been reported for homo

and heterodimers among bHLH family members⁴⁴. However, we did not detect alterations in E2A gene expression. Rather, our data support a role for MSC within iT_{regs} in repressing lineage defining TFs for other T cell subsets, specifically T_H2 cells. The specific interaction between MSC and GATA3 we observed may be critical for suppressing T_H2 development independently of other E2A proteins. However, whether other bHLH family members are induced in other effector T cells to repress alternate gene programs and promote their differentiation needs further evaluation.

Foxp3 programs T_{regs} for their regulatory function³. In addition, Foxp3 also orchestrates inhibition of non-T_{reg} transcriptional programs, such that unidirectional development of iT_{regs} is ensured⁴⁵. For example, a physical interaction between Foxp3 and GATA3 has been suggested to prevent T_H2 polarization^{25, 46}. In contrast, other studies have suggested that GATA3 can promote T_{reg} development by binding to CNS2 in the genomic Foxp3 locus in tT_{regs}, suggesting that GATA3 and Foxp3 may synergize to further promote Foxp3 expression⁴⁷. GATA3 was shown to exert its function via sequence-specific DNA binding⁴⁸ in that it exhibits various functions by cell type specific binding, even among the closely related T cell subsets. This suggests that GATA3 may display its functional specificity via interaction with other cofactors within a specific subset. In fact, our results identified MSC as a novel binding partner of GATA3 within iT_{regs}, indicating that different GATA3 protein complexes formed under various differentiation and developmental conditions could exert distinct effects. Whether MSC is critical for only iT_{reg} development and whether tT_{regs} utilize another mechanism for suppressing development of T_H2 and other effector cell programs cannot be elucidated from our data.

In conclusion, we show here that MSC plays an important role in suppressing T_H2 gene expression during iT_{reg} differentiation. Our study provides a novel perspective, in that Foxp3 alone does not suppress other lineage specific transcriptional programs, but rather cooperates with other TFs like MSC to achieve this goal. In iT_{regs}, MSC forms a complex with the key T_H2 TF GATA3 and thus acts as a repressor, favoring the linear chromatin configuration of the T_H2 cytokine locus and thereby actively inhibiting the T_H2 gene program. We propose that MSC functions as a negative regulator for the assembly of the three-dimensional chromatin architecture required for the opening of the T_H2 locus and that MSC is an important factor that suppresses development of T_H2 responses during iT_{reg} differentiation. Although the role of Foxp3 in inducing T_{reg} differentiation cannot be understated, our studies suggest that Foxp3 is not able to do this alone. In fact, the factors that induce Foxp3 also induce regulators that dominantly suppress the transcriptional programs of other T cell subsets, allowing Foxp3 to work unopposed. With this premise in mind, we identified MSC as a novel TF that assists Foxp3 in promoting iT_{reg} development while at the same time repressing the T_H2 gene program. Regulating MSC expression in CD4⁺ T cells may allow for therapeutic modulation of iT_{reg} responses in mucosal sites, in inflammatory bowel disease, asthma, atopy and allergies.

Online methods

Animals

C57BL/6 (B6), CD45.1⁺, OT-II, *Foxp3*^{Cre-YFP}, *Rosa26*^{RFP}, *Rag2*^{-/-} were purchased from Jackson Laboratory; and *Foxp3*^{GFP} mice have been described previously^{49, 50}; *Smad3*^{-/-} mice were from Dr. Lillian Maggio-Price. *Msc*^{-/-} mice were obtained from Dr. Eric N. Olson, the University of Texas Southwestern Medical Center at Dallas, and rederived by embryo transfer. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

Reagents and Antibodies

Unless otherwise stated all chemicals were purchased from Sigma Aldrich. The following antibodies were used in flow cytometry (FACS) and cell sorting: CD4 (H129.19, BD Pharmingen), CD8 (53-6.7, Biolegend), CD25 (7D4, BD pharmingen), IL-4 (11B11, Biolegend), IL-17A (TC11-18H10.1, BD Pharmingen), IFN- γ (XMG1.2, BD Pharmingen), CD44 (IM7, Biolegend), CD62L (MEL-14, Biolegend), CTLA4 (14D3, eBioscience), GITR (DTA-1, eBioscience), TIGIT (GIGD7, eBioscience), ICOS (C398.4A, Biolegend), Foxp3 (FJK-16s, eBioscience), IL-10 (JES5-16E3, Biolegend), Nr1p (FAB566A, R&D), IL-4R (I015F8, Biolegend), Ki67 (SolA15, eBioscience).

