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Stem cells for murine interstitial cells of Cajal suppress cellular immunity and colitis via prostaglandin E₂ secretion

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Abstract

Background & Aims—Following allogeneic transplantation, murine stem cells (SC) for interstitial cells of Cajal (ICC), electrical pacemaker and neuromodulator cells of the gut, incorporated into gastric ICC networks indicating *in-vivo* immunosuppression.

Immunosuppression is characteristic of bone marrow- and other non-gut-derived mesenchymal stem cells (MSC), which are emerging as potential therapeutic agents against autoimmune diseases including inflammatory bowel disease. Therefore, we investigated whether gut-derived ICC-SC could also mitigate experimental colitis and studied the mechanisms of ICC-SC-mediated immunosuppression in relation to MSC-induced pathways.

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Methods—Isolated ICC-SC were studied by transcriptome profiling, cytokine assays, flow cytometry, mixed lymphocyte reaction and T cell proliferation assay. Mice with acute and chronic colitis induced by dextran sulfate sodium and T cell transfer, respectively, were administered ICC-SC intraperitoneally and evaluated for disease activity by clinical and pathological assessment and for ICC-SC homing by live imaging.

Results—Unlike strain-matched dermal fibroblasts, intraperitoneally administered ICC-SC preferentially homed to the colon and reduced the severity of both acute and chronic colitis assessed by clinical and blind pathological scoring. ICC-SC profoundly suppressed T cell proliferation *in vitro*. Similarly to MSC, ICC-SC strongly expressed cyclooxygenase 1/2 and basally secreted prostaglandin E₂. Indomethacin, a cyclooxygenase inhibitor, countered the ICC-SC-mediated suppression of T cell proliferation. In contrast, we found no role for regulatory T cell-, programmed death receptor- and transforming growth factor- β -mediated mechanisms reported in MSC; and transcriptome profiling did not support a relationship between ICC-SC and MSC.

Conclusion—Murine ICC-SC belong to a class different from MSC and potentially mitigate experimental colitis via prostaglandin E₂-mediated immunosuppression.

Keywords

immunosuppression; IBD; ICC

INTRODUCTION

The inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis are chronic idiopathic disorders affecting over 1 million people in the United States.¹ IBD results from inappropriate inflammatory responses to intestinal microbes in a genetically susceptible host.² Recent studies have shown that autoimmune inflammation can be mitigated by systemically administered bone marrow-, adipose tissue- or umbilical cord blood-derived mesenchymal stromal/stem cells^{3–5} (MSC; defined broadly as ubiquitous progenitors of all nonepithelial, nonhematopoietic cells derived from the mesoderm^{6,7}). There are several ongoing phase II–III clinical trials that are using MSC for treatment of IBD (clinicaltrials.gov). Although the immunoregulatory functions of MSC remain incompletely understood,⁶ available evidence indicates important roles for prostaglandin E₂ (PGE₂)-, programmed cell death 1 (PD-1)-, regulatory T cell (Treg)- and transforming growth factor- β (TGF- β)-mediated pathways.^{3–5,8–11}

Interstitial cells of Cajal (ICC) are mesodermally-derived cells of the gastrointestinal *tunica muscularis* that generate electrical pacemaker activity and mediate neuromuscular control.¹² Populations of ICC, identified by their characteristic expression of Kit receptor tyrosine kinase and anoctamin 1 (Ano1), a calcium-activated chloride channel, are depleted in several gastrointestinal neuromuscular disorders significantly contributing to their pathogenesis.^{13,14} In the murine gastric *tunica muscularis*, we also identified a small population of Kit^{low/-}Ano1⁺Cd44⁺Cd34⁺ cells, which are clonogenic and capable of extensive self-renewal and differentiation into ICC both *in vitro* and *in vivo*, thus fulfilling the criteria for *bona fide* stem cells (ICC stem cells, ICC-SC).^{15,16} When injected

intraperitoneally into adult, major histocompatibility complex (MHC)-mismatched, nonobese diabetic mice, an established model of ICC injury,¹⁴ ICC-SC (line 2xSCS70) homed to the stomach, differentiated and engrafted into ICC networks,¹⁶ indicating immunosuppressive capability. Therefore, we hypothesized that, similarly to systemic MSC to which they may be related, gut-derived ICC-SC have immunoregulatory potential and could mitigate experimental colitis. Furthermore, we also studied the mechanisms of ICC-SC-mediated immunosuppression in relation to MSC-induced pathways.

METHODS

Standard methods (RNA sequencing (RNA-seq), gene expression microarrays, cytokine assays, enzyme immunoassays, mixed lymphocyte reaction (MLR) and T cell proliferation assay by carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution) and additional details are described in the Supplementary Methods. Isolation and maintenance of the ICC-SC lines 2xSCS2F10 and 2xSCS70 were described previously.¹⁶ Only cells with diploid DNA content¹⁶ were used.

Ethics statements

Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic (A36211, A60011).

