1   **Ultrastructural changes in colonic epithelial cells in a rat model of inflammatory**

2   **bowel disease**

3

4   **Running title:** Ultrastructural changes in IBD-model rat (40/40 characters)

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29

30 **ABSTRACT**

31 Inflammatory bowel disease (IBD) is a global, chronic intractable disease. The functions

32 of drugs and food components have been evaluated in models of IBD induced by 2,4,6-

33 trinitrobenzene sulfonic acid (TNBS). Here, we used transmission (TEM) and osmium-

34 maceration scanning (SEM) electron microscopy to evaluate the ultrastructure of colonic

35 epithelial cells in rat models of IBD induced by TNBS. Histological evaluation revealed

36 that the intestinal crypts in the most regions of the IBD-model colons were deformed and

37 we classified them as having high cell migration rates (HMIG). The remaining regions in

38 the intestinal crypts retained a relatively normal structure and we classified them as

39 having low cell migration rates (LMIG). Osmium-maceration SEM revealed the mucosal

40 fluid flowing in spaces without secretory granules in crypt goblet cells of both HMIG and

41 LMIG regions, indicating the depletion of goblet cell mucin that is found in patients with

42 IBD. The Golgi apparatus in absorptive cells was stacked and curled in both regions.

43 Osmium-maceration SEM showed membrane network structures resembling

44 endoplasmic reticulum that were large and expanded in absorptive cells with HMIG rather

45 than with LMIG regions in IBD-model colons. These findings indicated that endoplasmic

46 reticulum stress is associated with susceptibility to IBD and that the effects of various

47 agents can be evaluated according to endoplasmic reticulum stress revealed by using

48 electron microscopy in models of IBD induced by TNBS. (224/250 words)

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53

RESEARCH HIGHLIGHTS

- ✓ Secretory granules are depleted in colonic crypt goblet cells of rat models of IBD.

- ✓ TNBS induces ER stress-like structures in colonic epithelial cells.

- ✓ Golgi apparatus is deformed in colonic epithelial cells.

(219/250 characters)

1. INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, intractable and costly disease with a prevalence of > 0.3 % in North America, Oceania and most countries in Europe (Ng et al., 2018). Furthermore, the incidence of IBD has increased in Asia, Africa and South America (Ng et al., 2018), indicating a global need for IBD prevention and treatment. Pathological assessment has shown that immune cells migrate into the colonic tissues and that crypt goblet cells are depleted of secretory granules in patients with IBD (Riddle, 2004). The mechanism of IBD onset is associated with endoplasmic reticulum (ER) stress of the intestinal epithelial cells (Ma et al., 2017), and it has been suggested that oral administration of substances depressing ER stress dramatically alleviated inflammatory process of IBD induced by dextran sodium sulfate (Cao et al., 2013).

Animal models are produced by introducing 2,4,6-trinitrobenzene sulfonic acid (TNBS) into the colon, where it induces colonic lesions like those of human IBD (Talapka et al., 2014; Morampudi et al., 2014). The functions of drugs and food components associated with IBD inhibition have been evaluated in such models (Pfeiffer, Sato, Qiu, Keith, & Evangelista, 1997; Valcheva-Kuzmanova, Kuzmanov, Kuzmanova, & Tzaneva, 2018). Although TNBS-induced colonic lesions have mostly been evaluated histologically, more detail ultrastructural evaluation of IBD models is required to understand the mechanism of IBD inhibition. Ultrastructural changes in colonic epithelial cells induced by TNBS have been described (Pfeiffer et al., 1997; Tian, Huang, Tian, Gao, & Chang, 2003; Bou-Fersen, Anim, & Khan, 2008). However, most of these were analyzed by using only transmission electron microscopy (TEM).

Osmium-maceration scanning electron microscopy (SEM) allows visualization of three-dimensional ultrastructural architectures after removing soluble protein (Tanaka &

84 Naguro, 1981; Bochimoto et al., 2017; Koga, Bochimoto, Kusumi, Ushiki, & Watanabe,

85 2017). The present study compared colonic epithelial cells in rat models of IBD induced

86 by TNBS and control rats using TEM and osmium-maceration SEM to identified detailed

87 ultrastructural changes.

88

89 **2. MATERIALS AND METHODS**

90 **2.1. Animals**

91 Twelve seven-week-old male Fischer 344 rats (Charles River Laboratories International,

92 Inc., Yokohama, Japan) were used in this study: six were administrated TNBS and six

93 saline. All of these rats were examined by histological and osmium-maceration SEM

94 analyses, and, among these, two rats for each group were also processed for TEM analysis.

95 This study proceeded according to the Regulations on the Management and Operation of

96 Animal Experiments, and the Animal Care and Use Committee of Obihiro University of

97 Agriculture and Veterinary Medicine approved the experimental protocol (Approval

98 number 18-86).

