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Enteric Glial Cells have specific immunosuppressive properties

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Abstract

Enteric glial cells (EGC) have trophic and neuroregulatory functions in the enteric nervous system, but whether they exert a direct effect on immune cells is unknown. Here, we used co-cultures to show that human EGC can inhibit the proliferation of activated T lymphocytes. Interestingly, EGC from Crohn's patients were effective at one EGC for two T cells whereas EGC from control patients required a ratio of 1:1. These data suggest that EGC contribute to local immune homeostasis in the gastrointestinal wall. They also raise the possibility that EGC have particular immunosuppressive properties in inflammatory bowel diseases such as Crohn's disease.

Keywords : Enteric Glial Cells, Immunoregulation, Inflammatory Bowel Diseases, T Cell.

Abbreviations :

PBMC, Peripheral Blood Mononuclear Cells; EGC, Enteric Glial Cells; ENS, Enteric Nervous System; PHA, Phytohaemagglutinin; CFSE, Carboxyfluorescein Succinimidyl Ester; CD, Cluster of Differentiation; FCS, Fetal Calf Serum.

INTRODUCTION

Enteric glial cells (EGC) are a particular class of glial cells that play a critical role in the function and homeostasis of the neurogastrointestinal tract (Gulbransen and Sharkey, 2012). Some are present in the lamina propria or associated with interganglionic nerve fiber tracts, but major populations are found in the myenteric and submucosal ganglia of the enteric nervous system (ENS). As central glial cells, intraganglionic EGC have neurotrophic functions and modulate neural transmission (Neunlist et al., 2013) but nothing is known about their impact on immune cells as reported for astrocytes and central neural stem cells (Bonnemain et al., 2012; Einstein et al., 2003; Gimsa et al., 2004; Meinel et al., 1994). Yet, control of the immune response is essential to ensure host defense without compromising gut integrity. Such a control is particularly critical in inflammatory bowel disorders as Crohn's disease characterized by intestinal chronic inflammation, abdominal cramping and persistent diarrhea (Baumgart and Sandborn, 2012). To investigate potential immunomodulatory properties of EGC, glial cells from human myenteric plexus were cocultured with activated T lymphocytes. Here, we show that hEGC inhibit T cell proliferation. Comparative analyses indicate a stronger immunosuppressive effect of EGC from patients with Crohn's disease as compared to control patients.

MATERIAL AND METHODS

Cell preparation

Patients undergoing colon resection (3 Cancer, 3 Crohn) gave their informed consent. The study was approved by the "Fédération des Biothèques" (biobank) of the University Hospital of Nantes, according to the guidelines of the French Ethics Committee for Research on Human, (no. DC-2008-402). Primary cultures of hEGC were prepared from the myenteric plexus of the patients and expanded (Soret et al., 2013). **As determined by immunocytochemistry, the cultures contained 94+/-3.4 % of GFAP⁺ cells and 89.8+/-4.6 % of S100b⁺ cells.** Blood cells were obtained by cytopheresis from healthy donors who gave their written informed consent (Etablissement Français du Sang, Nantes, France). Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences Europe GmbH, Milan, Italy), according to manufacturer's instructions.

Proliferation assay

The impact of hEGC on the proliferation of T cells was evaluated by incubating variable ratios of hEGCs (3-5 passages) with 10^5 PBMC labelled with 5 μ M of carboxyfluorescein-succinimidyl-ester (CFSE, Thermo Fisher Scientific, Massachusetts, USA) (Lyons and Parish,

1994). In some experiments, hEGC were replaced by 50% (vol/vol) of EGC-conditioned media (3 days). A ratio of one T cell for two hEGC was used to evaluate the allogeneic reaction. The proliferation assay was performed by plating hEGC in 96-well flat-bottomed culture plates. After one night, 10^5 PBMC stimulated or not with the phytohaemagglutinin (PHA, a lectin from the *Phaseolus vulgaris*, 5 $\mu\text{g}/\text{mL}$, Sigma-Aldrich, Missouri, USA), were added to hEGC. Cells were cocultured for 3 days in DMEM medium/ RPMI 1640 medium (1:1) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 5% inactivated FCS, 1% non-essential amino acids, 5 mM HEPES, 1 mM sodium pyruvate and 1 μM β -mercaptoethanol. Before flow cytometry analyzes, cells were stained with aqua Live/Dead reagent (Thermo Fisher Scientific) for 30 min on ice. To characterize T lymphocytes, cells were immunostained with HIT3a (CD3)-APC and RPA-T8 (CD8)-PECy7 (BD Pharmingen, San Diego, CA) for 30 min on ice. Analyses were performed using LSR II Flow cytometer (<http://www.bdbiosciences.com>) and data were analyzed with BD FACSDiva Software. The percentage of proliferating living (Aqua⁻) T lymphocytes (CD3⁺) was determined for each well. Six wells were tested for each cell density and the published results correspond to the mean values of six wells.

