FLORE
Repository istituzionale dell'Università degli Studi di Firenze

Age-associated modifications of intestinal permeability and innate immunity in human small intestine

Questa è la versione Preprint (Submitted version) della seguente pubblicazione:

Original Citation:

Age-associated modifications of intestinal permeability and innate immunity in human small intestine / Man, Angela L.; Bertelli, Eugenio; Rentini, Silvia; Regoli, Mari; Briars, Graham; Marini, Mario; Watson, Alastair J.M.; Nicoletti, Claudio. - In: CLINICAL SCIENCE. - ISSN 0143-5221. - STAMPA. - 129:(2015), pp. 515-527. [10.1042/CS20150046]

Availability:

The webpage https://hdl.handle.net/2158/1093597 of the repository was last updated on 2020-10-23T12:25:39Z

Published version:

DOI: 10.1042/CS20150046

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf)

Publisher copyright claim:

La data sopra indicata si riferisce all'ultimo aggiornamento della scheda del Repository FloRe - The above-mentioned date refers to the last update of the record in the Institutional Repository FloRe

(Article begins on next page)

02 May 2026

Age-associated modifications of intestinal permeability and innate immunity in human small intestine

Angela L. Man[1]* Eugenio Bertelli[2]*, Silvia Rentini[3], Mari Regoli[2], Graham Briars[4], Mario Marini[3], Alastair J. M. Watson[1, 5] and Claudio Nicoletti[1],

[1]Gut Health and Food Safety Program, Institute of Food Research, Norwich, United Kingdom; [2]Dept. Life Sciences, Univ. of Siena, Italy; [3]C.O.U. Gastroenterology and Digestive Endoscopy, A.U.O.S. University Hospital, Siena; Italy; [4]Dept of Paediatric Gastroenterology, Norfolk and Norwich Univ. Hospital, Norwich, UK; [5]Norwich Medical School, Univ. of East Anglia, Norwich, UK

Key words: Ageing, Intestine, Innate immunity, Interleukin 6, Intestinal permeability, Infection

Short title: Effects of ageing on the human gut

Correspondence to:
Dr. Claudio Nicoletti
Gut Health and Food Safety Institute Strategic Program
Institute of Food Research
Colney, Norwich NR4 7UA
United Kingdom
Tel: +44-1603 255233
e-mail: claudio.nicoletti@ifr.ac.uk

*These authors equally contributed to this work

Abstract

The physical and immunological properties of the human intestinal epithelial barrier in ageing are largely unknown. Ileal biopsies from young (7-12 years), adult (20-40y) and ageing (67-77y) individuals not showing symptoms of gastrointestinal pathologies were used to assess levels of inflammatory cytokines, barrier integrity, and cytokine production in response to microbial challenges. Increased expression of IL-6, but not IFNγ, TNF-α and IL-1β was observed during ageing; further analysis showed that $CD11c^+$ dendritic cells (DCs) are one of the major sources of IL-6 in the ageing gut and expressed higher levels of CD40. Up-regulated production of IL-6 was accompanied by increased expression of Claudin-2 leading to reduced transepithelial electric resistance (TEER); TEER could be restored in *in vitro* and *ex-vivo* cultures by neutralizing anti-IL-6 antibody. In contrast, expression of Zonula occludens-1, Occludin and Junctional-Adhesion Molecule-A1 did not vary with age and overall permeability to macromolecules was not affected. Finally, cytokine production to different microbial stimuli was assessed in a polarized *in vitro* organ culture. IL-8 production in response to flagellin declined progressively with age although the expression and distribution of TLR-5 on intestinal epithelial cells remained unchanged. Also, flagellin-induced production of IL-6 was less pronounced in ageing. In contrast, TNF-α production in response to probiotics (VSL#3) did not decline with age; however, in our experimental model probiotics did not down-regulate the production of IL- 6 and expression of Claudin 2. These data suggested that ageing affects properties of the intestinal barrier likely to impact on age-associated disturbances both locally and systemically.

Introduction

A hallmark feature of ageing is immunosenescence and the functional decline of the adaptive and innate immune system resulting in compromised immunity to microbial pathogens and increased frequency of cancer [1, 2]. These are accompanied by an imbalance between inflammatory and anti-inflammatory networks, resulting in low-grade chronic inflammation termed inflammaging [3]; converging evidence led to suggest that events in the gastrointestinal (GI)-tract that involve interaction between the various components of the epithelial barrier and intestinal microbiota might play a central role in this process [4]. However, currently our knowledge on the effects of ageing on the physical and immunological properties of the intestinal epithelial barrier is very limited. Most importantly the lack of knowledge is particularly profound in humans [5]. The gut is the primary and largest area of contact with environmental factors and antigens and contains the largest number of immune cells in the body and the intestinal barrier is integral to GI-defence in preventing or limiting exposure of the host and its immune system to luminal antigens. It is made up of several integrated and interactive components that are physical (the epithelium and mucus), biochemical (enzymes, anti-microbial proteins), immunological (secretory IgA and epithelia-associated immune cells), and microbial (the microbiota) in nature [6]. Maintaining barrier integrity is therefore essential for health and defects in intestinal barrier function can lead to persistent immune activation and are known to contribute to the pathogenesis of intestinal diseases including coeliac disease, colorectal cancer and inflammatory bowel disease [6]. However, disturbances of the barrier of the GI-tract might have consequences far beyond the gut. This notion is highlighted by the recent observations that systemic disorders, ranging from diabetes [7] to major depression [8] and also degenerative disorders of the central nervous system (CNS) such as Parkinson's disease [9] and multiple sclerosis (MS) [10] might be linked to events occurring in the intestine. The importance of a well-functioning intestinal barrier is further stressed by studies in *Drosophila* demonstrating that impairment of intestinal barrier function predicted age-onset mortality [11]. Among the various components of the epithelial barrier the gut epithelium plays a pivotal role. Although the primary task of the epithelium is to provide a barrier against macromolecules and pathogens it also plays a key role in establishing and maintaining the intestinal immune homeostasis and responding rapidly to microbial exposure, thus making it a central element of the innate immune system. In a steady-state situation intestinal epithelial cells (IECs) secrete cytokines that control the immune intestinal homeostasis by inducing anti-inflammatory dendritic cells (DCs) and T regulatory cells [12, 13]. In contrast, the presence of pathogenic stimuli causes IECs to release rapidly pro-inflammatory factors, such as IL-8 (CXCL-8), MCP-1 (CCL2) and MIP− 3α (CCL20) [14, 15] that provide the first line of defence against invading microorganisms. Thus, age-associated changes of epithelial innate immunity might have profound effects on both local and systemic immune responses. The aim of this study was to investigate age-associated changes of levels of inflammatory/regulatory cytokines and their impact on epithelial barrier integrity in the small intestine (terminal ileum) and to assess the effect of ageing on intestinal epithelial immunity to different types of microbial stimuli.