99

100 **2.2. Preparation of rat models of IBD**

101 The rat bowels were emptied by injecting 0.5 mL of 10% glycerin into the anus, then

102 either 0.25 mL of 50% ethanol containing TNBS (120 mg/mL) or sterilized saline was

103 delivered to the colon 45 min later by advancing the tip of a polypropylene catheter

104 (diameter 1.5 mm) to a point 80 mm from the anus. During TNBS treatment, the rats were

105 kept in a head-down position for 1 min under anesthetic condition by using an

106 intraperitoneal injection of pentobarbital (50 mg/kg body weight). Ten days later, the rats

107 were anesthetized by pentobarbital and then euthanized by cervical dislocation. The colon

108   near the site of administration was excised and trimmed into small blocks that were fixed

109   in 10% neutral formalin for histological procedures, or in 0.1 M phosphate buffer (pH

110   7.4) containing 0.5% paraformaldehyde and 0.5% glutaraldehyde for ultrastructural

111   analyses.

112

113   **2.3. Histological procedures**

114   Specimens embedded in paraffin using standard procedures were sliced into 5-μm-thick

115   sections and stained with hematoxylin-eosin to evaluate tissue injury.

116

117   **2.4. Transmission electron microscopy (TEM)**

118   Colon blocks were post-fixed with 1% OsO_4 for 30 min, dehydrated and then embedded

119   in LR White resin. Ultrathin sections (80 nm thick) were cut using a diamond knife and

120   examined using an HT7700 transmission electron microscope (Hitachi, Tokyo, Japan)

121   without uranyl acetate and lead citrate staining. Semi-thin sections (1 μm thick) were also

122   cut and stained with toluidine blue to confirm the degree of tissue injury in the specimens.

123

124   **2.5. Osmium-maceration scanning electron microscopy (SEM)**

125   Osmium maceration was applied as described (Bochimoto et al., 2017). Briefly, colon

126   blocks were immersed in 1% OsO_4 for six hours followed by 25% and 50% dimethyl

127   sulfoxide, frozen with a flat aluminum block precooled in liquid nitrogen, and broken into

128   two pieces using a screwdriver and a hammer. The specimens were immersed in 0.1%

129   OsO_4 for 96 h at 20°C, post-fixed in 1% OsO_4, stained with tannic acid and 1% OsO_4,

130   dehydrated and lyophilized in an ES2030 freeze-dryer (Hitachi) with t-butyl alcohol. The

131   specimens were then mounted onto a metal plate, lightly coated with platinum-palladium

132 in an E1010 ion sputter coater (Hitachi) and evaluated using an S4100 scanning electron

133 microscope (Hitachi).

134

135 **3. RESULTS**

136 **3.1. Histological findings**

137 Macroscopically broad inflammation was evident in the colons of the IBD rats (Figure

138 1A). Although the intestinal crypts in most regions of these colons were deformed or

139 absent (Figure 1D), the remaining regions retained the relatively normal structure of the

140 intestinal crypts found in the control colons (Figure 1B, C). The regions with the

141 deformed crypts contained many immune cells and were classified as having high

142 migration rates (HMIG). On the other hand, the number of immune cells in the regions

143 with the relatively normal crypts was comparable to that of controls, and such regions

144 were classified as having low migration rates (LMIG).

145

146 **3.2. Ultrastructural findings in crypt goblet cells**

147 Crypt goblet cells in control colons contained many small secretory granules (Figure 2A,

148 D), whereas most of those in the HMIG and LMIG regions of IBD colons contained a

149 large vacuolar structure (Figure 2B, C). Osmium-maceration SEM revealed that mucosal

150 fluid covering the surface of the epithelium flowed into spaces without secretory granules

151 to form large vacuolar structures in IBD colons (Figure 2E).

152

153 **3.3. Ultrastructural findings of absorptive epithelial cells**

154 Absorptive epithelial cells in control colons contained flat Golgi apparatus in perinuclear

155 regions (Figure 3A, D), whereas those in the HMIG region of IBD colons contained

156   stacked and curled Golgi apparatus (Figure 3C). Some Golgi apparatus in absorptive

157   epithelial cells in the LMIG region were also stacked and curled (Figure 3B, E). Analysis

158   using TEM revealed small vesicular structures in the apical region of epithelial cells.

159   These vesicles were respectively abundant and moderately abundant in the HMIG and

160   LMIG regions of IBD colons (Figure 4B, C), whereas control colons contained only a

161   few vesicles (Figure 4A). Osmium-maceration SEM induces precipitation of soluble

162   proteins revealing cellular membrane compartments where apical cytosol was removed

163   in the absorptive epithelial cells of control colons (Figure 4D). In IBD colons the apical

164   cytosol presented membrane network structures comprising many tubules (Figure 4E, F).