RESULTS

Human EGC inhibit the proliferation of activated T cells.

To characterize the properties of hEGC, primary cultures were prepared from the myenteric plexus of patients suffering from colorectal cancer (control EGC, away from the tumor) or Crohn's disease (Crohn EGC). Their effects on T cell proliferation were investigated by coculturing variable number of hEGC with 10^5 PHA-activated hPBMC. After three days, CFSE profiles were analyzed by flow cytometry (Fig. 1A) and the mean percentage of proliferating living (Aqua⁻) T lymphocytes (CD3⁺) was determined. As PBMC and EGC were collected from different individuals, the allogeneic response was controlled and no increase in the number of proliferating CD3⁺ cells was observed when incubating EGC with unstimulated PBMC (Fig. 1B and C). As expected, PBMC stimulation with PHA strongly stimulated the proliferation of CD3⁺ cells. Coculture of PBMC with small amount of EGC did not affect the rate of proliferation but a ratio of one control EGC for one stimulated T cell strongly inhibited T cell proliferation as illustrated by the decrease in the percentage of proliferating CD3⁺ cells (48.8 % decrease) (Fig. 1D). This inhibitory effect was also observed with Crohn EGC with an even more pronounced effect as only 1.1% of the CD3⁺ cells proliferated at a ratio of one Crohn EGC for one stimulated T cell (Fig.1 E).

Crohn EGC exert a stronger inhibition than control EGC.

To control the property differences between control and Crohn EGC, the results from three control and three Crohn's disease patients were averaged and statistically compared (Fig. 2A and B). The low percentage of proliferating CD3⁺ cells at a ratio of one EGC for one

stimulated T cell was confirmed with an inhibitory effect clearly lower for control EGC (64.1% decrease) as compared to Crohn EGC (95.8% decrease). Interestingly, only Crohn EGC inhibited significantly T cell proliferation at a ratio of one EGC for two stimulated T cells (82.2% decrease) (Fig. 2B). A tendency to a lower proliferation was observed at other ratios (1/5, 1/10) but the results were not statistically significant.

EGC inhibit the proliferation of both CD3⁺CD8⁻ and CD3⁺CD8⁺ lymphocytes.

To determine whether EGC had a selective effect on the CD8⁺ subpopulation, double immunostaining was performed. Flow cytometry analyses showed a reduced number of both proliferating CD3⁺CD8⁻ and CD3⁺CD8⁺ cells in the presence of EGC. A stronger inhibition of T cell proliferation was observed in all cases with Crohn EGC (Fig. 2C-F).

A moderate contribution of soluble factors

The contribution of soluble factors in the suppressive effect of EGC was determined by incubating PHA-activated T cells for three days with EGC-conditioned media. A low but significant inhibition of the percentage of CD3⁺ proliferating cells was observed with the supernatants from both control and Crohn EGC (Fig.3). Conditioned media from Crohn EGC had a more pronounced effect (19.7% decrease) than conditioned media from control EGC (13.5% decrease).

DISCUSSION

In the present paper, we provide the first evidence that human EGC may locally modulate immune responses by inhibiting the proliferation of activated T cells. These observations are in line with the demonstration that astrocytes and neural stem cells have immunosuppressive activity that can be important in case of inflammatory or immune reaction in the central nervous system (Bonnamain et al., 2012; Einstein et al., 2003; Gimsa et al., 2004; Meinel et al., 1994). The signal transduction pathways remain to be characterized but the modest inhibitory effects of EGC-conditioned media on T cell proliferation rather suggest the requirement of a direct cell-cell contact. **Several membrane-bound molecules are potential candidates to mediate this inhibitory effect** (Chen and Flies, 2013). **This includes PD-L1 as an immunomodulation of T cells was shown to be mediated by this ligand expressed by central glial cells** (Pittet et al., 2011). As inhibitory effects are observed on CD3⁺CD8⁻ and CD3⁺CD8⁺ cells, hEGC are most probably able to control the proliferation of CD8 and CD4 lymphocytes. This observation is important as both cell types are implicated in the intestinal immune homeostasis (Izcue et al., 2009).

