

## IL-9 enhances growth of ICC, maintains network structure and strengthens rhythmicity of contraction in culture

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### Abstract

Interstitial cells of Cajal (ICC) play a critical role in the control of gastrointestinal motility as pacemaker cells and as regulators of enteric innervation. ICC are one of the first cell types that are injured during an inflammatory process and maintenance of ICC health or promotion of growth and development maybe crucial in recovery after injury. The aim of this study was to evaluate the role of IL-9 in the growth, development and maintenance of ICC in culture. IL-9 in concentrations from 0.02 to 1 µg/ml promoted individual ICC growth and maintenance of the ICC network structure inside tissue explants under culture conditions. The number of ICC grown out of the explants increased significantly at day 4 of culture in the presence of 0.02, 0.5 and 1 µg/ml IL-9. In the presence of 0.5 µg/ml IL-9, explants in culture maintained a higher frequency and stabilized the frequency of spontaneous contractile activity. The ultrastructure of the ICC after 4 days in culture was similar to that *in situ*. Our data indicate that IL-9 promotes ICC growth in culture and it can be hypothesized that IL-9 is a critical factor in the maintenance of ICC health and ICC repair after injury.

**Keywords:** Interstitial cell of Cajal (ICC) • Auerbach's plexus • smooth muscle cells • IL-9 • growth factor • pacemaking • neurotransmission • ultrastructure • explant culture • myenteric plexus

### Introduction

Interstitial cells of Cajal (ICC) perform critical functions in the control of motility of the gastrointestinal (GI) tract both as pacemaker cells and as mediators of neurotransmission [10, 12, 26, 30]. In

addition, a lot of evidence has been obtained to support the notion that ICC pathology is an integral part of many motility disorders of the GI tract [33]. There is evidence that ICC are among the first cells to receive injury from an inflammatory insult [3] and loss of ICC has been associated with loss of GI motor function [25, 35, 36]. Hence it appears crucial to find mechanisms to support maintenance of the ICC network in intestinal pathophysiology and to elucidate the factors regulating growth, differentiation and maintenance of ICC.

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Little is known about the chemical environment that is needed for ICC development and repair. We do know that depletion of the c-kit receptor can result in loss of certain classes of ICC [11, 18, 37]. In the  $WW^v$  mouse which has a mutation in the c-kit gene, ICC-AP of the intestine develop through the embryonic period but do not develop further after birth [15]. ICC and mast cells are the only c-kit positive cells in the gut musculature and stem cell factor is critical for growth and differentiation of both cell types [21, 23]. Extensive research from Ordog and co-workers has revealed that insulin or insulin-like growth factor type-1 (IGF-I) are likely essential growth factors for ICC [8, 9, 22]. Recently interleukin-9 (IL-9), a multifunctional cytokine, was shown to be a potent growth factor for human mast cell progenitors. IL-9 acts together with stem cell factor to promote mast cell development [19] and megakaryocytopoiesis [4]. The role of IL-9 in the physiology and pathophysiology of gut function, specifically in the context of ICC, is not known, but parallels may exist with the role of IL-9 in the pathogenesis of asthma, in particular the development of muscular hyper-responsiveness with elevated levels of IL-9 [28]. It was recently shown that IL-9 plays a role in the development of intestinal muscle hypercontractility during enteric infection [14].

Considering the role of IL-9 in mast cell development and its synergy with stem cell factor we hypothesized that IL-9 might be one of the natural growth factors to promote the maintenance and repair of ICC *in vivo*. We show here that in explant cultures, IL-9 promotes ICC growth and it appears to stabilize ICC function as shown by a maintained robust frequency of spontaneous contractile activity.