.

Material and Methods

Subjects and biopsies

Terminal ileum biopsies (up to 8 biopsies/individual) were obtained, with fully informed consent and ethical approval during routine endoscopy of patients for preventive screening or diagnostic purpose. Donors, 31 adult (20-40 years old); 32 ageing (67-77y) and 19 young (7-12y) individuals were considered to be healthy when not showing symptoms suggesting gastrointestinal inflammatory disease/cancer during endoscopy and histology endoscopic inspection and later confirmed by routine histology excluded inflammation and neoplasia. Work has been carried out according to the Declaration of Helsinki (2008) and has been approved by the appropriate Ethics Committee (Comitato Etico Area Vasta Sud-Est, Italy and HRGC, Norwich, UK). All patients provided written informed consent (parental consent for children). Subjects had not been under medication for at least three months before the endoscopy (including antibiotics, immunosuppressants and steroids)**.** Details of how biopsies were processed for the different types of experiment can be found in Supplementary Materials Methods.

Paracellular permeability

Biopsies were mounted in adapted Ussing chambers exposing a surface of 2.0 mm^2. Tissues were immersed in Krebs' solution as described before [16] that was constantly oxygenated by a gas flow (95%; O_2; 5% CO2) and maintained at 37C**.** Two pairs of Ag/AgCl electrodes were used to monitor the transmucosal potential difference (PD) and short circuit current (I_{sc}) that were used to calculate transmucosal resistance according to Ohm's law. Subsequently the solution was replaced with Kreb's solution containing horseradish peroxidase (HRP type II, Sigma Aldrich) and transmucosal transport was carried out as described [16]. Samples were collected at 20 minutes intervals for 100 minutes; following incubation with the appropriate liquid substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma Aldrich) the reaction was stopped with H_2SO_4 and samples read at 450nm. TEER was also assessed in Caco-2 cells in the presence or absence of CM from cultures of biopsies from the three donor groups. The cells were seeded onto the upper face of Transwell inserts (6.5mm diameter, 3.0μm pore size, Corning, Costar) and grown on the filters for 14 days at 37°C, 5% CO_2, until fully differentiated. Transepithelial electric resistance (TEER) was monitored at various intervals (Millicell-ERS, Millipore) in the presence or absence of CM obtained from biopsy cultures.

In vitro organ cultures (IVOC)

Non polarized (np)-IVOC biopsies were mounted on foam support, the foam was saturated with a bicarbonate-buffered culture medium consisting of Dulbecco's minimum essential mediumand NCTC-135 medium (1:1) with 10% newborn calf serum plus 0.5% (wt/vol) D-mannose (all chemicals supplied by Sigma) [17]. Samples were then placed in a 24 well culture plate (Costar), covered, placed inside a larger container continuously gassed with 95% 0-5% C02, at 37C. The polarized (p)-IVOC was a modified version of previously described methods [18, 19]. Terminal ileum biopsies of standard size were placed mucosal side up on a IVOC-medium soaked nitrocellulose filter (3μm pore) and this was sandwiched between two 12 mm diameter acrylic glass (or PVC) disks; with the upper disk provided with a 2mm opening prepared in house by workshops at both University of Siena and Norwich Bioscience Institute. This was then accommodated into a modified Snapwell chambers (Corning, Corning, NY). To avoid antigen and microbial leakage to the basolateral side the upper disc was glued to the mucosal side of the biopsy by Histoacryl adhesive.

Immunohistochemistry

Frozen sections were fixed in 10% buffered formalin for 5 minutes and permeabilized with 0.5% tween-20 for 10 minutes. After rinsing sections were incubated overnight at 4°with rabbit anti-TLR5 (Invitrogen) (gift from S. Schueller), quenched with 10% donkey serum, and incubated with donkey Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch) for 60 minutes. Alternatively, sections were stained with rabbit anti-Claudin 2 antibody (Life Techologies) followed by incubation with FITC-labelled anti rabbit IgG antibody (Sigma) and counterstained with mouse anti-pan cytokeratin antibody (Sigma) followed by incubation with Cy3-conjugated anti-mouse IgG antibody (Jackson Immunoresearch). The primary antibody was not added to control sections. Sections were observed with a Zeiss LSM 710 or 510 confocal microscopes.

Preparation of conditioned medium (CM) from biopsy culture.