Interestingly, EGC isolated from the myenteric plexus of Crohn's disease patients were more potent than EGC isolated from patients unaffected by this inflammatory bowel disease. This stronger immunosuppressive effect was not related to an increase in EGC reactivity since no difference in the expression of glial reactivity markers such as GFAP was observed in EGC derived from control or Crohn patients (Coquenlorge et al.; Pochard et al., 2015). On the other hand, the increased efficiency of Crohn EGC in inhibiting the proliferation of activated T cells could be a compensatory mechanism to alleviate the damages caused by the disruption of the glial network. Indeed, the decrease in GFAP and S100 immunoreactive cells observed in the intestinal track of Crohn patients was suggested to contribute to the inflammation and propagation of Crohn disease (Bush et al., 1998; Cornet et al., 2001). Difference in the properties of Crohn and control EGC has already been observed by Pochard et al., who showed that Crohn EGC were unable to reduce the permeability of the intestinal epithelial barrier (Pochard et al., 2015). Whether increased immunosuppressive activity of EGC is specific of Crohn's disease remains to be determined but we may hypothesize that such a change is an adaptive response to control the immune response around the neuronal ganglia in case of inflammatory bowel diseases.

CONCLUSION

By demonstrating that EGC have the ability to inhibit the proliferation of activated T cells, our present data suggest effective crosstalk between EGC and immune cells that can vary in pathophysiological conditions.

Acknowledgments

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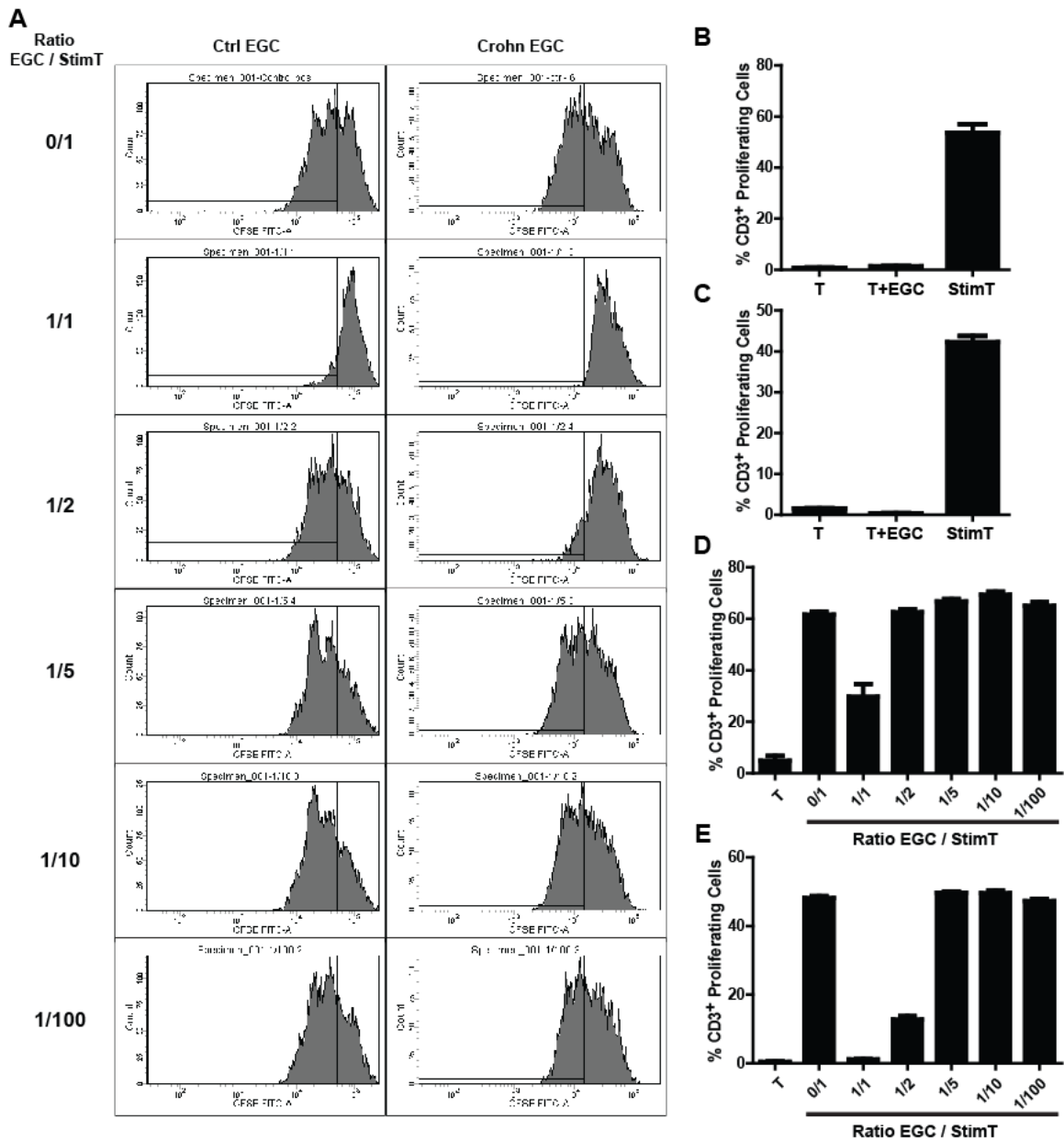