Histology

Mouse tissues were fixed in 10% formalin overnight, preserved in 70% ethanol, and embedded in paraffin before being processed for staining with hematoxylin and eosin (H&E). Scoring of the infiltrates was performed by blinded assessment of at least three sections of each tissue per mouse.

Assessment of intestinal inflammation

Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion. Score 0: No changes observed; Score 1: Minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; Score 2: Mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; Score 3: mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; Score 4: marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; Score 5: marked transmural inflammation with severe ulceration and loss of intestinal glands.

In vitro T cell differentiation

CD4⁺ Naive (CD44^{lo}CD62L⁺CD25⁻) T cells were obtained from spleens and lymph nodes of indicated mice by flow cytometric cell sorting. The purity of isolated T cell populations routinely exceeded 98%. Naive T cells were stimulated with plate-bound anti-CD3 (145-2C11, 1 μ g/ml) and anti-CD28 (PV-1, 1 μ g/ml) and polarizing cytokines (T_H1: 20 ng/ml

IL-12; T_H2: 20 ng/ml IL-4; T_H17: 20 ng/ml IL-6, 2 ng/ml TGF- β ; iT_{reg}: 2 ng/ml TGF- β ; all cytokines from R&D).

iT_{reg} generation *in vivo*

In vivo iT_{reg} development was induced by oral administration of OVA. Briefly, spleens and LN were harvested from *Rag2*^{-/-} OT-II and *Msc*^{-/-} *Rag2*^{-/-} OT-II mice. Purified CD4⁺ T cells were injected into congenic CD45.1 mice. The following day, drinking water was supplemented with 3% OVA (Sigma-Aldrich). After 5 d, mice were sacrificed, and the cells from Peyer's patches (PP) and Lamina propria (LP) were extracted and analyzed.

Flow cytometry

For intracellular cytokine staining, cells were cultured as described above and stimulated for 4 h at 37°C in culture medium containing PMA (50ng/ml; Sigma), ionomycin (1 μ g/ml; Sigma) and monensin (GolgiStop; 1 μ g/ml; BD Biosciences). Surface markers were stained in PBS with 1% FCS for 20 minutes at room temperature, then subsequently fixed in Cytoperm/Cytofix (BD Biosciences), permeabilized with Perm/Wash Buffer (BD Biosciences), and stained with cytokine antibodies diluted in Perm/Wash. For Foxp3 staining, cells were fixed and permeabilized with the Foxp3 Staining Buffer Set, according to the manufacturer's instructions (eBiosciences). All flow cytometry data were acquired on a FACS Calibur (Becton Dickinson) and analyzed with FlowJo software (TreeStar).

ELISAs

The cell supernatants or mouse serum were collected, and cytokines or IgG measurement was performed according to the manufacturer's recommendations (R&D).

Suppression assay

Naive CD4⁺ T effectors (5×10^5) were cultured with iT_{regs} (5×10^5 , titrated down as described) in 96-well round-bottom plates (Corning Inc.) in triplicate in the presence of soluble anti-CD3 (1 μ g/ml) and irradiated splenic APCs (1.2×10^5 /well). After 48 hr cells were pulsed with 1 μ Ci [³H]thymidine for an additional 18 hr and harvested and [³H]thymidine incorporation was analyzed to assess proliferation.

Suppression of Th Cell Differentiation

For in vitro experiments, CD4⁺CD62L⁺CD44⁻ naive T cell from CD45.1 mice were sorted and co-cultured with CD45.2⁺Foxp3⁺ WT or *Msc*^{-/-} iT_{reg} at ratio to 10:1. Cells were stimulated with anti-CD3/CD28 in the presence of polarizing. The cells and the supernatant were harvested and analyzed by flow cytometry or ELISA on day 5.