Immediately after removal biopsies were placed in a plastic tube containing 1 ml IVOC medium and kept in 95% O2/5%CO2 atmosphere at 37C for 5-6h ours. Following centrifugation (1100rpm x15 min) the conditioned medium (CM) was filtered sterile with centrifuge tube filters (0.22μm), aliquoted and stored for no more than 10 days at -80C° until used.

Effects of CM on biopsies and cell culture

The effects of mucosa-derived soluble mediators present in the CM on tight junction expression and intestinal permeability were then assessed in both np-IVOC of biopsies and Caco2 cells. Biopsies were cultured in np-IVOC for 10-12 hours in the presence of CM and the medium was replaced with fresh solution every 3-4h. Caco2 cells were cultured in the presence of CM for up to 36h and TEER monitored at various intervals. CM was added to both compartments of the transwell culture and its volume normalized to the weight of biopsies. In some case CM was pre-incubated with varying concentration of either anti-IL-6, anti-IL-1β or anti-TNF-α antibodies for 3 hours at 4C°. At the end of the culture both tissues and Caco2 cells were then used for RNA preparation

Gene expression

Total RNA was extracted from human terminal ileum biopsies or Caco2 intestinal epithelial cells TRIzol reagent (Invitrogen). Following evaluation of RNA integrity by gel electrophoresis RNA reverse transcription was carried out by iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The RT-cDNA reaction products were subjected to quantitative real-time PCR using the MyiQ single-color real-time PCR detection system (Bio-Rad) and iQ SYBR green supermix (Bio-Rad) according to the manufacturer's directions. All expression levels were normalized to β-actin or GADPH levels of the same sample. Relative quantity of target gene expression to housekeeping genes was measured by comparative Ct method. All real-time PCR reactions were performed in triplicate. Primers used are described in Supplementary Material Methods.

Western blot and ELISA

Western blot analysis and ELISA were carried out according to standard procedures. Details of these methods can be found in Supplementary Materials Methods.

Statistical analysis

Data were expressed as mean ± SD and analysed using Students' unpaired T-test or Tukey HSD test for multiple samples comparison. A p value < 0.05 was considered significant.

Results

Intestinal levels of IL-6 increased in the ageing human ileum.

Regulatory cytokines produced by IECs and gut-resident immune cells contribute to establish and maintain the intestinal immune homeostasis; however at this time very little is known on the effects of age on levels of regulatory cytokines in the human gut. Initially, two matched biopsies from eight individuals from the adult (20-40y) and ageing (67-77y) groups were used to monitor the expression of cytokine IL-6, IFN-γ, IL-1β and TNF-α some of which have been observed to increase significantly at systemic levels in ageing and are thought to underpin the development of frailty and increased mortality in the elderly [2, 3]. No age-associated changes were observed for TNF-α, IFN-γ and IL-1β among the two age groups. A different pattern was observed for IL-6 with the ageing group showing significantly higher levels (Figure 1A) ($p<0.01$). Levels of IL-6 mRNA was then further assessed in additional 5 individuals/age group that also included individuals between 7-12 years of age (young). IL-6 was not detected in two out of five young individuals and in one out of five adults and overall it was expressed at lower levels in biopsies from both young and adult individuals compared to ageing donors (Figure 1B). Increased tissue levels of IL-6 in the elderly were then confirmed at protein level by ELISA (Figure 1C). The amount of IL-6 reached 16.4±4.6 pg/mg/total protein in the ageing donors compared to both young (5.1±3.0 pg/mg/total protein) and adult (5.4.±2.2 pg/mg/total protein) individuals. Furthermore, gene expression analysis of cytokeratin (ctk)$^{+}$ CD45^{-} IECs, CD11c^{+} DCs and the remaining CD11c^{-} immune cell population showed that CD11c^{+} cell population displayed a significant increase in IL-6 expression in ageing ($p<0.05$) (Figure 2A). We observed a trend towards increased expression of IL-6 also in IEC although the increase did not reach statistical significance ($p=0.065$). We then focussed on CD11c^{+} cells. Subsequent analysis of isolated CD11c^{+} DCs showed that similar numbers of CD11c^{+} cells were recovered from biopsies from donors of different ages (Figure 2B). Phenotypic analysis showed that these cells from both groups expressed similar levels of CD86 and did not expressed E-Cadherin, a marker for inflammation-associated gut-DCs [20]; in contrast, expression of CD40 was higher in CD11c^{+} cells from ageing donors (Figure 2C). Also, isolated CD11c^{+} DCs from ageing donors produced a significant higher levels of IL-6 after 48h in culture (Figure 2D). Furthermore the percentage of IL-6-producing CD11c^{+} cells in the isolated fraction increased in ageing individuals (Supplementary Figure S1A-B). It has to be stressed that given the difficulties in recruiting patients in the young group (7-12y) we used, for this experiment biopsies from adult and ageing individuals only. These data demonstrated an age-associated increased production of IL-6; also suggested that, although the role of other cells such as IEC cannot be ruled out at this time, CD11c^{+} cells played an important role in this event.