165   The membrane structures were large and expanded in the HMIG region (Figure 4F).

166

167   **4. DISCUSSION**

168   The present study used TEM and osmium-maceration SEM to analyze the colons of rat

169   model of IBD induced by TNBS, and found three types of ultrastructural changes in

170   colonic epithelial cells of the models. Because colons with IBD are pathologically

171   inflamed, the LMIG region of IBD colons did not represent typical IBD lesions. However,

172   ultrastructural changes in colonic epithelial cells of the LMIG and HMIG regions were

173   more or less similar, and thus whether these changes are meaningful as models of IBD

174   should be addressed.

175   The present findings indicated that the depletion of crypt goblet cell mucin occurs

176   in IBD colons like in that of patients with IBD (Riddle, 2004). Although whether large

177   vacuolar structures in IBD colons are fusions or depleted spaces of secretory granules

178   was not evaluated by using only TEM analysis, osmium-maceration SEM significantly

179   revealed that it was the result of depletion of the granules. Because goblet cells in

180   intestinal crypts release mucin in response to endogenous secretagogues associated with

181   some neurotransmitters (Phillips, Phillips, & Neutra, 1984), these cells in the LMIG

182   region could also indirectly receive the inflammatory stimuli via enteric nerves and secret

183   the granules.

184   The absorptive epithelial cells of IBD colons contained small vesicular structures

185   in the apical region according to TEM analysis. Osmium-maceration SEM revealed that

186   these vesicles were a part of membrane network structures that were defined as ER based

187   on the morphological features. The ER-like structures were larger and expanded in the

188   HMIG region, indicating that ER stress with the expansion is induced by TNBS in colonic

189   epithelial cells, especially in severe inflammatory lesions, of rat models of IBD.

190   Endoplasmic reticulum stress is a critical factor associated with susceptibility to IBD (Ma

191   et al., 2018), and the present ultrastructural findings of epithelial cells seem useful and

192   important in term of using models of IBD induced by TNBS to evaluate the effects of

193   agents that will be used to treat IBD. The Golgi apparatus was deformed in both the HMIG

194   and LMIG regions of IBD colons. However, the degree of Golgi apparatus deformation

195   was similar between the HMIG and LMIG, and deformed Golgi apparatus is not a general

196   pathology of IBD. Therefore, it is suggested that deformation of Golgi apparatus is not

197   caused by inflammatory responses, and the structural changes seems caused by

198   cytotoxicity of TNBS itself with no relation to IBD.

199   Morphological changes among organelles in epithelial cells induced by TNBS have

200   been described. Pfeiffer et al. (1997) have also identified large amounts of vesicular

201   structures in the apical regions of absorptive epithelial cells induced by TNBS. Bou-

202   Fersen et al. (2008) described deformations of the ER and Golgi apparatus, as well as

203   swollen mitochondria in colons administrated with TNBS. Tian et al. (2003) also

204 described that TNBS induces mitochondrial swelling in colonic epithelial cells. The

205 present osmium-maceration SEM analysis revealed that the area mitochondria occupied

206 is lesser in IBD-model colonic epithelial cells (Figure 4D–F), suggesting the possibility

207 that the activity of mitochondria in colonic epithelial cells is associated with inducing

208 IBD. However, osmium-maceration SEM did not identify significant morphological

209 changes of mitochondria (Figure 4D–F), and thus the ultrastructural effects of various

210 agents should be evaluated using various means.

211

212 **5. CONCLUSION**

213 The functions of drugs and food components for treating IBD are considered to be

214 correctly determined from ultrastructural findings of the colons of animal models of IBD.

215 The present study found that some colonic ultrastructural changes induced by TNBS

216 seemed to be associated with inflammatory processes, whereas others seemed to reflect

217 only TNBS cytotoxicity and were not associated with inflammation. These findings

218 indicate that ultrastructural changes in animal models of IBD induced by TNBS can serve

219 as useful indexes, but whether they are significant in the context of IBD should be

220 carefully evaluated.

221

227 **AUTHORS' CONTRIBUTIONS**

228 Conceptualization: HB.

229 Investigation: HB, DK, RN, YI, JT, KHH, SS.

230 Supervision: NK, MF.

231 Writing – original draft: HB, DK.

232 Writing – review & editing: HB, DK, RN, YI, JT, KHH, SS, MS, NK, MF.

233

234 **CONFLICTS OF INTEREST**

235 The authors have no conflicts of interest to declare.

236

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283

284 **FIGURE LEGENDS**

285 **Figure 1.** Macroscopic and histological features of control and IBD colons.