Dextran sulfate sodium (DSS) model of acute colitis

C57BL/6J mice were given 5% DSS (36,000–50,000 Da; MP Biomedicals, Illkirch, France) in the drinking water for 7 days followed by water only for one day prior to euthanasia by CO₂ inhalation for tissue removal on day 8. 2xSCS2F10 ICC-SC derived from C57BL/6J mice or strain-matched dermal fibroblasts (see Supplementary Methods) (5×10^6 cells/250 μ L phosphate-buffered saline (PBS)/mouse; human equivalent dose:¹⁷ 16×10^6 /kg) or PBS vehicle were injected intraperitoneally 10 h prior to starting DSS and on day 4. Mice were weighed daily. Disease severity was assessed by colon length, disease activity index and histology on the day of euthanasia as described in the Supplementary Methods.

Cd4⁺Cd45RB^{high} T cell transfer model of chronic colitis

We performed adoptive transfer of 300,000 Cd4⁺Cd45RB^{high} (naïve) T cells from healthy C57BL/6J mice by intraperitoneal injection into syngeneic mice lacking T and B cells (RAG^{-/-} recipient mice; background: C57BL/6J) to induce T cell-dependent chronic colitis as described previously.¹⁸ 2xSCS2F10 ICC-SC (5×10^6 cells/250 μ L PBS/mouse) or PBS vehicle were injected intraperitoneally on day 7 and day 21. Disease severity assessment was performed at time of euthanasia 5–6 weeks after T cell transfer as described in the Supplementary Methods.

In-vivo tracking of ICC-SC homing in mice with DSS colitis

Mice were kept on purified, chlorophyll-free diet optimized for *in-vivo* imaging (OpenSource Diet D1001, Research Diets, Inc., New Brunswick, NJ) for >2 weeks before and throughout the study. 2xSCS2F10 ICC-SC and strain-matched dermal fibroblasts were

labelled with VivoTrack 680 near-infrared dye (Perkin Elmer, Waltham, MA) as per the application notes. 5×10^6 cells/250 μ L PBS/mouse or PBS vehicle were injected intraperitoneally 10 h prior to starting DSS administration. Following removal of abdominal hair and verification of lack of fluorescence from intestinal contents, live imaging was performed immediately after the injection (day 0) and on days 3, 5 and 8 (day of euthanasia) using the Xenogen IVIS 200 imaging system (Perkin Elmer). At each time point, we also performed postmortem imaging of colons and ceca isolated from two additional mice/group.

Cd4⁺ T cell proliferation assay (TPA)

Naïve Cd4⁺ splenocytes were isolated using magnetic separation kits (Miltenyi Biotec, Auburn, CA) from C57BL/6J mice as per the manufacturer's instructions. The T cells were stimulated with T cell-activator Cd3/Cd28 mouse Dynabeads (Life Technologies, Oslo, Norway) and cultured with 2xSCS2F10 ICC-SC irradiated at 3300 Gy. [³H]thymidine (Perkin Elmer) was added on day 4 and proliferation was determined after 18 hours of incubation by measuring the uptake of [³H]thymidine in a scintillation counter and expressed as counts per million. Some of the TPA assays were performed using Transwell permeable supports (Corning, Inc., Tewksbury, MA) with a pore size of 0.4 μ m separating the activated T cells from the ICC-SC and preventing their physical contact while permitting the exchange of soluble factors between the two compartments.

Statistical analyses

Data are expressed as means $\pm$ standard error and analyzed by Student's t test, one-way ANOVA or nonparametric alternatives.

RESULTS

ICC-SC mitigate DSS colitis in mice

We first investigated the immunosuppressive potential of ICC-SC in a murine model of acute DSS colitis. DSS causes injury to the intestinal epithelium permitting the exposure of the submucosa to bacteria and other luminal antigens resulting in inflammation with cells typically involved in innate immunity.¹⁹ Administration of 5% DSS resulted in severe colitis manifested by bloody diarrhea, weight loss, shortening of colon length and histologic features of inflammation including destruction of epithelium, abnormal crypts, edema, and infiltration of inflammatory cells (Figure 1A–F). Intraperitoneal administration of 5×10^6 2xSCS2F10 ICC-SC 10 h before and 4 days after the initiation of DSS exposure prevented the development of severe colitis: Relative to the PBS-injected DSS controls, mice treated with ICC-SC displayed lower body weight loss (from day 6 to euthanasia, $P=0.03$; Figure 1A–B) and disease activity scores ($P<0.001$; Figure 1C); had longer colons ($P<0.001$; Figure 1D), reduced epithelial damage and less infiltration with inflammatory cells (Figure 1E) resulting in a significant decrease in the blinded histologic scores ($P=0.034$; Figure 1F). In contrast, DSS colitis was not mitigated by dermal fibroblast administered in identical numbers and manner (Supplementary Figure S1). Thus, ICC-SC can specifically protect from the development of severe acute colitis.

ICC-SC mitigate chronic T cell-mediated colitis in mice

Next, we investigated the ICC-SC immunosuppressive potential in chronic T cell-induced experimental colitis, a mechanistically more relevant IBD model.¹⁸ Similarly to the DSS model, mice injected twice with 5×10^6 2xSCS2F10 ICC-SC lost less weight than mice injected with PBS ($P=0.04$; Figure 2A) and had lower disease activity scores ($P<0.001$; Figure 2B). Blinded histopathology analysis showed that ICC-SC-injected mice also had reduced severity of colitis ($P<0.03$; Figure 2C) manifesting in fewer abnormal crypts and crypt abscesses (Figure 2D). In contrast, the PBS-treated mice had severe mononuclear cell infiltrates with more transmural involvement (Figure 2D). Thus, ICC-SC were also strongly protective against inflammation in a chronic T cell-mediated model of colitis.