Permeability to solutes but not to macromolecules is increased during ageing in the small intestine

The notion that ageing is sometime associated with increased intestinal permeability (leaky gut) [21] prompted us to monitor the integrity of the epithelial barrier in biopsies from donors of all age groups. Also, given the direct effects of IL-6 on tight junctions, such as Claudin 2 [22, 23] we investigated the possibility that age-associated overexpression of IL-6 might directly affect intestinal permeability. First, we carried post-IVOC structural analysis of the terminal ileum by TEM and LM. These did not reveal major age-associated alterations of the overall morphology/structure of the TJs and intestinal mucosa and that tissue retained good morphology after 10 hour culture in pIVOC (Figure 3A-D). In contrast, subsequent functional and molecular analysis of TJs mRNA expression showed the presence of age-associated

alterations. First, transepithelial electric resistance (TEER), a measure of the ionic gradient across freshly collected ileal biopsies was determined in Ussing chamber. We observed that TEER was significantly reduced in biopsies from ageing individuals ($p<0.01$) while no difference was observed between the two younger groups (Figure 3E). Increase of permeability in the ageing small intestine appeared to be restricted to solutes, indeed flux of HRP (approx. 44kDA) did not vary between the different age groups (Figure 3F) showing that permeability to macromolecule was not affected by ageing. Permeability assay was paralleled by the analysis of mRNA levels of tight junctions. To this end, mRNA expression of Zonula occludens-1 (ZO-1), Occludin, Junctional Adhesion Molecule A 1 (JAMA-1) and Claudin-2 was assessed. In contrast to what has been observed previously in colonic biopsies of non-human primates [24] we did not observed any significant age-related effect on the expression of ZO-1, Occludin and JAMA-1 (Figure 4A-C). On the other hand, in agreement with the same report [24] we observed that levels of Claudin-2 were significantly increased ($p<0.01$) in the ageing group compared to adult individuals (Figure 4D) Further immunohistochemistry analysis showed absence of age-associated changes in the distribution of Claudin 2 in aged tissues (Figure 4F-H) .

Age-associated high levels of IL-6 affected intestinal permeability by up-regulating Claudin-2 expression.

To determine whether overexpression of IL-6 in the mucosa of the elderly was in fact responsible for the increased intestinal permeability to solutes we tested the effect of conditioned medium (CM) from cultures of biopsies of different age on the TEER and expression of Claudin-2 in human intestinal epithelial Caco2 cells and biopsies from adult donors. Treatment of Caco2 cells with CM from culture of aged biopsies (CM_{AG}) induced a significant fall in TEER; instead CM from culture of both young (CM_{Y}) and adult biopsies (CM_{AD}) did not have any effects on TEER (Figure 5A). Pre-incubation of CM_{AG} with anti-IL-6 antibody did restore TEER; however, incubation of CM with either anti-TNF-α or IL-1β antibody did not have any effect. The result of this functional analysis was confirmed at mRNA level. Caco2 cells treated with CM_{AG} showed a significant increase in the expression of Claudin-2 compared to untreated Caco2 cells whereas both CM_{AD} and CM_{Y} failed to up-regulate Claudin-2. CM_{AG}-mediated up-regulation of Claudin-2 was suppressed by pre-incubation with anti-IL-6 but not anti-IL1β or anti-TNF-α antibody (Figure 5B). Culturing Caco2 cells in the presence of blocking antibodies alone did not have any effects on both TEER and Claudin 2 expression (Supplementary Figure S2). The biological relevance of the above results was further confirmed in intestinal biopsies cultured in np-IVOC (Figure 5C). Biopsies from young donors showed significantly higher levels of levels of Claudin-2 when cultured in presence of CM_{AG} compared to age-matched untreated biopsies (AD-baseline); also in this case pre-incubation with anti-IL-6 but not anti-IL-1β prevented up-regulation of Claudin-2. Thus, the collation of *in vitro* and *ex-vivo* results showed that increased levels of IL-6 appeared to be a feature of the ageing gut that has a direct impact on the expression of Claudin-2 with direct bearing on intestinal permeability.

Production of IL-8 in response to flagellin progressively declined with age.

The production of cytokine, such as IL-8 in response to flagellin in the gut is an important factor in the early stages of the innate immune response to pathogens [25]; we then determined whether intestinal response to bacterial components was impaired in ageing. Terminal ileum biopsies were then challenged for 10-12 hours with flagellin using a pIVOC culture, a model that allows reproducing faithfully real-life host-pathogen interaction by restricting the antigenic challenge to the mucosal apical side and that has been already

utilized to assess colonic tissue response to soluble flagellin [19]. We observed that a significant variation of IL-8 production in response to flagellin occurred across the course of life (Figure 6A). Tissue levels of IL-8 reached its highest in the young group (range 35.2-89.8 pg/mg/total protein), it was significantly reduced ($p<0.05$) in the adult group (range 24.7-62.6 pg/mg/total protein) and it declined further in the aged group ($p<0.01$ and $p<0.05$ compared to young and adult groups respectively) (range 9.2-49.3 pg/mg/total protein). Age-associated decline of IL-8 production in response to flagellin was confirmed by western blot analysis and subsequent densitometry analysis (Figure 6B, D). Furthermore, although a direct effect of IL-6 on the production of IL-8 has not been shown we investigated whether suppression of IL-6 could restore IL-8 production in ageing; pre-treatment of biopsies with neutralizing anti-IL-6 antibody (IgA isotype) did not restore the ability to produce IL-8 in ageing subjects (data not shown). Also, expression of flagellin-specific TLR5 was determined at both gene and protein level. Quantitative rt-PCR analysis showed that the expression of flagellin-specific receptor TLR5 did not vary with age (Figure 6C) and immunohistochemistry analysis showed absence of age-associated changes in the distribution of mature TLR5 (Figure 6E, F). Also, we observed that stimulation with flagellin induced a significant production of IL-6 in biopsies from both young and adult individuals but failed to do so in ageing (Supplementary Figure S3A)

Ageing does not affect the ability of probiotics to elicit protective epithelial innate immunity.