286 Mucous membranes of colons (**A**) in control (upper) and IBD (lower) rats. IBD and

287 control rats were injected with TNBS or saline at 80 mm from anus. Hematoxylin-eosin

288 stain (**B–D**) of control (**B**) and of low (LMIG; **C**) and high (HMIG; **D**) migration regions

289 of IBD colons. Asterisk and arrowhead in (**D**) indicate deformed intestinal crypts and area

290 without epithelium, respectively. Bars = 10 mm (**A**) and 100 (**B, C**) and 50 (**D**) μm.

291

292 **Figure 2.** Ultrastructural features of crypt goblet cells in control and IBD colons.

293 Transmission electron microscopy (TEM; **A–C**) and osmium-maceration scanning

294 electron microscopy (SEM; **D, E**) findings of control (**A, D**), LMIG (**B, E**) and HMIG

295 (**C**). Abbreviations: sg, secretory granules; v, vacuolar structure. *Lumen (**A–C**). Red

296 highlights, mucous fluid covering epithelial surface; green highlights, secretory granules

297 in (**D, E**). Bars = 2 μm.

298

299 **Figure 3.** Ultrastructural features of perinuclear region of absorption epithelial cells of

300 control and IBD colons.

301 Findings of TEM (**A–C**) and osmium-maceration SEM (**D, E**) of control (**A, D**), LMIG

302 (**B, E**) and HMIG (**C**). Arrowheads (**A–C**) indicate Golgi apparatus. Red and blue

303 highlights (**D, E**) indicate Golgi apparatus and nuclei, respectively. Bars = 1 μm.

304

305 **Figure 4.** Ultrastructural features of apical region of absorption epithelial cells in control

306 and IBD colons.

307 Findings of TEM (**A–C**) and osmium-maceration SEM (**D–F**) of control (**A, D**), LMIG

308   (**B, E**) and HMIG (**C, F**). Arrowheads (**A–C**) indicate small vesicular structures. Red and

309   green highlights (**D–F**) indicate membrane network structures and mitochondria,

310   respectively. Abbreviations: mv, microvilli; *Space without cytosol (**D–F**). Bars = 1 μm.