Quantitative RT-PCR

For gene expression detection, total RNA was isolated from whole cells using the Qiagen miniRNA extraction kit following the manufacturer's instructions. RNA was quantified and complementary DNA was reverse-transcribed using the cDNA archival kit (Applied Biosystems) following the manufacturer's instructions. The cDNA samples were used at 20ng/well in a 384 well plate and run in triplicate. PCR reactions were set up using TaqMan

Universal PCR Master Mix (Applied Biosystems) on an ABI Prism 7500 Sequence Detection System. Quantification of relative mRNA expression was normalized to the expression of GAPDH. Primers-probe mixtures were purchased from Applied Biosystems: *Foxp3* (Mm00475162), *MSC* (Mm00447887), *Gata1* (Mm01352636), *Gata3* (Mm00484683), *Il4* (Mm00445259), *Il5* (Mm00439646), *Il13* (Mm00434204), *Tcf3* (Mm01188711), *Tcf12* (Mm00441699), *GAPDH* (4352339E).

Western blot analysis

$0.2-1 \times 10^6$ cells were lysed in whole cell extract buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.2 mM EDTA, 10 mM Na_3VO_4 , 10% glycerol, protease inhibitors). Proteins were separated by gel electrophoresis using 4–12% NuPAGE Bis-Tris gels (Invitrogen) followed by transfer to nitrocellulose membrane. Membranes were incubated with 5% milk in TBST (0.5 M NaCl, Tris-HCl, pH 7.5, 0.1% (v/v) Tween-20) for 60 min and washed once with TBST. Proteins of interest were detected by incubating membranes over night at 4°C in 5% BSA/TBST with anti-MSC (F-20, Santa Cruz), anti-GATA3 (HG3–31, Santa Cruz) anti-p-Smad3 (C25A9, Cell Signaling), anti-Smad3 (9513, Cell Signaling) or anti- β -Actin (sc-47778, Santa Cruz), washing with TBST three times for 10 min and incubating with horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibody (7074 and 7076, Cell Signaling). Bound antibody was detected by using Immobilon Western chemiluminescent HRP substrate (Millipore).

Immunoprecipitation

Cell lysates were prepared as described above and proteins were immunoprecipitated by incubation of lysates with 3 μg antibody anti-MSC (F-20, Santa Cruz) or anti-GATA3 (HG3–31, Santa Cruz) over night at 4°C and pull-down of antibody-protein precipitates with Protein A/G Dynabeads (Invitrogen). Beads were washed extensively and proteins eluted with NuPAGE LDS sample buffer (10% β -mercaptoethanol). The presence of immunocomplexed proteins was determined by Western blot analysis with the indicated antibodies.

Proximity Ligation Assay

PLA detection was performed with a Duolink II kit (Olink Bioscience). The *in vitro* differentiated iT_{regs} were fixed in 1% paraformaldehyde for 15 min and incubated with a blocking solution at 37°C for 30 min, and then washed with Wash Buffer. The procedures for administering the primary antibodies, anti-MSC (F-20, Santa Cruz) and anti-GATA3 (HG3–31, Santa Cruz), PLA probes, hybridization, ligation, amplification, detection, and mounting followed the manufacturer's recommended protocol. The cells were observed using a confocal microscope.

Retroviral transduction

Retroviral particles were produced by transiently transfecting HEK 293T cells with retroviral packaging constructs pcl and gag/pol and expression plasmids MSCV-IRES-GFP or MSCV-IRES-GFP-Smad3 using Fugene HD (Roche). 72 hours after transfection, viral culture supernatants were harvested, supplemented with polybrene (8 mg/ml) and added to

previously stimulated T cells (5×10^5 /well, plate-bound anti-CD3/CD28 and different combination of cytokines for 24 h). Cultures were centrifuged at 800 G for 45 min at 25°C.