It has been shown that probiotics promoted gut health via stimulation of epithelial innate immunity, more specifically via the production of TNFα rather than its suppression. Indeed, production of TNFα by IEC following challenge with VSL#3 probiotics was found to be a critical event in probiotic-mediated prevention/suppression of ileitis [26]. Thus we determined as to whether ageing did affect the ability of the gut epithelium to produce TNFα in response to VSL#3. The pattern appeared to be different compared to that observed for IL-8; the production of TNF-α in response to challenge with live VSL#3 probiotics did not vary with age (Figure 7A) and challenge with probiotics induced a similar increase in the production of TNFα irrespective of the donor's age. In contrast, it would appear that exposure to VSL#3 did not affect the production of IL-6 in biopsies from all age groups (Supplementary Figure S3B). It has also been suggested that probiotics may beneficially impact on intestinal permeability. However, we observed there was a trend towards increased expression of Claudin 2 possibly as a consequence of increased level of TNFα (Figure 7B); this suggested that at least in this experimental setting VSL#3 did not restore the compromised intestinal permeability.

Discussion

Compared with immunosenescence of systemic immunity, age-associated changes in the mucosal immune system are less well understood. In particular, at intestinal level a major gap is represented by the lack of knowledge on events that pertain to the intestinal epithelial barrier and early events of the innate immune response. The main aim of the study was to investigate the impact of ageing on several aspects of the intestinal barrier including levels of inflammatory cytokines, barrier integrity and intestinal innate immunity to different types of microbial challenges in humans. Our study showed that ageing affects important physical and immunological functions of the intestinal epithelial barrier. Our first objective was to determine the levels of inflammatory cytokine in the ageing small intestine (terminal ileum). Production of inflammatory cytokine is an important attribute and effector function of IECs that influences the activity of various cell types in the intestinal mucosa; also increased levels of certain cytokines may have a direct bearing on some of the features of the ageing gut, such as increased permeability of the epithelial barrier ("leaky gut") [21]. Recently it was shown that colonic biopsies from aged non-human primates showed increased levels of IL-6, IL-1β and IFNγ and reduced expression of TJs such as ZO-1, Occludin and JAMA 1 and increased levels of Claudin-2 [24]. Also, alteration of tight junctions led to increased permeability to macromolecules (HRP). The pattern appeared to be different in biopsies from the terminal ileum of humans. Indeed, we observed that the ageing human small intestine is characterized by a significant increase in the level of IL-6 but not of IL-1β, IFNγ or TNF-α as observed in primates. Also, we failed to detect major alterations of ZO-1, Occludin and JAMA 1 and no changes in permeability to macromolecules (HRP) were observed. It is possible that these discordant observations reflected intrinsic differences between humans and non-human primates. Alternatively, this might be linked to intrinsic differences between distinct areas of the intestine, such as the colon and terminal ileum. The latter hypothesis is supported by the recent observation that in humans the regulatory features of DCs varied according to their geographical location in the gut (27). Thus, it is possible that while terminal ileum DCs are characterized by increased production of IL-6 in ageing, DCs located in the large intestine may display a different array of age-related modifications that might lead to a more significant alteration of the local inflammatory status and intestinal permeability. One consequence of increased intestinal levels of IL-6 is an enhanced permeability to solutes that was brought about by up-regulation of the tight junction Claudin-2. *In vitro* studies have shown that IL-6 affects barrier integrity by up-regulating the expression of Claudin 2 [22, 28] that in turns promotes the formation of pores that allow paracellular movement of cations and small molecules with radii less than 4 Å [23]. It is important to highlight that overexpression of Claudin-2 in IECs has been observed in animal model of colitis [29] and patients with inflammatory bowel disease [30]. Currently, the contribution of increased expression of Claudin-2 to the aetiology or progression of diseases has not yet been determined but it is possible that its up-regulation in the elderly could be linked to age-associated disturbances. Also, although other cytokines, such as TNF-α can regulate the expression of Claudin-2 [31], the observation that the addition of anti-IL-6, but not anti-TNF-α antibody to CM from ageing biopsies prevented both Claudin-2 over-expression and decline of TEER strongly suggested that IL-6 is the main factor underlying Claudin-2 up-regulation in ageing. Age-associated increase of IL-6 in the gut raises a series of questions, the most notable being, what is the triggering event and what are the potential consequences at systemic level. Indeed, although it has been known for some time that ageing in humans is associated with increased levels of circulating IL-6 and that this has a strong association with markers of physical frailty [32, 33, 34] the cause(s) underlying its increase is still unknown. It has been hypothesized that events in the gut might play a critical role in age-associated inflammatory