311

Figure S1. Distribution of immune cells and polysaccharide in control and model colons. Anti-Iba1 (Code 019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan) immunostain (**A–C**) and toluidine blue stain (**D–F**) to detect macrophages and mast cells, respectively, in control (**A, D**), LMIG (**B, E**) and HMIG (**C, F**). Arrowheads (**A–C**) indicate Iba1-positive macrophages; arrowheads (**D–F**) indicate metachromatic mast cells. Periodic acid-Schiff reaction (**G–I**) and Alcian blue (pH 2.5) reaction (**J–L**) to detect neutral and acidic polysaccharide, respectively. Inserts in (**I, L**) show high magnification of intestinal crypts. Bars = 100 (**A, B, G, H, J, K**), 50 (**C, I, L**), 20 (**D–F**) μm.
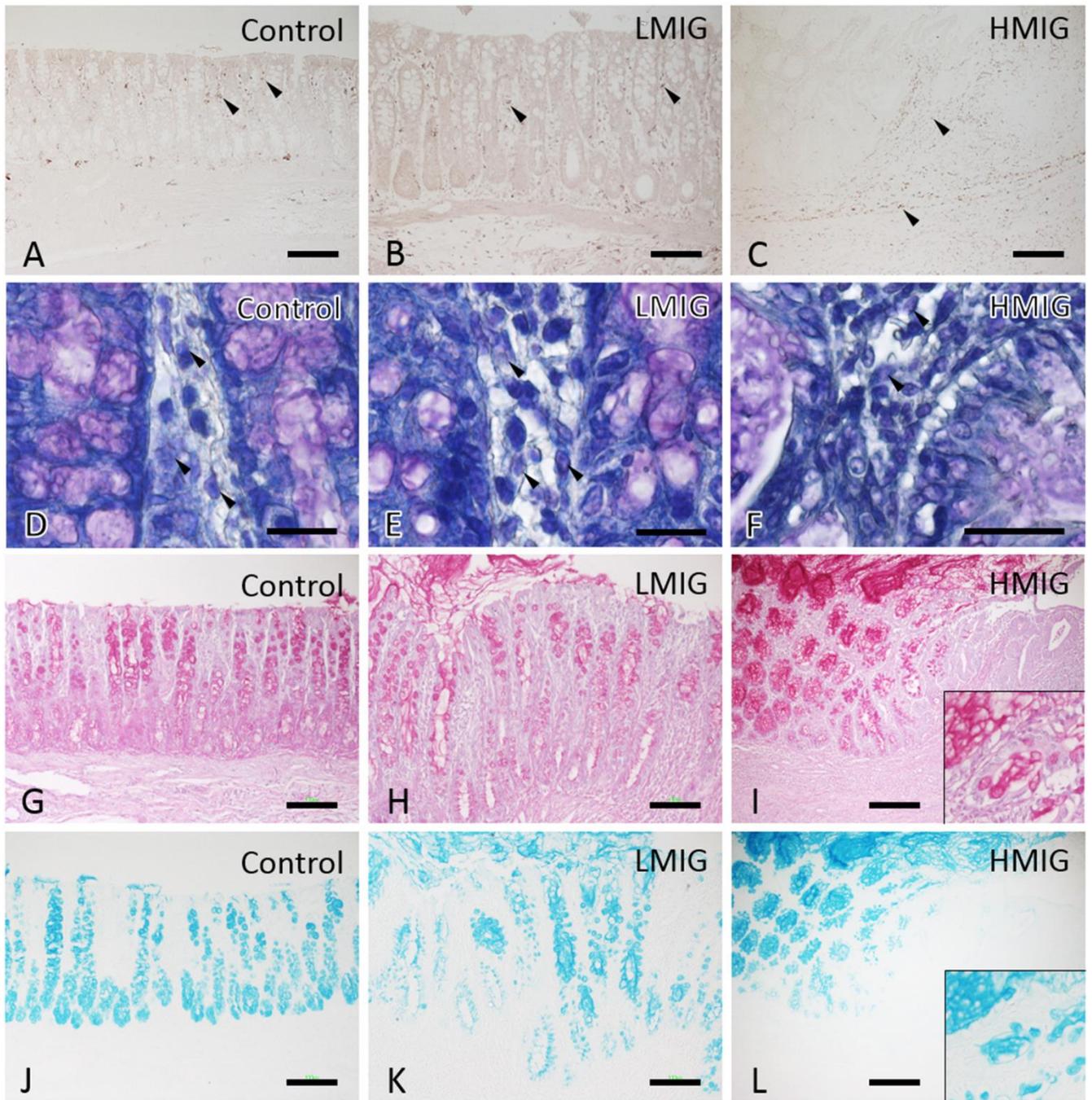
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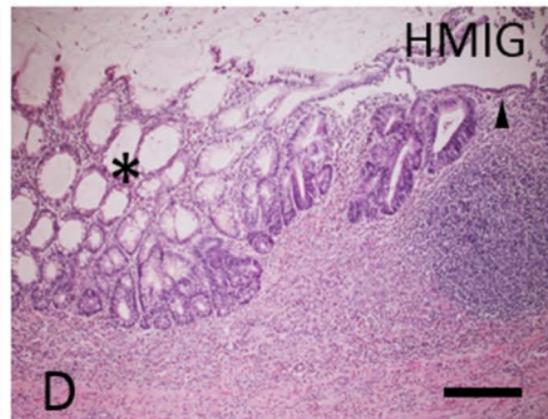