ICC-SC migrate to the inflamed colon

Previously we demonstrated the incorporation of intraperitoneally administered 2xSCS70 ICC-SC into gastric ICC networks in an allogeneic transplantation model.¹⁶ To address migration in the setting of inflammation, we injected 5×10^6 2xSCS2F10 ICC-SC or strain-matched dermal fibroblasts labeled with a near-infrared fluorescent dye into mice 10 h prior to the initiation of DSS treatment and performed live mouse imaging on days 0, 3, 5 and 8. We verified the absence of free dye in the injection by demonstrating lack of fluorescence in the last wash after labeling (Supplementary Figure S2). Evidence of ICC-SC persisting in the abdominal compartment, often clearly outlining intestinal structures, existed in the whole-animal images up to the day of euthanasia on day 8 (Figure 3A). On day 8, we also imaged the excised colons of ICC-SC-, fibroblast- and PBS-injected mice to ensure specific association of the injected cells with the colon. These experiments showed that ICC-SC had indeed migrated to the colon, whereas only very little or no colon-associated fluorescence was seen in mice injected with dermal fibroblasts or PBS (Figure 3B). Thus, ICC-SC-mediated suppression of acute and chronic colitis was associated with specific homing of these cells to the gut.

ICC-SC suppress T cell proliferation by cell proximity-dependent and -independent mechanisms

We next investigated whether the mitigation of experimental colitis by ICC-SC seen *in vivo* was due to the suppression of T cell responses and whether the effects were mediated by cell proximity-dependent mechanisms including direct cell-to-cell contact and evanescent paracrine factors. In MLR, co-culturing of mixed cultures of lymphocytes (C57Bl/6J splenocytes isogenic to the 2xSCS2F10 cells stimulated by irradiated allogeneic, BALB/cJ-derived splenocytes) with 10^4 – 10^5 irradiated 2xSCS2F10 ICC-SC resulted in a dose-dependent inhibition of T lymphocyte proliferation as measured by [3 H]thymidine incorporation (10^4 ICC-SC: $P<0.05$; 10^5 ICC-SC: $P<0.001$; Figure 4A). In the reverse experiment utilizing BALB/cJ splenocytes allogeneic to 2xSCS2F10 cells as responders and irradiated C57BL/6J cells as stimulators, we obtained even more robust repression of T lymphocyte proliferation (10^4 ICC-SC: $P<0.001$; 10^5 ICC-SC: $P<0.001$; Figure 4B), further demonstrating the ability of ICC-SC to suppress immune responses across MHC barriers.

To focus on mechanisms as they relate to Cd4⁺ T helper cells we performed TPA by stimulating Cd4⁺ naïve T cells using Dynabeads coated with anti-Cd3 and anti-Cd28

monoclonal antibodies; an approach that mimics *in-vivo* T cell proliferation. ICC-SC also profoundly suppressed T cell proliferation in this assay as measured by [³H]thymidine incorporation ($P<0.001$) and simultaneously downregulated the secretion of pro-inflammatory cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) by cytometric bead assay (Figure 4C). Using the CFSE dye dilution method we confirmed that increasing amounts of ICC-SC co-cultured with activated T cells dose-dependently reduced the percentages of proliferating Cd4⁺ T cells (Supplementary Figure S3). To determine whether the mechanism of immunoregulation by ICC-SC is dependent on cell proximity or stable soluble factors we performed TPA using Transwell chambers wherein we separated the responder T lymphocytes from ICC-SC by a membrane with a pore size of 0.4 μ m. In these experiments we detected a partial but statistically highly significant suppression of the proliferative response ($P<0.001$; Figure 4D). Consistent with this observation, conditioned media from ICC-SC-containing wells in both standard and Transwell TPA were able to suppress T cell proliferation, albeit to a lesser degree than co-cultured ICC-SC, particularly in the case of supernatants from the Transwell-separated ICC-SC (Figure 4E). Together, these findings indicate that cell-to-cell proximity is important but not strictly required for the immunosuppressive effect of ICC-SC.

PGE₂- but not PD-1-, Treg- or TGF- β -mediated mechanisms underlie ICC-SC-induced immunosuppression

ICC are derived from the mesoderm¹² and their precursors may be related to broadly defined MSC found in many tissues.^{6,7,15,16} MSC-induced immunosuppression is mediated by multiple mechanisms including PGE₂-, PD-1-, Treg- and TGF- β -mediated pathways.^{3-5,8-11} Therefore, we next examined the contribution of these mechanisms to the ICC-SC-mediated immunosuppression.