dysregulation [4] and it has been also shown that certain components of the intestinal microbiota can induce the production of IL-6 [35]. This together with the notion that intestinal microbiota undergoes significant changes in the elderly [36] makes it plausible to hypothesize that age-associated alteration of the microbiota that resulted in increased presence of IL-6-inducing bacteria species might be one of the triggering events. Furthermore, elevated intestinal levels of IL-6 may also directly contribute to establish and maintain the age-associated low grade chronic inflammation, or inflammageing [3] at systemic level by contributing to promote the differentiation of pro-inflammatory TH17 [37], the circulating number of which are significantly higher in the elderly [38]. Although at this time we cannot rule out the possibility that other cell types, such as IECs may contribute to increased levels of IL-6 in ageing we observed that CD11c^{+} DCs played a role in this event. Also, these cells are characterized by increased expression of CD40 that together with IL-6 secretion is highly relevant to the generation of TH17 *in vivo* [39]. Furthermore, by extending our study to the immunological features of the intestinal barrier in response to microbial antigen of different nature we observed that the production of cytokines in the gut in response to different microbial challenges in ageing may or may not decline, possibly depending on the nature of the antigenic stimulus. Indeed, while epithelial production of IL-8 in response to flagellin progressively declined across life and it is significantly compromised in ageing the production of TNFα in response to exposure to probiotics did not decline in the elderly. Interestingly, in contrast to a previous report [40] elevated levels of IL-6 did not affect the expression of flagellin-specific TLR5 on IECs that remained unchanged in ageing. Thus, it would seem that increased levels of IL-6 alone did not suffice to inducing major changes of the expression of TLR5 and dysregulation of the major tight junctions. This would suggest that a simultaneous up-regulation of several pro-inflammatory cytokines is required to induce significant detrimental effects on the intestinal epithelial barrier. Also, the notion that levels of TLR5 did not change with age also strongly suggested that the progressive age-dependent decline in production of IL-8 is due to alteration of intracellular signalling pathways following the engagement of flagellin with TLR5. Ultimately it is likely that reduced levels of IL-8 may play an important role in the increased susceptibility of the elderly to infections. In contrast, TNF-α production in response to a more complex microbial challenge, such as live VSL#3 probiotic mixture did not show significant variation between the age groups. The latter finding is of potential interest. First, although studies conducted in mice and cell lines have shown that probiotics promoted gut health by inducing the production of the pro-inflammatory cytokine TNFα, rather than its suppression [26], their effect on the human gut was still unknown. Second, very little attention has been given so far to how the intestinal response to probiotics varies between individuals of different age. Our results demonstrated that VSL#3 induced the production in the human gut of TNFα, which plays an important role in preventing/ameliorating ileitis in mice [26] and that age does not influence the production of a cytokine required for the beneficial effects of probiotics. On the other hand, we have shown that *in vitro* challenge with VSL#3 probiotic mixture did not down-regulate the expression of Claudin 2 thus suggesting that a short term exposure to VSL#3 as carried out in our *ex-vivo* experimental model was not enough to beneficially affect the partially compromised intestinal permeability. We believe that the identification of the triggering events affecting aspects of the epithelial barrier integrity and intestinal epithelial innate immune response to certain antigenic stimuli, such as flagellin is a goal of certain medical relevance, and may provide us with the tool to impact on local and possibly systemic age-associated disorders.

Clinical Perspectives

The effects of ageing on physical and immunological properties of the intestinal epithelial barrier in humans are largely unknown. We report that the ageing gut is characterized by higher levels of the cytokine IL-6 that in part affects intestinal permeability. Furthermore, ageing is associated with an impaired intestinal innate immunity to microbial challenge in the small intestine that might lead to the increased susceptibility to infections typical of the aged organisms. This study suggests a pivotal role of the gut in the generation of the chronic low-grade inflammatory status (termed 'inflammaging') typical of the aged organism; it also provides the basis to hypothesize that manipulating the composition of the intestinal microbiota in the elderly may represent an important strategy to intervene on age-associated disorders both locally and systemically.

Author contribution

Claudio Nicoletti, Eugenio Bertelli and Alastair Watson designed the study; Mario Marini and Graham Briars designed the study from a clinical point of view, produced ethical approval and provided human samples; Angela Man, Eugenio Bertelli, Mari Regoli and Silvia Rentini conducted the research and analysed data; Claudio Nicoletti, Eugenio Bertelli and Alastair Watson wrote the paper.

Acknowledgements

The authors wish to thank S. Schueller and S.R. Carding for helpful comments and discussion and P. Pople for computer artwork.

Funding. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), UK, Institute Strategic Programme Grant [BB/J004529/1: The Gut Health and Food Safety-ISP] (to CN) and intramural funds from University of Siena, Italy (to EB).

References

1- Ershler W.B. (2003) Biological interactions of aging and anemia: a focus on cytokines, J. Am. Geriatr. Soc. **51**, S18-21.
2- Gardner I.D. (1980) The effect of aging on susceptibility to infection, Rev. Infect. Dis. **2**, 801-810.
3- Franceschi C., Campisi J. (2014) Chronic inflammation (inflammageing) and its potential contribution to age-associated diseases. J Gerontol. **69,** Suppl1:S4-9 doi: 10.1093/gerona/glu057 .
4- Guigoz Y., Dore J. Schiffrin E.J. (2008) The inflammatory status of old age can be nurtured from the intestinal environment, Curr. Opin. Clin. Nutr. Metab. Care. **11**, 13-20
5- Man AL, Gicheva N, Nicoletti C. (2014) The impact of ageing on the intestinal epithelial barrier and immune system. Cell Immunol. **289**, 112-118
6- Turner JR. (2009) Intestinal mucosal barrier function inhealth and disease. Nature Rev Immunol. **9**, 799-809
7- Bleau C, Karelis AD, St-Pierre DH, Lamontagne L. (2014). Crosstalk between intestinal microbiota, adipose tissue and skeletal muscle as an early event in systemic low-grade inflammation and the development of obesity and diabetes. Diabetes Metab Res Rev. **2,** doi: 10.1002/dmrr.2617
8- Maes M, Kubera M, Leunis JC (2008) The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. Neuro Endocrinol Lett. **9**, 117-24.
9- C.B. Forsyth C.B., K.M. Shannon K.M., J.H. Kordower J.H., Voigt RM, Shaikh M, Jaglin JA, Estes JD, Dodiya HB, Keshavarzian A.(2011) Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease, PLoS One **6**, e28032.
10- A. Vrieze A., P.F. de Groot P.F., R.S. Kootte R.S., Knaapen M, van Nood E, Nieuwdorp M (2013) Fecal transplant: a safe and sustainable clinical therapy for restoring intestinal microbial balance in human disease? Best Pract. Res. Clin. Gastroenterol. **27**, 127-137.
11- Rera M., Clark R.I., Walker D.W. (2012) Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila, Proc. Natl. Acad. Sci. U. S. **109**, 21528-21533.
12- Rimoldi M, Chieppa M, Salucci V., Avogadri F, Sonzogni A, Sampietro GM, Nespoli A, Viale G, Allavena P, Rescigno M. (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol. **6**, 507-514
13- Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M (2009) Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning Mucosal Immunol. **2**, 340-50
14- Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. (2003) Nat Rev Immunol. **4**, 331-341
15- Sansonetti P.J. War and peace at mucosal surfaces. (2004) Nat Rev Immunol. **4**, 953-964
16- Greenwood-Van Meerveld B, Tyler K, Keith JC Jr. (2000) Recombinant human interleukin-1 modulates ion transport and mucosal inflammation in the small intestine and colon.Lab Invest. **80**:1269-1280.