## Material and methods

### Explant culture

The animal experiments were performed following the guidelines of the Animal Ethics committee at McMaster University. 2–4 day old CD1 mice were killed by cervical dislocation and the GI tract, starting from the esophagus to the colon, was removed with intact mesenteric vascular bed to minimize stretch when transferred into a dissection dish, which was filled with M199 medium (Gibco). After removing the gut from the mesenteric vascular bed under a dissec-

tion microscopy, the jejunum (1.5 cm length) was obtained and mounted without stretch onto a Sylgard surface by insect pins (0.1 mm in diameter). According to the method developed by Lars Thuneberg [29], the musculature was cleaved along the deep muscular plexus, leaving the outer circular muscle layer, the myenteric plexus, and the longitudinal muscle layer intact. The muscle strip was transferred into a Falcon Petri dish (VWR) with medium M199 and then cut into millimetre-size pieces. These “explants” (5–10) were gently placed on collagen-coated (rat-tail collagen, Roche Diagnostics Corporation) glass coverslips in 4 well dishes (Nunc Serving Life Science) by using curved forceps, and immersed in M199 culture medium with or without IL-9 (0.002–2  $\mu\text{g/ml}$ ). An equal number of explants with and without IL-9 were incubated in 95%  $\text{O}_2$ -5%  $\text{CO}_2$  at 37°C for 4 days. The IL-9 was generously provided by the Ludwig Institute for Cancer Research, the culture medium contained 10% Fetal Bovine Serum (Gibco), 1% L-Glutamine (Gibco), and 1% Antibiotic-Antimycotic (Gibco).

### Statistical analysis

The total cell number outside of the explants was determined by propidium iodide counterstaining. Data were expressed as means  $\pm$  SE;  $n$  = number of culture dishes containing 5–10 explants. A single observation ( $n = 1$ ) constitutes the average value of observations from 5–10 explants or cells associated with these explants from one culture dish. The paired Student's  $t$ -test was used to evaluate differences between mean values.  $P$  values of 0.05 or less were considered to indicate statistical significance.

### Immunohistochemistry

After 4 days of explant culture, the culture medium was removed from the coverslips, and all the cultured cells with or without IL-9 were fixed with 4% paraformaldehyde for 10–15 min at 4°C. Before staining with c-kit antibody, the samples were incubated in 10% FBS (Gibco) at room temperature for 30 min to reduce non-specific staining before addition of conjugated c-kit antibody (ACK4). After the incubation with 1:500 anti-c-kit 4 antibody (FITC anti-mouse CD117 c-kit monoclonal antibody Cedarlane) at 4°C over night, the samples were washed in PBS for 3  $\times$  10 min. Propidium iodide (15  $\mu\text{g/ml}$  Sigma) was added to the cells for 15–20 min at room temperature to mark all cells. The cells were then washed in PBS for 3  $\times$  5 min before being examined with

a confocal microscopy (LSM 510, Zeiss, Germany). Quantification of immune positive cells external to the explants was done by manual counting and expressed as percentage of total cells in the area. The immunopositive cells inside the explants were measured with pixel intensity of fluorescence calculated using software provided by Zeiss (KS400), imageJ and Adobe programs.

## Electron microscopy

Electron microscopy of 4 day explant cultures was performed in Falcon Petri dishes (VWR) coated with rat-tail collagen (Roche Diagnostics Corporation). The cells were fixed *in situ* with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) containing 1.2 mM CaCl<sub>2</sub> for 2 h at room temperature. After fixation, the samples were washed overnight in 0.1 M cacodylate buffer (pH 7.4) containing 1.2 mM CaCl<sub>2</sub>, post fixed with 1% OsO<sub>4</sub> in 0.05 M sodium cacodylate buffer (pH7.4) for 1 h at room temperature. The cells were stained with 2% uranyl acetate for 30 min at room temperature. After dehydration in graded ethanol and epoxy resin, cells and explants while still at the bottom of the dishes were embedded in Tabb 812 resin overnight on an orbital shaker by using the "inverted capsule embedding technique" [2, 6] To avoid damage to the cells, the hardened blocks were removed from the bottom of the dishes with a sharp pull without applying dry ice or liquid nitrogen. Ultrathin sections were cut and stained with lead citrate for 4 min before viewing with a transmission electron microscope (Jeol 1200EX Biosystem, Tokyo, Japan)

## Contractile activity

Contraction was observed using a phase-contrast microscope (Zeiss Axiovert 100, Germany). The images were captured with a Sony CAD solid-state color video camera installed on the microscope and counted manually in real time with Northern Elipse 7.0 (Empix Imaging, Inc). Assessment was done under continuous surperfusion with Medium 199 at 32°C.