By RNA-seq performed in 2xSCS2F10 and 2xSCS70 ICC-SC (Gene Expression Omnibus (GEO) accession number: GSE60854; library preparation: Illumina (San Diego, CA) TruSeq; sequencing platform: Illumina HiSeq 2000) we verified the expression of Ano1 (an ICC marker), Cd44, Cd34, insulin receptor, insulin-like growth factor-1 receptor and platelet-derived growth factor receptor- α previously reported in these cells.¹⁶ We also detected mRNA expression for a number of genes involved in immunoregulatory pathways including the PG pathway (genes expressed in both lines: prostaglandin-endoperoxide synthase 1/cyclooxygenase 1, Ptgs1/COX-1; prostaglandin-endoperoxide synthase 2/cyclooxygenase 2, Ptgs2/COX-2; prostaglandin E synthase 2, Ptgs2, prostaglandin E synthase 3, Ptgs3, and prostaglandin E receptors 1 and 4, Ptger1/EP1, Ptger4/EP4), the PD-1 pathway (expressed at very low levels: PD-1 ligand 1, PD-L1/B7-H1/Cd274) and the TGF- β pathway (Tgfb1, Tgfb2 and Tgfb3) (Table 1 and Supplementary Figure S4). Furthermore, by gene expression microarray analysis (GSE60854; platform: Affymetrix (Santa Clara, CA) Mouse Genome 430.2) Ptgs1, Ptgs2, prostaglandin E synthase (Ptgs) and Tgfb1 transcripts were upregulated in 2xSCS2F10 cells and Ptgs2, Ptger2/EP2, Cd274/PD-L1 and Tgfb1 were upregulated in 2xSCS70 cells relative to strain-matched gastric *tunica muscularis* source tissues (cut-off: log₂ fold change>1 and false discovery rate Q value<0.05; Table 1). Even though PD-1 and PD-1 ligand 2 (PD-L2, Pdcd1lg2) were not expressed in either cell line and PD-L1 was only expressed at very low levels, flow

cytometry analysis indicated elevated surface expression of PD-L1 after stimulation with IFN- γ , a cytokine abundantly found in inflammatory milieu (Supplementary Figure S5A). Therefore, we further studied the potential contribution of all three pathways to ICC-SC-mediated immunoregulation.

Using neutralizing antibodies to PD-1 and PD-L1 we did not see abrogation of ICC-SC-mediated suppression of T cell proliferative responses in MLR and TPA experiments (Supplementary Figure S5B,D,E). We also used PD-1 and PD-L1 knock-out mice to set up MLR and TPA but still could not detect any change in ICC-SC capacity to suppress T cell proliferation (Supplementary Figure S5C,D,E). These results strongly indicate lack of involvement of the PD-1 pathway in ICC-SC-mediated immunosuppression.

We next performed experiments to determine the role of Treg- and TGF- β -mediated pathways. By flow cytometry we detected no significant generation of Foxp3⁺ Treg cells²⁰ upon co-culture with ICC-SC (Supplementary Figure S6A), and ICC-SC suppressed the proliferation of T cells from KLF10^{-/-} mice, which cannot upregulate Treg cells in response to stimulation e.g. by TGF- β ²¹ (Supplementary Figure S6B). Furthermore, ICC-SC did not secrete interleukin-10 (IL-10), an anti-inflammatory cytokine required for Treg functions,²² and supernatants from T cells co-cultured with ICC-SC did not contain IL-10 (Supplementary Figure S6C). To evaluate TGF- β -dependent, Treg-independent mechanisms, we performed TPA with Cd4⁺ cells derived from mice expressing the dominant-negative form of the human TGF- β receptor II under the direction of the mouse *Cd4* promoter. Neither in these studies, nor in experiments utilizing neutralizing anti-TGF- β antibodies did we detect abrogation of ICC-SC immunosuppressive activity (Supplementary Figure S7A-B). Together, these results do not support involvement of TGF- β or Treg cells in the immunoregulatory function of ICC-SC.

Finally, we examined the role of PGE₂ as the genes related to its biosynthesis were highly expressed in ICC-SC. By enzyme immunoassay we determined that ICC-SC basally secrete PGE₂ (Figure 5A). In both MLR and TPA, the supernatants from suppressed T cells co-cultured with ICC-SC contained higher amounts of PGE₂ than proliferating T cells cultured without ICC-SC ($P < 0.001$; Figure 5A-B). Indomethacin, a COX-1/2 inhibitor (10 μ M) significantly reduced the suppression of T cell proliferation by ICC-SC in TPA ($P < 0.05$; Figure 5C). Furthermore, mice with chronic T cell-mediated colitis and injected with ICC-SC had higher levels of PGE₂ in their colon as compared to mice treated with PBS ($P = 0.001$; Figure 5D). Collectively, these data provide strong evidence for a major role for PGE₂ secretion in the ICC-SC-induced immunosuppression.

ICC-SC are different from MSC by transcriptional profiling

The experiments described above indicated that only some, but not all, mechanisms of immunoregulation described in MSC contribute to the suppression of T cell responses by ICC-SC. Therefore, we compared the gene expression profiles (obtained by microarrays) of 2xSCS2F10 cells and 2xSCS70 cells with those of murine bone marrow-derived MSC from two different sources^{23,24} and MSC isolated from the murine colonic mucosa.²⁴ As reference we also included data from two different classes of postnatal ICC (myenteric ICC, ICC-MY, and deep muscular plexus ICC, ICC-DMP) from the murine small intestines²⁵ and

murine bone marrow-derived neural crest stem cells (NCSC).²³ Previously published data were downloaded from the National Center for Biotechnology Information GEO database. Unsupervised K-means clustering analysis showed that gene expression profiles of ICC-SC were closely related to each other and postnatal ICC but did not cluster with MSC or neural crest stem cells (Figure 6). This result, besides confirming the relationship between 2xSCS2F10 and 2xSCS70 cells and ICC, suggests that ICC-SC and MSC belong to distinct SC classes.