17- Hicks S., Candy DC, Phillips AD (1996) Adhesion of enteroaggregative Escherichia coli to pediatric intestinal mucosa in vitro. Infect Immun. **64**:4751-60
18- El Asmar R, Panigrahi P, Bamford P, Berti I, Not T, Coppa GV, Catassi C, Fasano A (2002) A. Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. Gastroenterology. **123**,1607-15
19- Schuller S., Lucas M. Kaper J.B., Girón JA, Phillips AD (2009) The ex-vivo response of human intestinal mucosa to enteropathogenic E. coli infection. **11**:1607-1615
20- Siddiqui K R., Laffon S, Powrie F. (2010) E-Cadherin Marks a Subset of Inflammatory Dendritic Cells that Promote T Cell-Mediated Colitis Immunity. **23**, 32: 557–567
21- Hollander D., Tarnawski H. (1985) Aging-associated increase in intestinal absorption of macromolecules Gerontology. 31, 133-137
22- Suzuki T, Yoshinaga N, Tanabe S. (2011) Interleukin-6 (IL-6) regulates Claudin-2 expression and tight junction permeability in intestinal epithelium. J Biol Chem. **286**:31263-31271
23- Van Itallie C.M., Holmes J., Bridges A, Gookin JL, Coccaro MR, Proctor W, ColegioOR, Anderson JM (2008) The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci. **121**, 298-305
24- Tran L., Greenwood-Van Meerveld B. (2013) Age-associated remodeling of the intestinal epithelial barrier. J. Gerontol. A Biol. Sci. Med. Sci. **68**, 1045-1056.
25- Eckmann L, Kagnoff MF, Fierer J (1993) Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun. **61**:4569-74
26- Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F (2010) Probiotics promote gut health through stimulation of epithelial innate immunity. Proc Natl Acad Sci U S A. **107**:454-459
27- Mann ER, Bernardo D, English NR, Landy J, Al-Hassi HO, Peake ST, Man R, Elliott TR, Spranger H, Lee GH, Parian A, Brant SR, Lazarev M, Hart AL, Li X, Knight SC (2015). Compartment-specific immunity in the human gut: properties and functions of dendritic cells in the colon versus the ileum. Gut. pii: gutjnl-2014-307916. doi: 10.1136/gutjnl-2014-307916
28- Al-Sadi R, Ye D, Boivin M, Guo S, Hashimi M, Ereifej L, Ma TY. (2014) Interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by JNK pathway activation of claudin-2 gene. PLoS One. **9**, e85345. doi: 10.1371/journal.pone.0085345.
29- Heller F, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, Mankertz J, Gitter AH, Bürgel N, Fromm M, Zeitz M, Fuss I, Strober W, Schulzke JD. (2015) Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology. **129**, 550–564
30- Fujino S., Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y (2003) Increased expression of interleukin 17 in inflammatory bowel disease. Gut **52**, 65–70
31- Mankertz J., Amasheh M., Krug S.M, Fromm A, Amasheh S, Hillenbrand B, Tavalali S, Fromm M, Schulzke JD (2009) TNF-α up-regulates claudin-2 expression in epithelial HT-29/B6 cells via phosphatidylinositol-3-kinase signaling Cell Tissue Res. **336**, 67-77
32- Ershler W.B., Keller ET..(2000) Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med. **51**, 245-70
33- Roubenoff R. (2014) The cytokine for gerontologist has some company. J. Gerontol. A Biol. Sci. Med. Sci. **69**:163-64

34- Sanders J.L., Ding V., Arnold A.M, Kaplan RC, Cappola AR, Kizer JR, Boudreau RM, Cushman M, Newman AB (2014) Do changes in circulating biomarkers track with each other and with functional changes in older adults?. J. Gerontol. A Biol. Sci. Med. Sci. **68**:174-81
35- Ivanov I.I., Atarashi K., Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. **139**:485-498
36- Claesson .J., Jeffery .IB., Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW(2012) Gut microbiota composition correlates with diet and health in the elderly Nature. **488**:178-84
37- Kimura A., Kishimoto T. IL-6: regulator of Treg/Th17 balance (2010) Eur J Immunol. 40, 830-835
38- Schmitt V., Rink L., Uciechowski P. (2013) The Th17/Treg balance is disturbed during aging. Exp Gerontol. **48**:1379-1386
39- Perona-Wright, Jenkins SJ, O'Connor RA, Zienkiewicz D, McSorley HJ, Maizels RM, Anderton SM, MacDonald AS. (2009). A pivotal role for CD40-mediated IL-6 production by dendritic cells during IL-17 induction in vivo. J Immunol. **182**:2808-15
40- Sánchez-Muñoz F., Fonseca-Camarillo G., Villeda-Ramírez M.A., Miranda-Pérez E, Mendivil EJ, Barreto-Zúñiga R, Uribe M, Bojalil R, Domínguez-López A, Yamamoto-Furusho JK . (2011) Transcript levels of Toll-Like Receptors 5, 8 and 9 correlate with inflammatory activity in Ulcerative Colitis. BMC Gastroenterol. **11**:138-142

Legends

Figure 1- Levels of regulatory cytokines in the small intestine of individuals of different age. A pair of terminal ileum biopsies/subject collected from adults (n=10; 20-40y; grey bars) and ageing (n=10; 67-77y; black bars) individuals were used to assess expression of cytokine genes (A). IL-6 showed a significant increase in the ageing group ($p<0.01$), no changes were seen for TNFα, IL-1β or IFNγ. Statistical analysis was carried using Student's unpaired t-test. Age-associated increase of IL-6 was subsequently confirmed in additional 5/individual groups that included also young (7-12y) subjects (B). Finally, increased tissue levels of IL-6 were confirmed at protein levels by ELISA in a pair of terminal ileum biopsies/subject (n=10/group) in all age group (C); IL-6 tissue levels are expressed relative to amounts of total protein. ND= not detected.