Figure 1. Impact of hEGC on T cell proliferation. EGC prepared from the myenteric plexus of one control (ctrl) or one Crohn's patient were plated in sextuplicate (six replicate wells per cell density) and cultured for three days with 10^5 CFSE-labeled PBMC stimulated (Stim T) or not (T) with PHA. CFSE profiles were analyzed by flow cytometry as shown in (A). The percentage of proliferating living (Aqua⁻) T lymphocytes (CD3⁺) was determined for each well and the mean was calculated for each cell density. The absence of allogeneic reaction was controlled by coculturing EGC with unstimulated T (B, C). Analyses were performed with various ratio of control (D) and Crohn (E) EGC. Data are expressed as mean values \pm SEM using the non-parametric Kruskal-Wallis followed by Dunns post-hoc tests; *, $p < .05$; **, $p < .001$; ***, $p < .0001$.

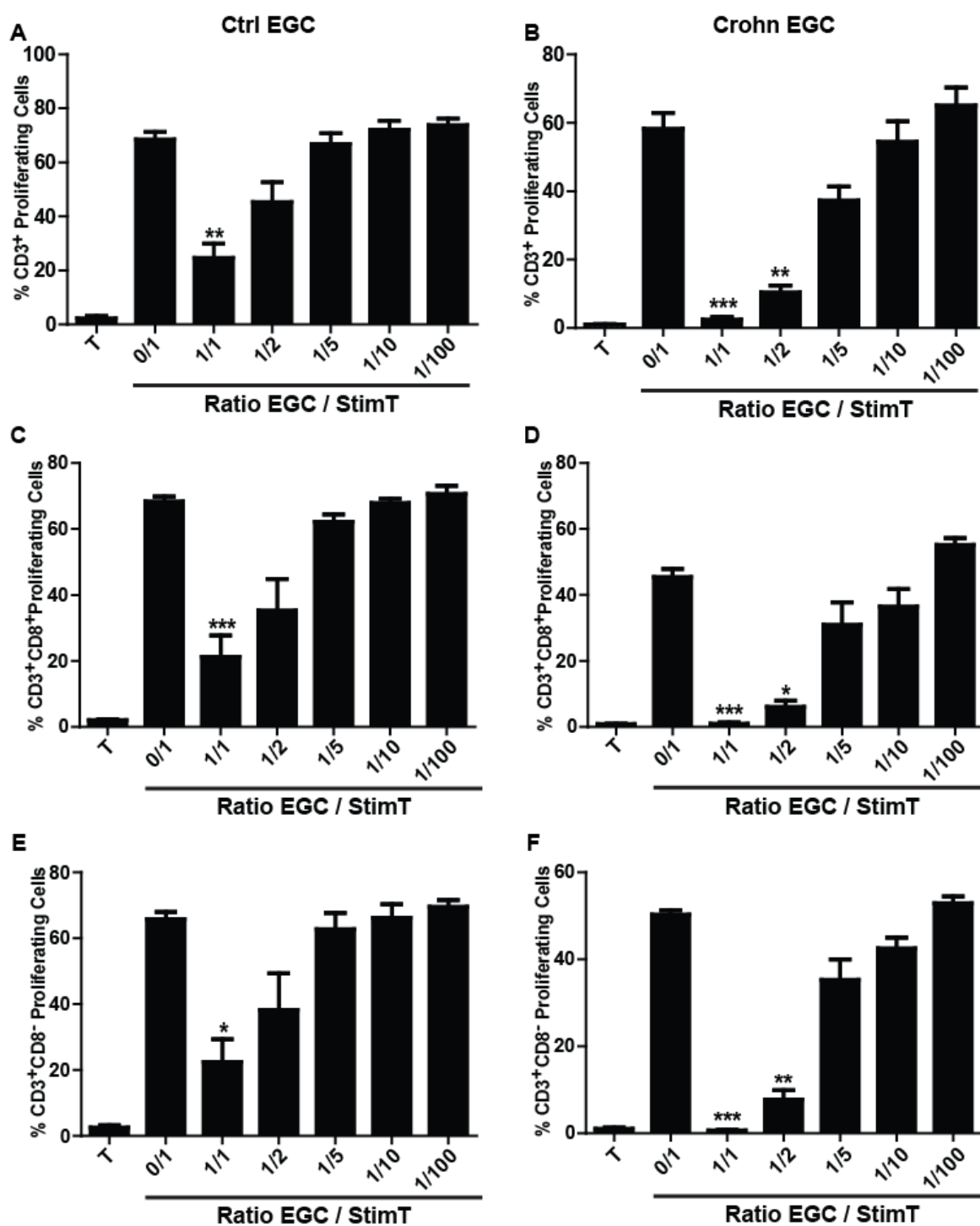