DISCUSSION

Previously we found that Kit^{low/-}Ano1⁺Cd44⁺Cd34⁺ ICC-SC isolated from the murine stomach were able to home to, and incorporate into, gastric ICC networks in an allogeneic transplantation model.¹⁶ Here we show that this capability reflects a potent immunosuppressive potential demonstrable both *in vivo* and *in vitro*: ICC-SC suppressed inflammation and T cell responses in severe acute, 5% DSS-induced and chronic, adoptive T cell transfer-induced colitis models, as well as in MLR (utilizing either isogenic or allogeneic responder cells) and TPA experiments. Following intraperitoneal injection, ICC-SC preferentially homed to the inflamed colon and reduced the severity of colitis by both clinical and histological criteria and mitigated weight loss, indicating that their immunomodulatory capacity not only facilitates their survival in the host, but can also ameliorate autoimmune disease in the latter. These were specific effects as they could not be reproduced with strain-matched dermal fibroblasts, which also lacked tropism toward the inflamed colon when administered in an identical dose and manner. By transcriptome profiling, protein expression studies and extensive mechanistic experiments utilizing knock-out mice, neutralizing antibodies and pharmacological inhibition we show that ICC-SC employ PGE₂ biosynthesis and secretion as their main mechanism of immunosuppression. We also found this mechanism is most effective across short distances, likely affecting the host tissues to which the injected ICC-SC have migrated without exerting major systemic actions. Furthermore, transcriptome profiling by RNA-seq and microarrays not only verified key phenotypic characteristics of ICC-SC^{15,16} but also indicated that these cells are unlikely to be a manifestation of systemic or colonic MSC.^{23,24}

In this study we administered ICC-SC to mice with DSS- or T cell-mediated colitis in a total dose representing ~1/3 of the dose employed in the studies demonstrating ICC-SC incorporation into ICC networks¹⁶ but 1.6–2.7-fold higher (following appropriate interspecies conversion¹⁷) than the MSC doses utilized in ongoing clinical trials (NCT00482092 and NCT01090817²⁶). While precise dose-response relationships will have to be determined in future studies, our *in vitro* data suggest that lower doses can also be expected to elicit significant immunosuppressive effects (Figure 4A,B).

The importance of PGE₂ in the ICC-SC-mediated immunomodulation was supported by several lines of evidence including the expression of relevant biosynthetic enzymes including COX-1/2, the reduction of ICC-SC effects on T cell responses by pharmacological COX-1/2 blockade and the detection of secreted PGE₂ in ICC-SC-conditioned media and in the colon of ICC-SC-injected mice. Numerous studies have documented the suppressive role of PGE₂ on T cell function, which are mediated by multiple signaling pathways.²⁷ For

example, binding of PGE₂ to Ptger2/EP2 and Ptger4/EP4 receptors on Cd4⁺ T cells stimulates the production of cyclic AMP (cAMP),²⁸ which interferes with IL-2-mediated gene expression and inhibits the activation and expansion of T cells through IL-2 production and responsiveness.^{29,30} Furthermore, Ptger4/EP4-mediated mechanisms are also important for maintaining mucosal barrier integrity,³¹ which likely explains the efficacy of ICC-SC treatment against both T cell transfer- and DSS-induced colitis. Our data on PGE₂ as a major mechanism of immunosuppression are also congruent with studies in adipose tissue-, bone marrow- and umbilical cord-derived MSC.^{4,5,8}

TPA experiments suggested that inhibition of COX-1/2-mediated PGE₂ synthesis did not fully account for the ICC-SC-induced immunosuppression. Apart from PGE₂, MSC have been shown to exert immunosuppressive effects through multiple mechanisms including TGF-β, Treg/IL-10 and the PD-1 pathway.^{4,9,10,32} We used RNA-seq and gene expression microarrays to identify immunoregulatory genes expressed in ICC-SC. By employing T cells derived from knock-out mice and antibodies to block these pathways, we demonstrated that the PD-1 and TGF-β pathways were not involved in immunoregulation by ICC-SC. MSC may also enhance the induction and differentiation of Cd4⁺ T cells to adaptive Foxp3⁺ T regulatory cells.^{5,11} Therefore, we used Cd4⁺ *Foxp3* reporter cells and Cd4⁺ T cells derived from KLF10 knock-out mice and determined that ICC-SC effects were not dependent on induction of T regulatory cells, either. Other molecular mechanisms reported to contribute to MSC-induced immunosuppression include nitric oxide, indoleamine 2,3-dioxygenase 1, TNF-α-induced protein 6 (Tnfaip6) and complement component factor h.³ Of these genes, Tnfaip6 is expressed in both ICC-SC cell lines and its potential role will be investigated in future studies.

Since ICC are thought to originate from mesodermal tissues surrounding the primitive gut,¹² their adult precursors may be related to broadly defined MSC.^{6,7} Surprisingly, gene expression profiling and unsupervised cluster analysis did not support ICC-SC being related to bone marrow- and colonic mucosa-derived MSC or bone marrow-derived NCSC, whereas ICC-SC were clustered with differentiated ICC in the same analysis. Thus, the developmental origin of ICC-SC remains unclear and will be addressed in future studies.