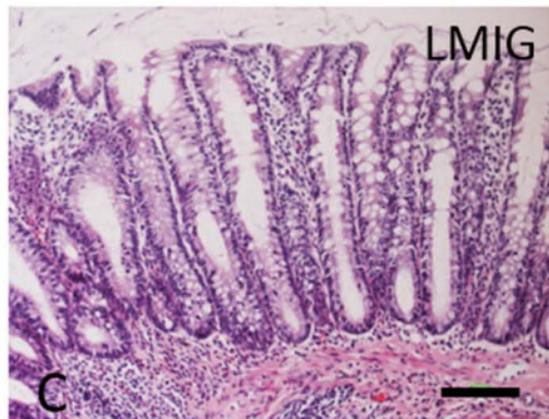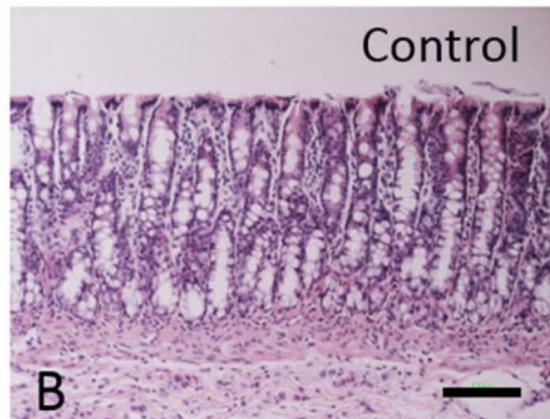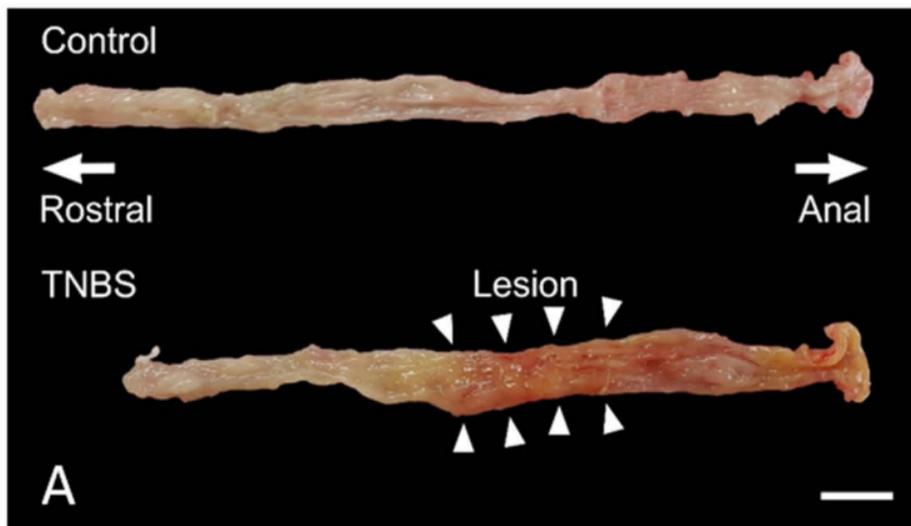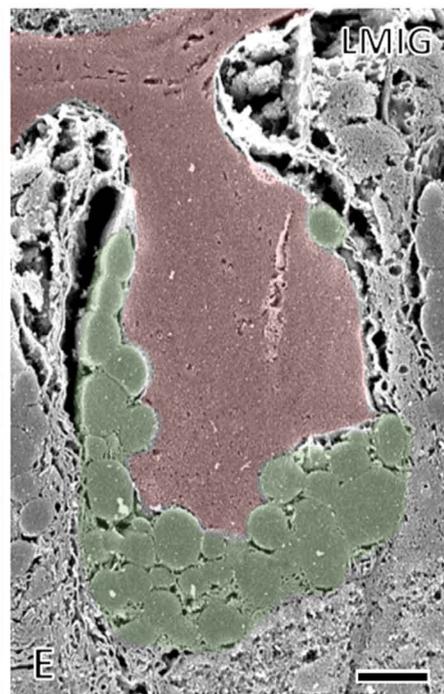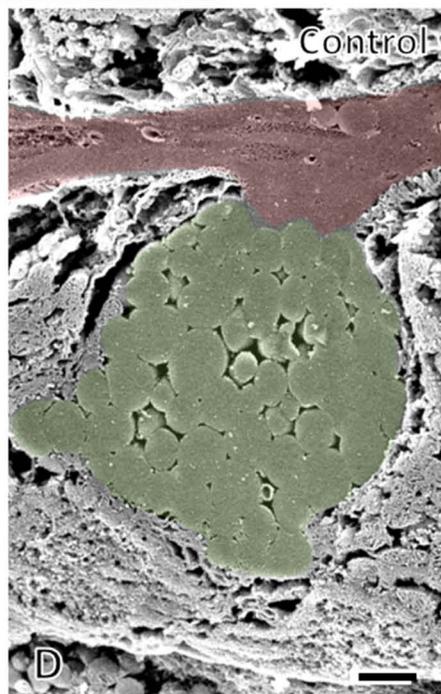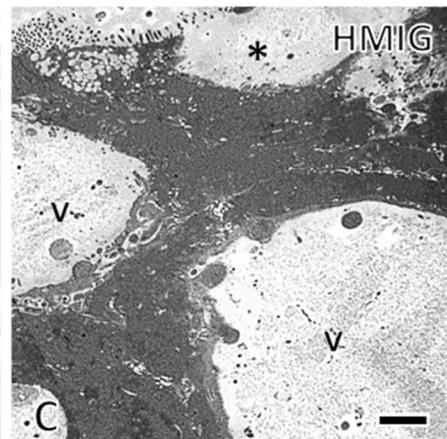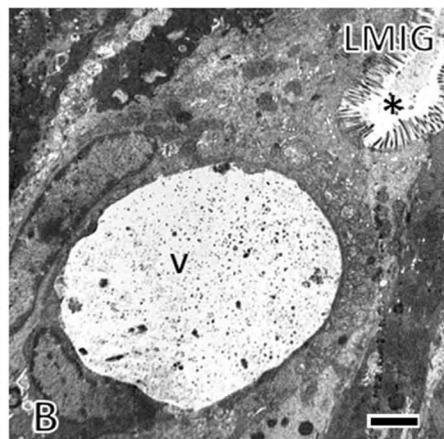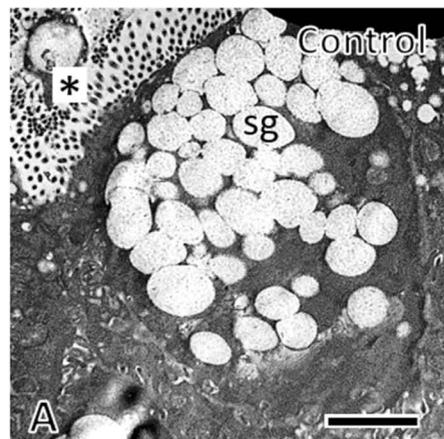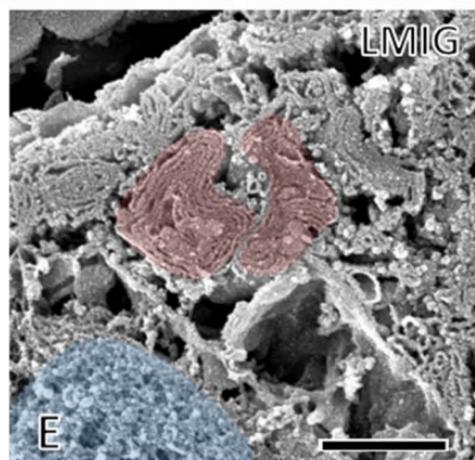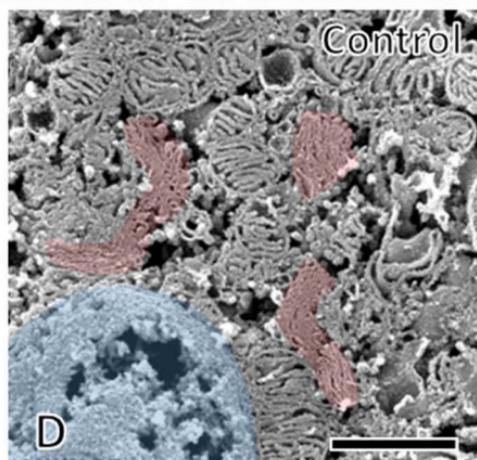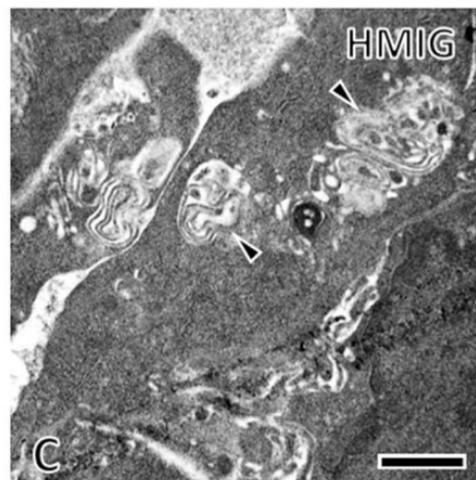