MSC promoter luciferase reporter construction

The murine Muscudin promoter region (−2000bp~+150bp) was cloned into the pGL3 basic vector at KpnI and SmaI site. The following primers were used for cloning: 5'-GAAGGTACCGGTAGTCAGTCGCAGTGAAGGGACAG-3' and 5'-TCTGACAGAGGATGGGGTGT-3'

Reporter assays

HEK 293T cells (1×10^5 cells/well, 48 well plate) were transiently transfected with the indicated expression vectors, empty vector controls as well as the promoter firefly luciferase-reporter constructs and Renilla luciferase reporter vector (Promega) with Fugene HD (Promega). 48 h after transfection luciferase expression was determined by measuring luminescence with the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to Renilla luciferase activity. Data is representative of at least two independent experiments, each data point represents duplicate values. The following vectors were used; pCMV5, pCMV5-Smad3 (a gift from Dr. Akihiko Yoshimura), pGL3-MSC reporter.

Bone marrow transfer

Bone marrow from the WT and *Msc*^{−/−} mice was depleted of CD4⁺ cells with anti-CD4 microbeads (Miltenyi Biotech), and was injected intravenously into *Rag2*^{−/−} mice after lethal irradiation (1,000 rads). Mice were further analyzed after 6–8 weeks.

T Cell-Induced Colitis

Naive (CD44^{lo}CD62L⁺GFP[−]) CD4⁺ T cells were purified from WT or *Msc*^{−/−} Foxp3-GFP-KI mice. 7×10^5 naive T cells were injected intraperitoneally (i.p.) were injected into age- and sex-matched *Rag2*^{−/−} mice, and weight loss was monitored over 6–8 weeks.

HDM antigen-induced allergic asthma

1×10^6 Foxp3.GFP⁺ sorted WT or *Msc*^{−/−} iT_{regs} were injected intravenously into the mice. To generate HDM antigen-induced allergic asthma, 25µg house dust mites (HDM) protein (Greer Laboratories) in 25µl PBS was injected into each mouse nasally every day for 10 consecutive days. The injections were performed in anesthetized mice (2.5% Isoflurane) using a VetEquip Inhalation Anesthesia System (VetEquip, Inc).

Chromatin immunoprecipitation (ChIP)

ChIP was undertaken following manufacturer's instructions (Cell signaling) followed by PCR amplification of the immunoprecipitates in defined genomic regions. The following antibodies were used for ChIP: anti-Smad3 (ab28379, Abcam), anti-GATA3 (D-16, Santa Cruz), anti-MSC (F-20, Santa Cruz), anti-CTCF (D31H2, Cell signaling), anti-Rad21 (ab992, Abcam), anti-acetylated H4 (06–866, Millipore), anti-H3K9me3 (C5B11, Cell Signaling) and anti-H3K27me3 (07–449, Millipore). The regions that were identified by

ChIP PCR were amplified by predefined primers for the genes including *CBS*, *Foxp3*, *Il-4*, *Il-5*, *Il-13* and *Msc*. The primer sequences were listed below.

ChIP-Re-ChIP

The initial ChIP was performed as described above using beads bound to the GATA3 antibody. Beads were washed in low salt and high salt buffer and then incubated at room temperature for 30 min in 100 µg of the peptide used to generate the GATA3 antibody (D-16, Santa Cruz). The sample was vortexed every 5 min. After 30 min the eluted material was removed and elution was repeated with fresh peptide. A second round of ChIP was performed using antibodies against MSC under the conditions described. Enrichment was quantified by quantitative PCR.

Formaldehyde-assisted isolation of regulatory elements (FAIRE) assay

iT_{regs} were cross-linked and sonicated as described for the ChIP assay. Samples were centrifuged and the aqueous phase was isolated by three consecutive extractions with phenol–chloroform–isoamyl alcohol (25:24:1). The DNA was precipitated by ethanol. After the final extraction, the FAIRE samples were purified and reverse-cross-linked as described for the ChIP assay. The enrichment of fragmented genomic DNA in the FAIRE samples relative to each input DNA was measured by quantitative PCR.