## Results

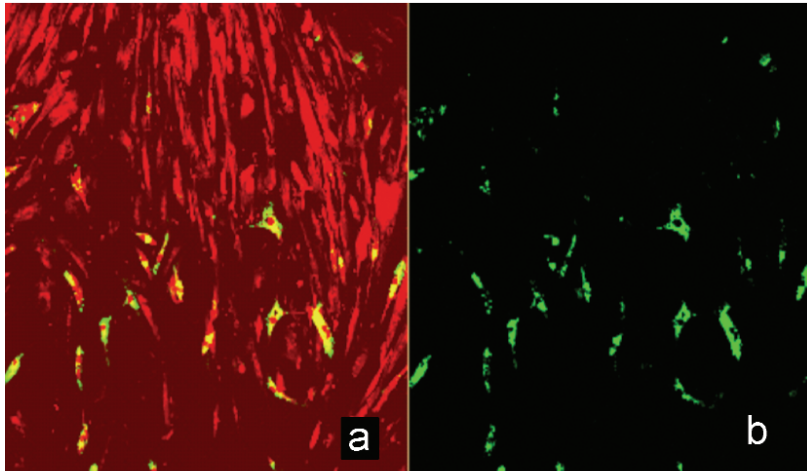
After incubation of explants in culture medium, the first cells started to grow out of the explants between

24 and 30 hrs ( $27.0 \pm 5.3$  h;  $n = 35$ ) under control conditions. Smooth muscle cells formed muscle bundles growing perpendicular to the explants and ICC formed networks associated with these smooth muscle cells. Explants maintained contractile activity and rhythmic contractile activity was also observed in isolated ICC, often independent of the explants. Rhythmically contracting ICC networks (three or more ICC connected to each other and contracting simultaneously) outside of the explants were only seen occasionally after 4 days without IL-9 but regularly in the presence of IL-9. In the presence of 0.5  $\mu\text{g/ml}$  IL-9, the first cells appeared between 18 and 25 hrs after incubation ( $22.1 \pm 3.7$  h;  $n = 35$ ;  $P < 0.05$ ). Typical branching ICC were observed within 48 hrs in IL-9 ( $n = 10$ ) but only after 72 hrs in control ( $n = 10$ ). After 4 days in culture, the density of ICC (percentage of ICC related to total number of ICC and smooth muscle cells combined, observed outside the explants) was markedly increased in the presence of IL-9 (Fig. 1) in a dose dependent manner (Fig. 2). The total cell number was determined by staining all cells with the nuclear stain propidium iodide (Fig. 1). The total number of cells present outside the explants at day 4 of culture was not significantly different with or without IL-9.

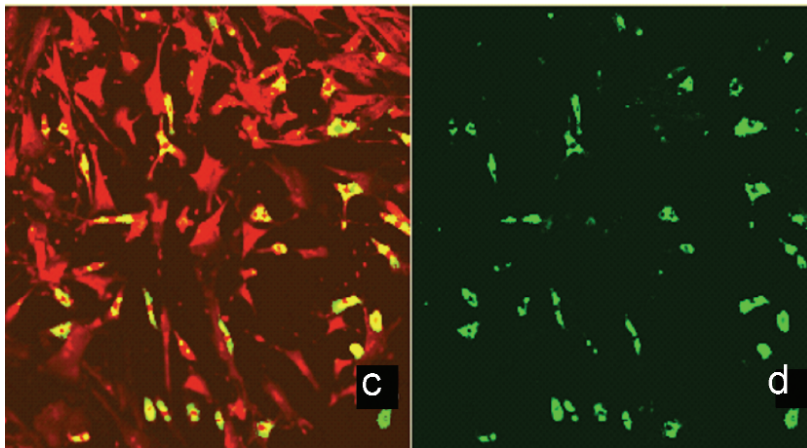
The number of ICC could not be determined inside the explants because the network structure and overlapping of cells made identification of single ICC impossible, hence dye intensity and area were assessed at day 4 of culture (Fig. 3). The intensity of fluorescence was indicated by a fluorescent brightness value given in ImageJ software by measuring the summation of the fluorescent areas within each explant. The average value of all explants per culture dish was taken as  $n = 1$ . Fluorescence intensity for cultures incubated with 0.5  $\mu\text{g/ml}$  IL-9 was  $202.5 \pm 51.4$  ( $n = 25$ ) compared to  $141.0 \pm 42.4$  in control ( $n = 25$ ;  $P < 0.001$ ). The pixel values, reflecting the area of fluorescence of the explants were  $662 \pm 238$  in control,  $1379 \pm 246$  with IL-9 ( $n = 25$ ;  $P < 0.001$ ).