Figure 2. Impact of control and Crohn EGC on the proliferation of stimulated CD3⁺ (A, B), CD3⁺CD8⁺ (C, D) and CD3⁺CD8⁻ (E, F) T cells. The proliferation test was performed with EGC from three control patients (n = 3) and three patients with Crohn's disease (n = 3). Data are expressed as mean values ± SEM using the non-parametric Kruskal-Wallis followed by Dunns post-hoc tests; *, p < .05; **, p < .001; ***, p < .0001.

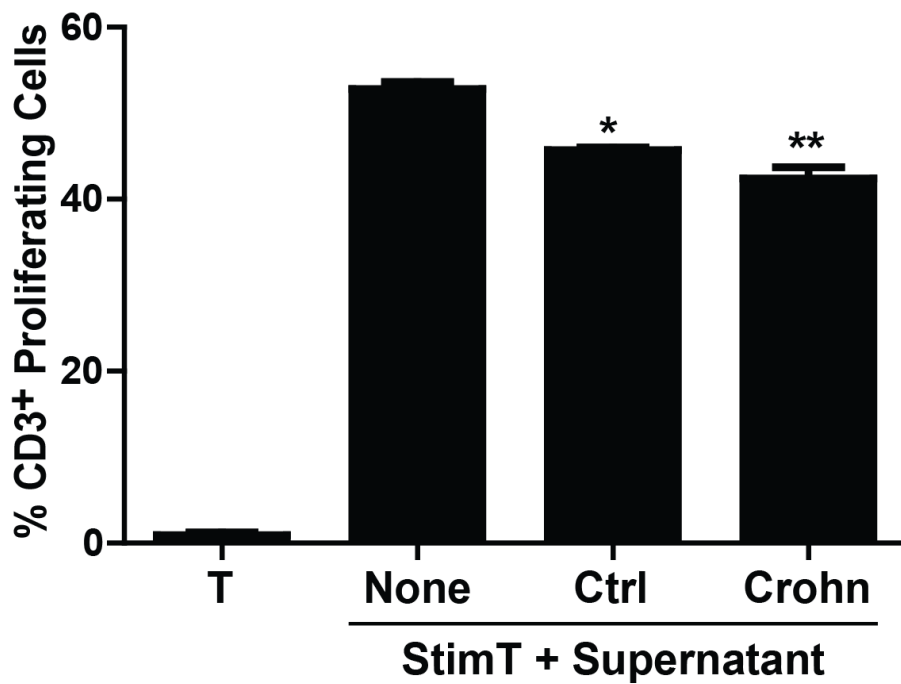


Figure 3. Impact of EGC-conditioned media on T cell proliferation. 10^5 CFSE-labeled PBMC stimulated (Stim T) or not (T) with PHA were incubated with conditioned media (vol/vol : 50/50) from cultures of control or Crohn EGC. Data are expressed as mean values \pm SEM using the non-parametric Kruskal-Wallis followed by Dunns post-hoc tests; *, $p < .05$; **, $p < .001$; ***, $p < .0001$.