Chromosome conformation capture (3C)

iT_{regs} were fixed with 1% formaldehyde, lysed in 10 mM Tris pH 8.0, 10 mM NaCl, 0.2% NP-40 with complete protease inhibitors (Roche). Nuclei were collected, suspended in 1× restriction enzyme buffer in the presence of 0.3% SDS and incubated at 37°C for 1 h. 2% Triton X-100 was then added to sequester the SDS. The sample was digested with BglII (New England Biolabs) at 37°C overnight. After stopping the reaction by adding 1.6% SDS and incubating the mixture at 65°C for 30 min, chromatin DNA was diluted with ligation reaction buffer in the presence of 1% Triton X-100. DNA was ligated with T4 DNA ligase (New England BioLabs) at 16°C. After treatment with 10 mg/ml proteinase K at 65°C overnight to reverse cross-links and with 0.4 µg/ml RNase A for 30 min at 37°C, DNA was extracted with phenol-chloroform, ethanol precipitation, and used for quantitative PCR. The control template for the 3C assay was prepared as the same protocol with a bacterial artificial chromosome clone (RPCI – 23, CHROI).

For ChIP, FAIRE and 3C assay, the PCR reactions with purified DNA were carried out using SYBR green mix (Applied Biosystems) on ABI Prism 7500 Sequence Detection System. The primers used were listed as the following,

CBS-1F	GGTCTTAGCAGGTTCCCAA;
CBS-1R	CGTTCGG TAAGACAAGCAC;
CBS-2F	CACTCAGCACCTTACCTG;
CBS-2R	CCTGGGCTAAATGAATCAGT;
CBS-3F	AGGCA CAGTGTAGAAGTGT;

CBS-3R	GTCTCTCTTCCAGTCCAGTT;	
CBS-4F	GGCACTTGTAACGCTCTAA;	
CBS-4R	CCCTG ACCAACATCTCCAA;	
CBS-5F	ATTGTGGAGGCTGGCAAG;	
CBS-5R	GGTGACAGCCCAAATAAGT;	
CBS-6F	CCA CATCCACCTGTCACTT;	
CBS-6R	CTGTTTCACATCCATC GCA;	
CBS-7F	CAGGCTTGATCATCACCA;	
CBS-7R	TTCTTGAGGGACAGCACT;	
Foxp3 ProF	CCTTTGTGATTTGACTTATT;	
Foxp3 ProR	CAGAGGTAAAAGGTATCAAT;	
Foxp3 CNS1F	GTTTTGTGTTTTAAGTCTTTTGCACTTG;	
Foxp3 CNS1F	CAGTAAATGGAAAAATGAAGCCATA;	
Foxp3 CNS2F	GTTGCCGATGAAGCCCAAT;	
Foxp3 CNS2R	ATCTGGGCCCTGTTGTCACA;	
Foxp3 CNS3F	CAGGAAGTGTTTATGGGTC;	
Foxp3 CNS3R	ATGAGGATTGGGAGGGGTG;	
IL4 ProF	GCAGGATGACAACTAGCTGGG;	
IL4 ProR	ACGGCACAGAGCTATTGATGG;	
IL5 ProF	TTTCCTCAGAGAGAGAATAAATTGCTT;	
IL5 ProR	GCTGGCCTTCAGCAAAGG;	
IL13 ProF	GGGTCAATTGGCTAGGCA;	
IL13 ProR	CTTTGCCAGGTCAGTCAGTA;	
RHS7 F	GCCAAGGTACACAGAGAAA;	
RHS7 R	GTCTCAATACTCCAGCTCCTTC;	
CGRE F	GTCTCTTATCGACCCCATC;	
CGRE R	AAAGGCTTGGGGAAACAC;	
HS2 F	AGGCCTCTTCTGGTCACCTAACA;	
HS2 R	GTCCCTCCCACTACAAATGGA;	
RHS6 F	ATGCCTGCCCTAGCTACC;	
RHS6 R	CCAGAAGATCACGGAATG;	
MSC	-1996bp~-1847bp	GTCAGTCGCAGTGAAGGGAC;
MSC	-1996bp~-1847bp	GGGGTATGCGGCATTCTCT;
MSC	-1696bp~-1627bp	TCCATCTTCTGCCTGCACC;
MSC	-1696bp~-1627bp	TTGACTGTAGGCAAGCCTGG;
MSC	-1463bp~-1343bp	CCCTACCCCCAACCAAAGAC;
MSC	-1463bp~-1343bp	GCTTCCCTCCACCTTGTCTC;
MSC	-1236bp~-1078bp	ATCCGCGTATCTGCTTGACC;
MSC	-1236bp~-1078bp	TGCTCCAGACGGAGGAATTG;
MSC	-4bp~144bp	AGTCAAGTTCAGCAGAGCCC;
MSC	-4bp~144bp	TCTGACAGAGGATGGGGTGT;
MSC	152bp~253bp	AGCCAGAGAAAGCGAGACTG;
MSC	152bp~253bp	GAGTCTTCGGGGTCACTCAC;