To investigate whether ICC ultrastructure was maintained under IL-9 incubation, we studied ICC under an electron microscope after obtaining thin sections using the "pop-off" technique [2]. The cultures with 0.5  $\mu\text{g/ml}$  IL-9 at day 4 were processed as mentioned in the methods section. As shown in Fig. 4, ICC maintained excellent ultrastructure in the presence of IL-9. The "pop-off" technique was perfected such that single cells

without IL-9



with IL-9



**Fig. 1** Effect of IL-9 on ICC grown out of explants. ICC were identified by c-kit immuno-fluorescence at day 4 of explant culture. All cells were stained with propidium iodide. **a:** control with c-kit immuno-fluorescence (green) and propidium iodide (red). **b:** control, c-kit immuno-fluorescence only. **c:** in the presence of IL-9 (0.5  $\mu\text{g/ml}$ ) with c-kit immuno-fluorescence (green) and propidium iodide (red). **d:** in the presence of IL-9 (0.5  $\mu\text{g/ml}$ ), c-kit immuno-fluorescence only.

grown on collagen could be cut longitudinally. Single ICC showed a triangular- or stellate-shaped cell body with multiple, thin processes that extended to smooth muscle cells, large prominent nuclei, numerous mitochondria, endoplasmic reticulum and caveolae. No obvious abnormality of ultrastructure was seen and thick filaments were absent.

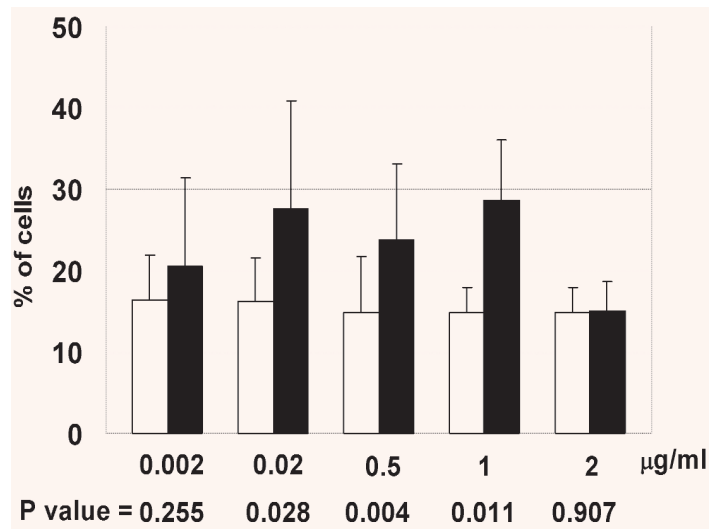
Explants, cultured in the absence or presence of 0.5  $\mu\text{g/ml}$  IL-9, maintained rhythmic contractile activity. In the absence of IL-9, the contraction was often sluggish (contractions and subsequent relaxations were relatively slow and were completed between 1 and 2 s) with an average frequency of  $20 \pm 5$  contractions per minute. In the presence of IL-9, contractions were robust (contractions were completed in less than 0.5 s) with little variability in frequency assessing all single explants and occurred at  $28 \pm 5$  contractions per minute ( $n = 24$ ,  $P < 0.001$ ), measured at  $32^\circ\text{C}$ .

## Discussion

The present study provides evidence that IL-9 promotes ICC development in culture and maintains ICC phenotype according to ultrastructural features. In addition, survival of ICC networks inside explants in culture improved based on the increased c-kit positivity and maintenance of robust rhythmic contractile activity.