The ability of ICC-SC to differentiate into ICC *in vitro* and *in vivo*¹⁶ opens up their potential for use in the treatment of a wide variety of diseases that are mediated by either damage or loss of adult ICC including diabetic gastroparesis,¹⁴ achalasia, constipation, intestinal pseudo-obstruction, Chagas disease, Hirschsprung's disease and congenital hypertrophic pyloric stenosis.¹³ ICC have been shown to develop in gut organoids derived from embryonic stem cells (ESC)³³ and induced pluripotent stem cells (iPSC).³⁴ However, in some studies, syngeneic ESC and iPSC – the latter even after *in-vitro* differentiation – have been found to generate an immune response (mediated by T cells and macrophages) when transplanted leading to their rejection.^{35,36} Unlike ESC and iPSC-derived cells, which may potentially be immunogenic, ICC-SC are in fact immunosuppressive and thus may be better suited to treat disorders mediated by injury or loss of ICC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ano1	anoctamin1
cAMP	cyclic AMP
CFSE	carboxyfluorescein diacetate succinimidyl ester
CI	confidence interval
COX-1	cyclooxygenase 1 (prostaglandin-endoperoxide synthase 1, Ptg1)
COX-2	cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2, Ptg2)
DSS	dextran sulfate sodium
ESC	embryonic stem cell(s)
GEO	Gene Expression Omnibus
IBD	inflammatory bowel disease
ICC	interstitial cell(s) of Cajal
ICC-DMP	deep muscular plexus ICC
ICC-MY	myenteric ICC
ICC-SC	ICC stem cell(s)
IFN-γ	interferon-γ
IL	interleukin
MLR	mixed lymphocyte reaction
iPSC	induced pluripotent stem cell(s)

MHC	major histocompatibility complex
MSC	mesenchymal stem cell(s)
NCSC	neural crest stem cell(s)
PD-1	programmed cell death 1 (Pcd1)
PD-L1	programmed cell death 1 ligand 1 (B7-H1, Cd274 antigen)
PD-L2	programmed cell death 1 ligand 2 (Pcd1lg2)
PGE₂	prostaglandin E ₂
Ptger1	prostaglandin E receptor 1 (subtype EP1)
Ptger2	prostaglandin E receptor 2 (subtype EP2)
Ptger3	prostaglandin E receptor 2 (subtype EP3)
Ptger4	prostaglandin E receptor 4 (subtype EP4)
Ptges	prostaglandin E synthase
Ptges2	prostaglandin E synthase 2
Ptges3	prostaglandin E synthase 3 (cytosolic)
RNA-seq	RNA deep sequencing
TGF-β	transforming growth factor- β (Tgfb1, Tgfb2, Tgfb3)
TNF-α	tumor necrosis factor- α
Tnfaip6	tumor necrosis factor alpha induced protein 6
TPA	T cell proliferation assay
Treg	regulatory T cell(s)
SC	stem cell(s)

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Figure 1. ICC-SC alleviate DSS-induced colitis in mice

11–13 mice/group were studied in two independent cohorts. See control experiments utilizing dermal fibroblasts in Supplementary Figure S1. (A) Time-course of changes in body weights expressed as percentages of pre-DSS body weights on day 0. (B) Changes in body weights at euthanasia expressed as percentages of pre-DSS body weights on day 0. ICC-SC administration mitigated DSS-induced body weight loss (PBS: $18.6 \pm 2.4\%$ vs. ICC-SC: 12.3 ± 1.2 ; $P=0.03$). (C) Macroscopic disease score based on clinical signs of colitis (wasting and hunching and the physical characteristics of stool) and an ordinal scale of colonic involvement (thickness and erythema). Mice treated with ICC-SC had lower disease activity scores than PBS-injected mice (PBS: 7.4 ± 0.2 vs. ICC-SC: 3.5 ± 0.3 ; $P<0.001$). (D) Assessment of colon length shortening used as an index of disease. Mice treated with ICC-SC had longer colons than PBS-injected mice (PBS: 6.3 ± 0.2 cm vs. ICC-SC: 7.3 ± 0.2 ; $P<0.01$). (E) Representative histologic sections of mouse colons. Note reduced overall tissue thickness in the ICC-SC-treated mouse in the low-power images of the “Swiss rolls” (upper

panels; magnification: 10×) and normal tissue architecture in the close-ups (lower panels; magnification: 100×). Also note abnormal crypt morphology, polymorphonuclear (PMN) cell infiltration and edema in the control DSS mouse. (*F*) Histologic grading of colitis. ICC-SC administration reduced histological signs of DSS-induced colitis (PBS: 14.8 ± 1.2 vs. ICC-SC: 10.8 ± 1.2 ; $P=0.034$).

Figure 2. ICC-SC alleviate T cell-induced chronic colitis in mice

8 mice/group were studied in two independent cohorts. (A) Body weight loss expressed as percentages of initial body weights on day 0. Mice treated with ICC-SC had less weight loss than PBS-injected mice (PBS: $18.6 \pm 2.1\%$ vs. ICC-SC: 10.2 ± 3 ; $P = 0.04$). Cd4⁺Cd45⁺RB^{high}, T-cell induced colitis. (B) Macroscopic disease score based on clinical signs of colitis (wasting and hunching and the physical characteristics of stool) and an ordinal scale of colonic involvement (thickness and erythema). Mice treated with ICC-SC had lower disease activity scores than PBS-injected mice (PBS: 7 ± 0.3 vs. ICC-SC: 4 ± 0.6 ; $P < 0.001$). (C) Histologic grading of colitis. ICC-SC decreased colitis activity in the T cell transfer model (PBS: 11.1 ± 1.0 vs. ICC-SC: 7.5 ± 1.2 ; $P = 0.03$). (D) Representative histologic sections of mouse colons. Note reduced overall tissue thickness (upper panels; magnification: 10 $\times$) and normal tissue architecture and lack of mononuclear infiltrates (lower panels; magnification: 100 $\times$) in the ICC-SC-treated mouse.