chr11	53647348~53647367	TTCCCAGATACACAAGGCTA;
chr11	53652416~53652434	TGAACCGAGTGTGGTAGTG;
chr11	53719432~53719450	TGCTGAGGGAGAGCTGTAG;
chr11	53721013~53721032	AGATCAGGACCTTGCTATAC;
chr11	53701722~53701741	CAAACCTCATAGGAATGCACC;
chr11	53709891~53709910	AAGAAGAAATTTTGAGGGCTG;
chr11	53683649~53683649	GTACAGTAAGAGGAGCAAAT;
chr11	53686056~53686075	ATACCATCTCATGACAATGA;
chr11	53636054~53636071	GGGTCAATTTGGCTAGGCA;
chr11	53641510~53641529	CTTTGCCAGGTCAGTCAGTA;
chr11	53617858~53617876	CCTTGCTTGATACGGTATC;
chr11	53621624~53621641	CTCCTGTGACCACAAGTC;
chr11	53635822~53635845	TTCTTTCTCAGACTCTTTCCCCAC;
chr11	53634698~53634719	TTCAACAGGCTAAGGCCACAAG;
chr11	53656801~53656823	TCACAAGCATTTCTTCTTTCAGT;
chr11	53658698~53658720	TTAAAGTAACCCGAGGCTGGAGA;
chr11	53621691~53621710	CTTTTCCTTAGTCTTGTCG;
chr11	53628403~53628422	AAACAGCCTTGGATGTTGTT.

RNA-Sequence

Total RNA was used for preparing RNA-seq libraries with the Illumina TruSeq RNA Sample Preparation Kit. Libraries were sequenced with single-end 36bp reads on an Illumina GAI. RNA-seq reads were aligned to the NCBI Build 37 (UCSC mm9) of the mouse genome using. The resulting alignments were processed by Scripture to evaluate the expression of transcripts from RefSeq. We use quantile normalization to further normalize the FPKM values. As differentially expressed transcripts, we define all cases with >1.5 fold change. To avoid spurious fold levels due to low expression values we add to the expression values a small constant of 4.2 (the value of the 3rd quantile [out of 10] of all FPKM values analyzed).

Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.0 (Graph Pad software, La Jolla, CA, USA) using an unpaired two-tailed Student's t-test. Statistical significance was defined as $P < 0.05$.

Data availability

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TGF- β -dependent early induction of MSC in CD4⁺ T cells

(a) Microarray analysis of CD4⁺ T cells to reveal earlier higher induction of TGF- β -dependent genes for transcription factors at 10 hours than at 48 hours by comparison of TGF- β alone, TGF- β with IL-27 or TGF- β with IFN- γ ; (GSE38686); (b) Naive CD4⁺CD44^{lo}CD62L⁺CD25⁻ T cells from WT mice were stimulated under T_H0, T_H1, T_H2, T_H17, iT_{reg} conditions and harvested at 10 and 48 hours for q-PCR. Relative mRNA levels of *Msc* genes for different subsets were shown; (c) Naive T cells from WT mice were differentiated into indicated T cell subsets and harvested at 48 hours. MSC protein in different subsets was detected by immunoblotting; (d) The level of MSC was assessed in *in vitro* differentiated iT_{regs} and isolated spleen tT_{regs} from WT mice by immunoblotting; (e) tT_{regs} (CD4⁺Foxp3⁺Nrp1⁺) and iT_{regs} (CD4⁺Foxp3⁺Nrp1⁻) were isolated from indicated

organs of WT mice and the expression of indicated genes was analyzed by q-PCR. Data are representative of three independent experiments (c, d) or are pooled from three independent experiments (b, e). * $P < 0.05$ (Student's *t*-test, error bars, SD).