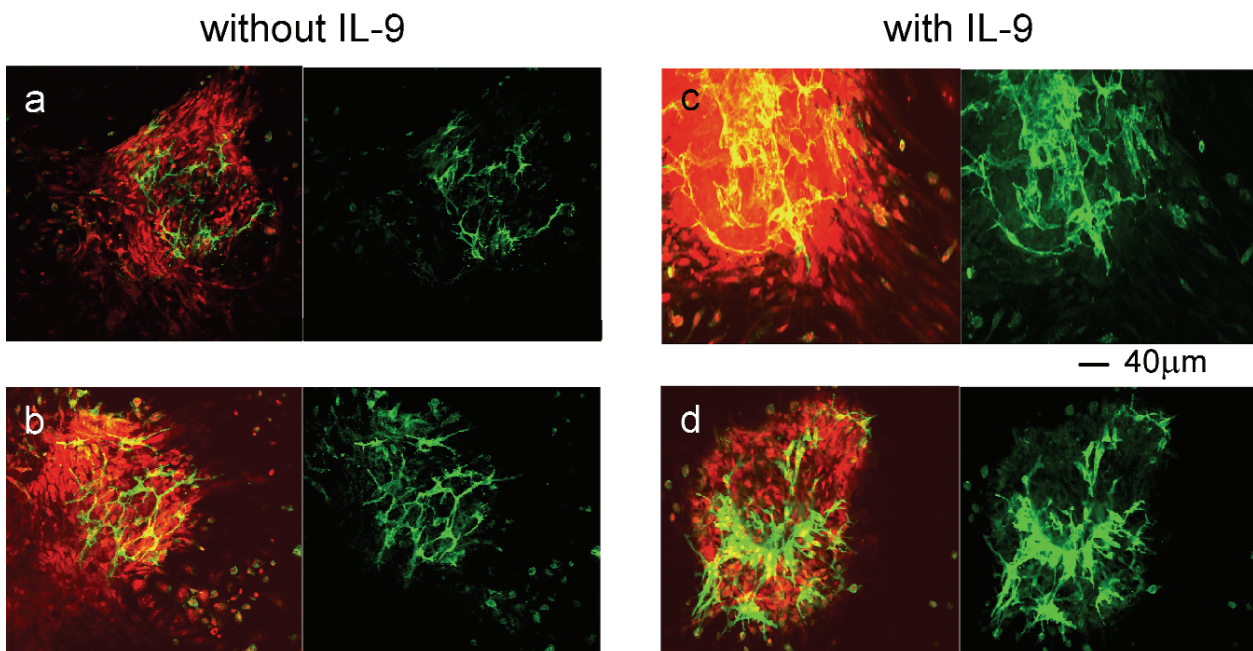
It is assumed that ICC need stem cell factor as a growth factor since c-kit antibodies reduce ICC viability [18]. The experience with added stem cell factor to ICC culture is not consistent between laboratories. Thuneberg did not add stem cell factor to his cultures [29]. In our hands, adding stem cell factor did not improve ICC viability [17]. The Mayo group published a slightly different experience [24]: ICC grew well without stem cell factor but improved by adding stem cell factor. A possible explanation for

**Fig. 2** Effect of IL-9 on density of c-kit positive ICC in culture. IL-9 addition to explant cultures increased the number of c-kit positive cells measured at 4 day of culture associated with individual explants. Data were obtained from 32 cultures from 8 neonatal mice, quantifying individual c-kit positive cells outside the explants expressed as % of total cells. Each culture dish containing IL-9 was compared to a culture dish without IL-9 maintained under the same conditions. The number of culture dishes examined (each culture dish contained 5–10 explants and the average was taken from these explants) were (control/ IL-9) for the concentrations 0.002 (6/17), 0.02 (9/11), 0.5 (13/26), 1 (4/5) and 2  $\mu\text{g/ml}$  (4/10). White bars represent control data, black bars represent data in the presence of IL-9.