Figure 3. ICC-SC migrate to the inflamed intestine

Representative images of 3–4 mice/group are shown. (A) Serial live imaging of mice after injection of labeled ICC-SC or PBS on days 0, 3 and 8. Note strong fluorescence, often clearly associated with intestinal structures, in ICC-SC-injected but not in control (CTRL) mice. (B) Colons excised from mice injected with ICC-SC, dermal fibroblasts (DFib) or PBS imaged on day 8. Note strong fluorescent foci in the colons of ICC-SC-injected mice and minimal or no fluorescence in the colons of animals injected with dermal fibroblasts or PBS. Scale bars indicate units of radiant efficiency ($(p/s/cm^2/sr)/(μW/cm^2)$).

Figure 4. ICC-SC suppress T cell proliferation by cell proximity-dependent and -independent mechanisms

[^3H]thymidine uptake experiments and cytokine assays were carried out in triplicate. Data were analyzed using ANOVA with *post-hoc* multiple comparison tests and are presented as 95% confidence interval (CI) of difference or means $\pm$ SEM. B/c and B6 denote splenocytes derived from BALB/cJ mice and C57BL/6J mice, respectively. Irradiated cells marked by * also served as negative controls. (A) MLR using C57BL/6J splenocytes isogenic to 2xSCS2F10 ICC-SC as responders and BALB/cJ splenocytes as stimulators. ICC-SC dose-dependently inhibited T cell proliferation measured by [^3H]thymidine incorporation (95% CI of difference: 547.7–7844 cpm; $P < 0.05$ for 10^4 ICC-SC and 3,485–10,780 cpm; $P < 0.001$ for 10^5 ICC-SC). (B) MLR using BALB/cJ splenocytes allogeneic to 2xSCS2F10 ICC-SC as responders and C57BL/6J splenocytes as stimulators. ICC-SC dose-dependently inhibited T cell proliferation measured by [^3H]thymidine incorporation (95% CI of difference: 2,618–10,660 cpm; $P < 0.001$ for 10^4 ICC-SC and 8,648–16,690 cpm; $P < 0.001$ for 10^5 ICC-SC).

(C) TPA using Cd3/Cd28 mouse Dynabeads to stimulate Cd4⁺ naïve T cells. 2xSCS2F10 ICC-SC profoundly suppressed T cell proliferation as measured by [³H]thymidine incorporation (95% CI of difference: 14,510–22,500 cpm; $P<0.001$) and suppressed the production of proinflammatory cytokines IFN- γ (98.5 ± 38.3 vs. 4.6 ± 1.4 pg/mL; $P<0.05$) and TNF- α (89.8 ± 21.3 vs. 1.1 ± 0.7 pg/mL; $P<0.01$) by Cd4⁺ T cells. (D) TPA performed in a Transwell configuration (T) to physically separate 2xSCS2F10 ICC-SC (top well) from the responder Cd4⁺ T cells (bottom well). T lymphocyte proliferation was only partially but significantly inhibited (95% CI of difference: 34,850–44,940 cpm; $P<0.001$). (E) TPA wherein ICC-SC were replaced by conditioned media harvested from TPA experiments performed in mixed 2xSCS2F10 ICC-SC and T cells (CM) or TPA experiments where the medium was obtained from ICC-SC cultured in Transwell inserts, i.e., not in physical contact with the responding T cells (CM(T)). CM(T) suppressed T cell proliferation but to a lesser degree than the co-cultured ICC-SC (co-cultured: 95% CI of difference: 1886–7201 cpm; $P<0.01$ vs. CM-T: 7,284–12,600 cpm; $P<0.001$) or CM (95% CI of difference: 4,773–10,090 cpm; $P<0.001$).

Figure 5. ICC-SC mediate immunosuppression by secreting PGE₂

Experiments were carried out in triplicate and analyzed as described in Figure 4. (A–B) PGE₂ measured in MLR (A) and TPA (B) culture supernatants by enzyme immunoassay. Data are means±SEM. Unlike splenocytes, 2xSCS2F10 ICC-SC basally secreted PGE₂ at 486.7±52.3 pg/ml/10⁵ cells. In both MLR and TPA, the supernatants from suppressed T cells co-cultured with ICC-SC contained higher amounts of PGE₂ than proliferating T cells cultured without ICC-SC (MLR: 491.2±14.6 vs. 0 pg/mL; $P < 0.001$; TPA: 544.5±9.3 vs. 117.8±10 pg/mL; $P < 0.001$). (C) In TPA, indomethacin (IN; 10 μM), a COX-1/2 inhibitor, countered the suppression of T cell proliferation by ICC-SC (95% CI of difference: –10,240 to –602.4 cpm; $P < 0.05$ for 10⁵ ICC-SC and –10,100 to –455.6 cpm; $P < 0.05$ for 5×10⁴ ICC-SC). In the absence of ICC-SC, Cd4⁺ cell proliferation was not significantly inhibited by indomethacin (95% CI of difference: –2,587 to 5,127 cpm; $P > 0.05$). (D) PGE₂ measured by enzyme immunoassay in the colon of mice with T cell-mediated chronic colitis. Mice treated

with ICC-SC had higher levels of PGE₂ in their colon as compared to mice treated with PBS (PBS: 40.9±2.3 pg/mg vs. ICC-SC: 52.7±2; $P=0.001$). Data are means±SEM.