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Figure 2. Smad3 regulates MSC during iTreg differentiation

(a) The binding of Smad3 to the *Msc* promoter in T_H0 and iT_{reg}s was assayed by ChIP-PCR. Six horizontal bars represent the locations of Smad3 binding sites on the *Msc* locus detected by qPCR; (b, c) Luciferase assay using an *Msc* promoter-driven reporter in HEK293T cells transfected with a control or Smad3-expressing vector; (d, e) Naive CD4⁺ T cells transduced with retrovirus expressing control vector (Ctrl RV) or Smad3-expressing vector (Smad3 RV) and differentiated into T_H0 or iT_{reg}s. mRNA (d) and protein (e) expression of Smad3 and Foxp3 were determined; mRNA (f) and protein (g) expression of Foxp3 and MSC within WT and *Smad3*^{-/-} iT_{reg}s. Data are representative of three independent experiments. **P* < 0.05 (Student's *t*-test, error bars, SD).

Figure 3. MSC promotes Foxp3 expression during iT_{reg} differentiation

Flow cytometry analysis (a) and quantification (b) of CD44 and CD62L expression on CD4⁺ T cells from peripheral lymph nodes (LN) of WT and *Msc*^{-/-} mice at 8 or 40 weeks of age; (c) Representative hematoxylin and eosin staining of small intestine (SI), lung, liver and kidney sections. Scale bar, 200 μm; (d) Quantification of histological analysis from Fig 3c of mice aged 40 weeks old. 0–3 score: 0, no mononuclear infiltration; 3 high degree of mononuclear infiltration; Flow cytometry analysis (e) and quantification (f) of Foxp3 expression in CD4⁺ cells in mesenteric (mLN) and lamina propria (LP); (g) Naive CD4⁺ T cells from WT and *Msc*^{-/-} mice were stimulated with TGF-β and IL-2 for 72 hours. Flow cytometry analysis of the frequency of Foxp3⁺ cells; Data are representative of three independent experiments (a, c, e, g) with n = 5 mice each group or are pooled from three independent experiments (b, d, f). **P* < 0.05. NS: not statistically significant. (Student's *t*-test, error bars, SD).

Figure 4. MSC-deficient iTreg exhibits enhanced TH2 response

(a) Scatterplot of the average signal of WT versus *Msc*^{-/-} iTreg gene expression in RNA-Seq data. The red line indicates gene expression change by a factor of 1.5; data normalized from two replicates are shown. (b) Naive CD4⁺ T cells from WT and *Msc*^{-/-} mice were stimulated under iTreg conditions with TGF-β and harvested at 72 hours. Relative mRNA expression levels of indicated genes are shown by q-PCR; (c) Flow cytometry analysis of Foxp3 and TH2 associated cytokines in WT or *Msc*^{-/-} iTreg; Flow cytometry analysis (d) and quantification (e) of IL-17, IFN-γ and IL-4 in CD4⁺ T cells from gut LP of 10 month old WT and *Msc*^{-/-} mice. Data are pooled from three independent experiments (b, e) or are representative of three independent experiments (c, d). **P* < 0.05, ***P* < 0.01, (Student's *t*-test, error bars, SD).

Figure 5. T_H2 response is primarily responsible for the reduction of Foxp3 in the absence of MSC

(a) Naive CD4⁺ T cells from WT and *Msc*^{-/-} mice were stimulated with TGF- β for 4, 12, 24 and 48 hours. mRNA expression of indicated genes was assessed by qPCR analysis; (b) FAIRE-qPCR analysis at indicated sites on T_H2 and *Foxp3* loci in WT or *Msc*^{-/-} naïve T cells stimulated with TGF- β for 12 and 48 hours; (c) Flow cytometry analysis of Foxp3 expression during the differentiation of naïve T cells from indicated mice into iT_{regs} in the presence of TGF- β ; (d) Flow cytometry analysis of CD4⁺ T cells from dLN and LP of indicated mice. Data are pooled from three independent experiments (a, b) or are representative of three independent experiments (c, d) with n = 4 mice each group. *P < 0.05, **P < 0.01 (Student's t test, error bars represent SD).