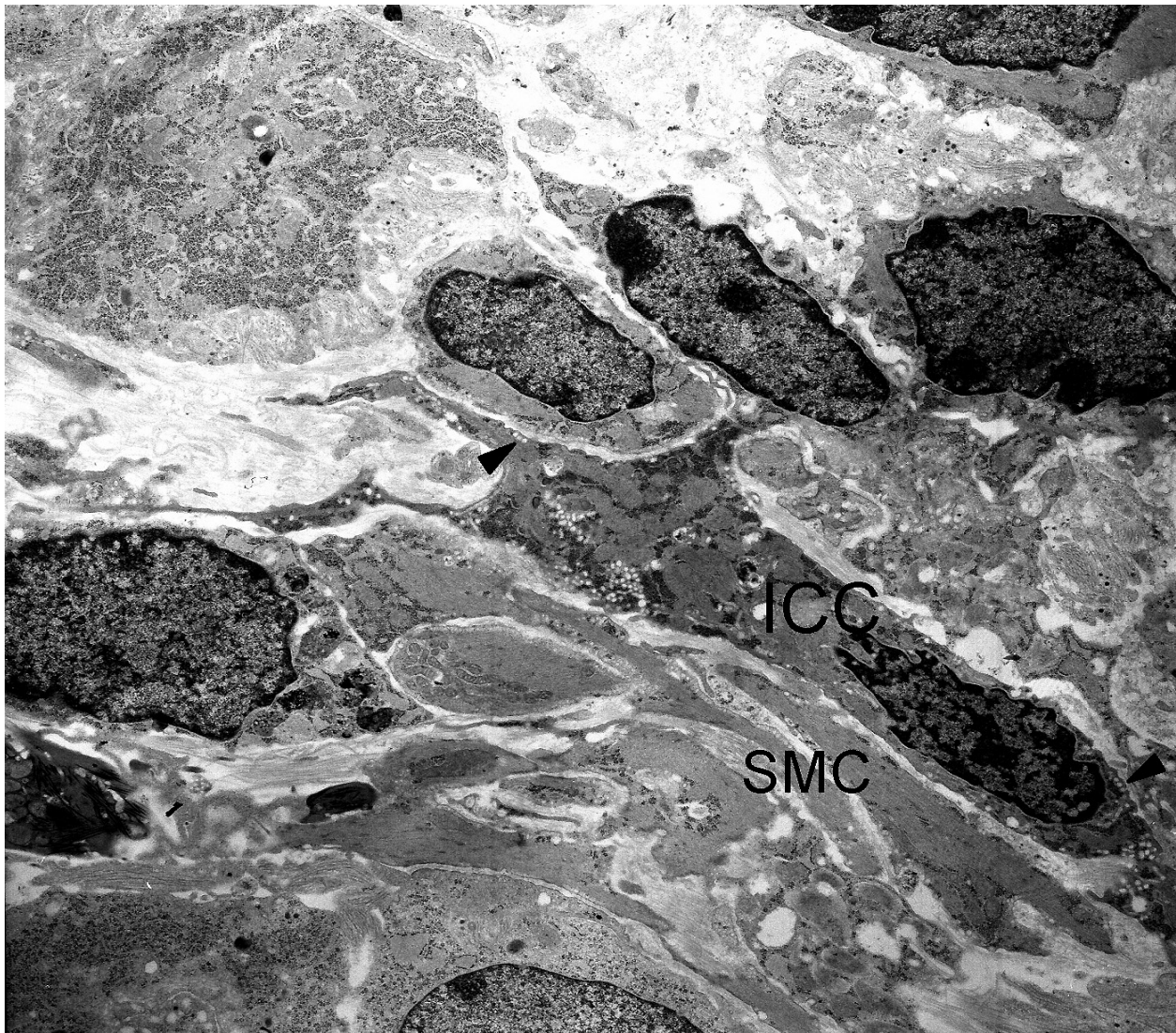


this difference is that Thuneberg and our laboratory keep cells in a very small volume with adequate cell density where presumable stem cell factor secreted from explants (muscle cells) can build up to a sufficient concentration for ICC to survive. Another growth factor for ICC survival is likely insulin since depletion of ICC networks over time in organ culture (murine gastric muscle) was prevented by

insulin or IGF-I [9]. The  $\text{TGF}\beta/\text{smad3}$  signaling pathway may also be involved in the development and differentiation of ICC [34]. Here we show that the addition of IL-9 to ICC in culture improves their survival and development. This is consistent with the combination of IL-9 and stem cell factor being a superior condition for the proliferation of mast cells [19]. Mast cells and small intestinal pacemaker ICC



**Fig. 3** Effect of IL-9 on ICC in culture within explants. ICC were identified by c-kit immuno-fluorescence (green) at day 4 of explant culture. All cells were stained with propidium iodide (red). **a, b**: controls; **c, d**: in the presence of IL-9 (0.5  $\mu\text{g/ml}$ ). Left panels in **a, b, c, d**: c-kit immuno-fluorescence (green) and propidium iodide (red). Right panels in **a, b, c, d**: c-kit immuno-fluorescence only.