Figure 6. ICC-SC and MSC have different transcriptional profiles

Affymetrix Mouse Genome 430.2 data from 2xSCS2F10 (1) and 2xSCS70 ICC-SC (2) (GEO accession number: GSE60854), postnatal mouse ICC-DMP (3) and ICC-MY (4) (GSE7809),²⁵ bone marrow-derived NCSC (5; GSE30419),²³ bone marrow-derived MSC (6; GSE30419²³ and GSE19233²⁴) and colonic mucosal MSC (7; GSE19233²⁴) were compared by unsupervised K-means clustering following preprocessing with robust multiple-array analysis. Heat map shows 3643 probe sets with >8-fold average difference between 2xSCS2F10 (1) and colonic MSC (7). Note that ICC-SC lines were closely related to each other and mature ICC but not to MSC or NCSC.

Table 1

ICC-SC gene expression related to PGE₂-, PD-1- and TGF-β-mediated pathways of immunosuppression.

Annotation					2xSCS2F10				2xSCS70			
Chr.	Gene ID	Gene Name	Common Name	Coding Length	RNA-seq		Microarray		RNA-seq		Microarray	
					Read Count	RPKM	Log ₂ Fold Change	B-H Q	Read Count	RPKM	Log ₂ Fold Change	B-H Q
2	Ptgs1	prostaglandin-endoperoxide synthase 1	cyclooxygenase 1, COX-1	2864	12940	89.53*	2.76*	0.00069*	3132	17.38*	0.70	0.51553
1	Ptgs2	prostaglandin- endoperoxide synthase 2	cyclooxygenase 2, COX-2	4145	14150	67.64*	5.829*	0.00048*	17072	65.45*	5.87*	0.00030*
2	Ptges	prostaglandin E synthase		3647	42	0.23	1.001*	0.00362*	86	0.37*	-0.28	0.57472
2	Ptges2	prostaglandin E synthase 2		1952	716	7.27*	0.537	0.01819	1272	10.36*	0.39	0.07199
10	Ptges3	prostaglandin E synthase 3 (cytosolic)		1954	9576	97.11*	N/A	N/A	13922	113.22*	N/A	N/A
8	Ptger1	prostaglandin E receptor 1 (subtype EP1)	EP1	2082	820	7.80*	-0.446	0.05449	1174	8.96*	-0.280	0.23150
14	Ptger2	prostaglandin E receptor 2 (subtype EP2)	EP2	3768	4	0.02	-1.046	0.03408	10	0.04	2.195*	0.00126*
3	Ptger3	prostaglandin E receptor 3 (subtype EP3)	EP3	2018	4	0.04	-3.590	0.00642	48	0.38*	-0.185	0.64704
15	Ptger4	prostaglandin E receptor 4 (subtype EP4)	EP4	2966	276	1.84*	-1.472	0.05174	84	0.45*	0.349	0.65987
1	Pdcd1	programmed cell death 1	PD1	1930	0	0	0.295	0.25966	0	0	0.10	0.63904
19	Cd274	CD274 antigen	B7-H1, PD-L1	3638	134	0.73*	0.671	0.21609	124	0.54*	1.89*	0.04736*
19	Pdcd1lg2	programmed cell death 1 ligand 2	PD-L2	3415	0	0	0.193	0.41008	0	0	-0.01	0.95412
7	Tgfb1	transforming growth factor, beta 1		2094	724	6.85*	3.665*	0.00029*	2514	19.08*	1.40*	0.02537*
1	Tgfb2	transforming growth factor, beta 2		4725	4654	19.52*	-1.202	0.00373	8376	28.17*	-1.37	0.06065
12	Tgfb3	transforming growth factor, beta 3		3385	4612	27*	0.317	0.44533	3570	16.76*	-1.19	0.16751

RNA-seq: Expression of selected genes in 2xSCS2F10 and 2xSCS70 cells quantified as reads/kilobase exon/megabase mapped reads (RPKM). Read counts for all transcript variants for a gene were considered. Microarray: Differential expression of the same genes in 2xSCS2F10 cells (n=3) vs. C57BL/6J gastric *tunica muscularis* source tissues (n=2) and in 2xSCS70 cells (n=3) vs. Immortomouse gastric *tunica muscularis* source tissues (n=3). B-H Q: Benjamini-Hochberg false discovery rate. In the case of more than one probe set per gene, the probe set with the lowest Q value was selected. N/A, gene not represented on the microarray.

* significant expression defined as RPKM>0.3³⁷ or log2 fold change>1 AND Q<0.05.

Chr., chromosome. RNA-seq read distribution histograms, exon-exon junctions and correlation between gene expression detected by RNA-seq and microarrays are shown in Supplementary Figure S4.