Figure 6. MSC suppresses the GATA3 activity within iT_{regs}

(a) Co-immunoprecipitation (with control IgG, anti-MSC or anti-GATA3) of lysates of WT and $Msc^{-/-}$ iT_{regs}, followed by immunoblotting analysis with the indicated antibodies; (b) MSC and GATA3 association was visualized in iT_{regs} with an in situ proximity ligation assay. Punctate staining (red) indicates a MSC-GATA3 interaction as detected by the assay. Scale bar, 5 μ m; (c) Schematic representation of the T_H2 cytokine locus with the positions of indicated genes location, conserve non-coding sequences (CNS) and DNase I-hypersensitive sites (RHS and HS); (d) The binding of MSC to the T_H2 locus on WT and $Msc^{-/-}$ iT_{regs} was determined by ChIP-PCR; (e) GATA3 ChIP was performed followed by re-ChIP using

antibodies against MSC and the binding to the indicated sites on the T_H2 locus was determined by qPCR; (f) 12 and 48 hours after TGF- β stimulation, the binding of GATA3 to the T_H2 locus from WT and *Msc*^{-/-} iT_{regs} was determined by ChIP-PCR. Data are representative of three independent experiments (a, b) or are pooled from three independent experiments (d–f). *P < 0.05, **P < 0.01 (Student's t test, error bars represent SD).

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Figure 7. MSC suppresses T_H2 response within iT_{reg} s via regulating T_H2 chromatin conformations

(a) Schematic representation of the T_H2 cytokine locus with the positions of indicated genes location conserve non-coding sequences (CNS) and DNase I-hypersensitive sites (RHS and HS). Below, *Bgl*II restriction fragments used in the chromosome conformation capture analysis assay; Relative cross-linking frequencies between a fixed anchor fragment bearing the *Il4* gene promoter (upper) or the RHS7 site (lower) and other *Bgl*II fragments via (b) naive T, T_H2 , and iT_{reg} s. (c) WT and *Msc*^{-/-} iT_{reg} s. The positions of anchor fragments are shown as black shading. The positions of the *Bgl*II fragments cross-linked to the anchor fragments are shown as gray shading; (d) Schematic representation of the T_H2 cytokine

locus and CTCF binding sites (CBS-1 to CBS-7); The binding of (e) CTCF or (f) Rad21 to the T_H2 locus on WT and *Msc*^{-/-} iT_{regs} was determined by ChIP-PCR; Data are pooled from three independent experiments. **P* < 0.05 and ***P* < 0.01 (Student's *t*-test, error bars, SD).

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Figure 8. MSC promotes the suppressive activity of iTregs on TH2 response

(a) Cell tracer violet-labelled naive WT CD4⁺ T cells in the presence of WT or *Msc*^{-/-} GFP⁺ (Foxp3⁺) iTregs with indicated ratio were stimulated with anti-CD3/CD28 for 3 days, followed by flow cytometry analysis for violet dilution; (b) The expression of indicated co-inhibitory molecules on *Foxp3*^{GFP} and *Foxp3*^{GFP}*Msc*^{-/-} iTregs was determined by flow cytometry; (c) Naive T cells, *Foxp3*^{GFP} and *Foxp3*^{GFP}*Msc*^{-/-} iTregs were sorted and cocultured at a ratio of 1:10 under TH1, TH2, or TH17 cell polarizing conditions. On day 3 cytokine production from the culture media was determined by ELISA; (d) Hematoxylin and eosin staining of lung tissue sections from the recipient mice without transfer (Ctrl) or transferred with WT or *Msc*^{-/-} iTregs. (Magnification, Upper panel ×5, Lower panel ×20) Scale bar, 200μm; (e) Inflammatory cells in BAL fluid 10 d after HDM administration with indicated treatments. Eos, eosinophils; Neu, neutrophils; Mac, macrophages; Lym, lymphocytes; (f) ELISA analysis of IgE and IL-4 production in BAL fluid of HDM administrated mice with indicated treatments; (g) Quantification of intracellular cytokines in gated CD4⁺ T cells from the lung of HDM administrated mice with indicated treatments. Data are pooled from three independent experiments (c, e, f, g) or are representative of three

independent experiments (a, b, d) with $n = 4$ mice each group. $*P < 0.05$, (Student's t -test, error bars, SD).

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