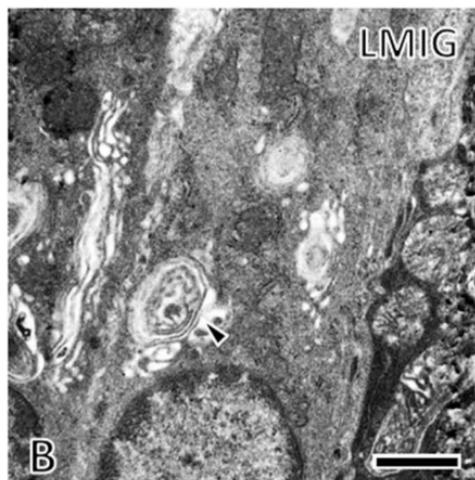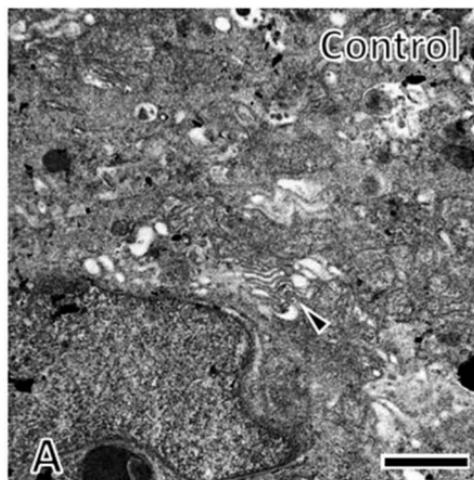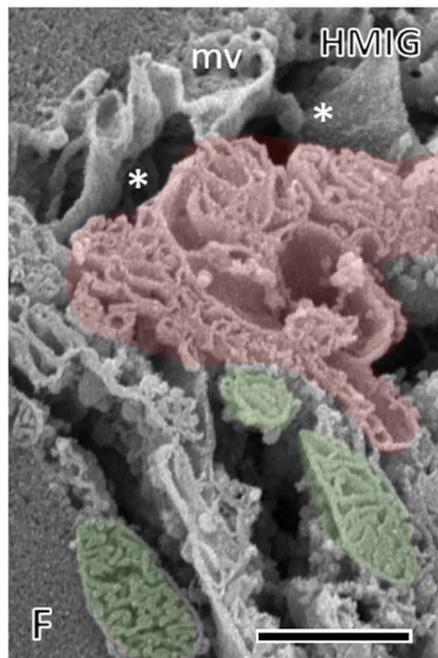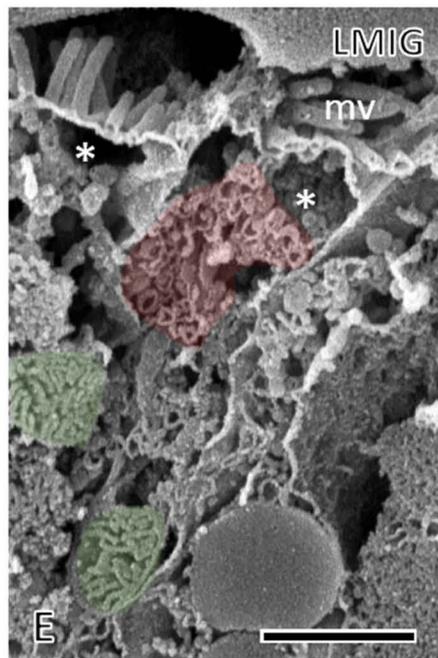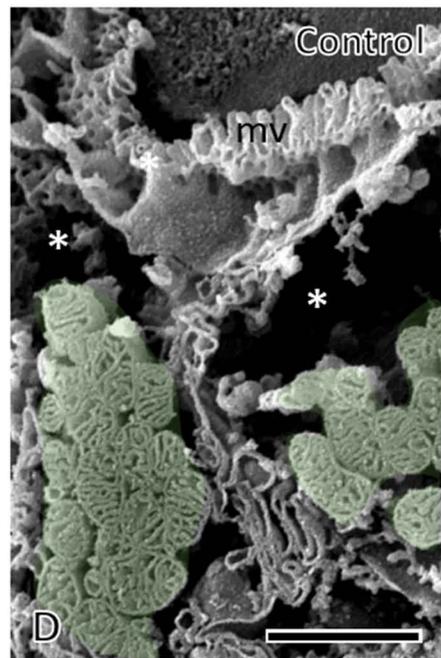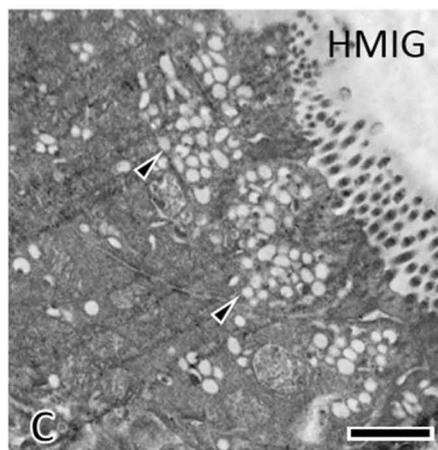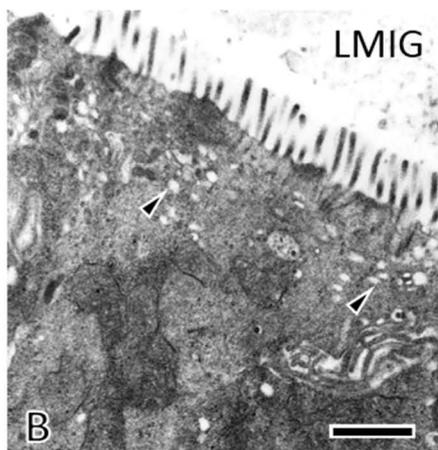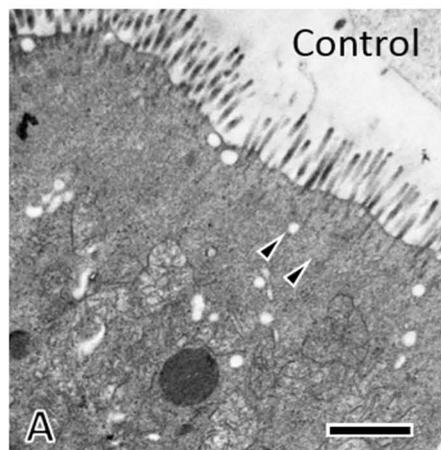
Control rats

Models of IBD induced by TNBS

Lesion

Secretory granules

Cell migration

ER stress

Mucous fluid