**Fig. 4** Ultrastructure of ICC in presence of IL-9. ICC ultrastructure is maintained after 4 days in the presence of 0.5  $\mu\text{g/ml}$  IL-9. ICC displayed numerous mitochondria, endoplasmic reticulum and caveolae (arrowheads). Multiple branches were a hall mark of ICC. ICC were always in the neighbourhood of smooth muscle cells (SMC).

have growth requirements in common since both are absent from mice that have a mutation in the *c-kit* gene [1, 5, 11, 18, 37].

The mechanisms by which IL-9 promote ICC growth are not known. It is possible that IL-9 is a natural growth factor. This could be evaluated by examining IL-9 knockout mice. It is also possible that IL-9 promotes mast cell development in our cultures and through that mechanism promotes ICC survival. However, mast cells were rarely encountered. The influence of IL-9 on frequency and contraction could be due to IL-9 maintaining or inducing ion channels

in ICC that strengthen pacemaking. IL-9 induces the calcium activated chloride channel *mCLCA3* in epithelial cells in mice and increased expression of IL-9 [40] and *hCLCA1* in human epithelial cells from asthmatic patients [31]. Chloride channels take part in ICC pacemaking [13, 41] although the identity of the channels has not been clarified; the possibility exists that the CLCA1 protein is involved [16]. In the present study, IL-9 in the incubation medium is shown to be beneficial for ICC survival and maintenance of rhythmic contractile activity of the explants and isolated ICC. At this moment it cannot be stated

that routine addition of IL-9 is recommended when the purpose of the study is to evaluate normal properties of ICC. IL-9 may induce changes in ICC that may not be part of normal ICC but might be part of normal repair processes.

Data on murine ICC in short term culture cannot be extrapolated to human ICC *in vivo*. However, the current data further develop a hypothesis derived from a recent study on ICC development during a disease process. The hypothesis is that IL-9, secreted from mast cells, is one of the factors that promote ICC survival in human disease. We observed recently in esophageal tissue from patients with achalasia that there is a linear relationship between the number of surviving ICC and the number of mast cells that accumulate in the musculature [39]; piecemeal degranulation of mast cells making contact with ICC was abundant. Since mast cells can secrete IL-9 and stem cell factor [38] a positive influence of mast cells on ICC survival and/or repair seems possible. Infection with *T. spiralis* is well characterized as an inducer of mastocytosis within the small intestine, and expulsion of the parasite from the intestine is dependent on mast cells [32], in particular through the secretion of IL-9 [20]. ICC in this model are severely injured but show repair within days [3, 35, 36] possibly related to secretion of IL-9 by mast cells and other immune cells. Hence worm expulsion in a nematode infection may be facilitated by IL-9 through markedly increased epithelial secretion [20], muscle hypercontractility [14] and maintenance and repair of ICC. In asthma, hyper-responsiveness appears to be associated with high levels of IL-9. A highly significant difference in the expression of IL-9 mRNA and protein was detected in the airways of asthmatic subjects compared with those of all other groups. Both the degree of airflow obstruction and airway responsiveness to metacholine were significantly correlated with IL-9 mRNA expression levels [27]. Interestingly, IL-9 receptors are expressed on smooth muscle cells from asthmatics but not healthy individuals [7]. Although not investigated, it is possible that during inflammatory conditions ICC will express the IL-9 receptor as well.

In summary, IL-9 is a growth promoting factor for ICC, possibly relevant under normal condition, likely relevant under pathological conditions. Interaction between immune cells that secrete IL-9 and ICC may be an important factor for maintenance of ICC in motor disorders of the gut.

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