Figure 2- Expression of IL-6 in isolated intestinal cell populations. Three cell populations, IEC ($Ctk^{+}CD45^{-}$), $CD11c^{-}CD45^{+}$ and $CD11c^{+}CD45^{+}$ were isolated from biopsies (6-8 biopsies/individual) of donors (5 individuals/group) of different age and assessed for IL-6 expression (A). A trend towards increase of IL-6 expression was observed in IEC although the difference did not reach statistical significance (*, $p=0.06$); whereas IL-6 production was significantly increased in $CD11c^{+}$ DCs (**). The purified $CD11c^{+}$ cells (B) were then assessed for the expression of co-stimulatory molecules (C); DCs from both age groups displayed similar expression of CD40, lack of expression of E-Cadherin while higher expression of CD40 was observed in the ageing group. Increased production of IL-6 by $CD11c^{+}$ cells from ageing donors was further confirmed by ELISA using culture (48h) supernatants. This set of experiments was performed using only biopsies from adult (n=5/group; 20-40y) and ageing (67-77y) individuals for difficulties to recruit donors in the young group. Statistical analysis was carried out using Student's unpaired t-test.

Figure 3- Intestinal permeability to solutes and macromolecules in ageing. Post-IVOC (10 hours) TEM micrographs of the structure of tight junctions in freshly isolated biopsies from young (A), adult (B) and ageing (C) individuals did not show any visible age-associated morphological alteration; also, intestinal tissues retained good morphology after pIVOC. (D). The overall integrity of the structure of the ageing gut also appeared to be intact at lower magnification (D) where a series of adjoining TJs are illustrated (boxes). TEER declined in ageing as established in Ussing chambers (E); however, permeability to macromolecules (HRP, approx. 44kDA) remained unchanged (F). Statistical analysis was carried using Tukey HSD test for multiple samples comparison

Figure 4- Expression of tight junctions in the ageing gut. Level of expression of ZO-1, Occludin and JAMA-1 (A-C) were similar in biopsies from all age groups. In contrast, Claudin 2 was significantly up-regulated in ageing individuals (D) ($p<0.01$) compared to younger individuals. Each symbol represents the average from two biopsies/individual (6-8 individuals/group). Statistical analysis was carried using Tukey HSD test for multiple samples comparison; asterisk (*) indicates significance difference. Also, expression of Claudin 2 did not change with age (E-H). Cross section of biopsies (apical area) from adult (E) and ageing (F) individuals showed that Claudin-2 (green) is restricted to the tight junction complex between adjacent epithelial cells (IEC). Similar distribution was seen in longitudinal section of biopsies from adult (G) and ageing (H) individuals; also in this case Claudin 2 (green), arrow heads) was restricted at the apical domain (L=lumen) as part of the tight junction complex. Sections were counterstained with anti-pan cytokeratin antibody (red). As average, 3-5 sections from 3 biopsies/donor (3 donors/group) were examined.

Figure 5- Conditioned medium from cultures of ageing biopsies affected intestinal permeability and expression of Claudin 2. Caco2 cells (A) were cultured in the presence of conditioned medium (CM) from young (CM_{Y}), adult (CM_{AD}) and ageing (CM_{AG}) individuals and TEER monitored for at least 36h. Both CM_{AD} and CM_{AG} did not have a significant effect on intestinal permeability, in contrst, addition of CM_{AG} to the culture had a significant impact on permeability that became evident after 18h. Pre-incubation of CM_{AG} with anti-IL-6 antibody but not anti-IL1β or anti-TNF-α prevented CM_{AG}-mediated increased permeability. rt-PCR analysis carried out after 24h culture in the presence of CM_{AG} showed that IL-6 underpins the up-regulation of Claudin-2 expression (B). In (C) biopsies from adult donors cultured in the presence of CM_{AG} (AD-CM_{AG}) showed higher expression levels of Claudin-2 compared to untreated biopsies from adults (AD-baseline) and comparable to what observed in biopsies from ageing donors (AD). Addition of anti-IL6 antibody (AD-CM_{AG}-αIL6) but not anti-IL1β (AD-CM_{AG}-αIL1β) prevented upregulation of Claudin-2. Statistical analysis was carried using Tukey HSD test for multiple samples comparison; different letters indicate statistical difference; equal letters indicate lack of statistical difference. Asterisk (*) indicates significance difference.

Figure 6- Intestinal response to flagellin. Tissue levels of IL-8 in response to flagellin were assessed after 10 hours p-IVOC. Levels of IL-8 progressively declined from young individuals to adult and continued to do further so in ageing subjects (A) as seen by ELISA. IL-8 tissue levels are expressed relative to amounts of total protein. Age-dependent variation of tissue levels IL-8 following challenge with flagellin is shown also by western blot analysis (B) and associated densitometric analysis (D); data are representative of three independent experiments with similar results. mRNA levels of TLR5 remained unchanged throughout life (C) and expression of TLR5 did not increase after stimulation compared to unchallenged tissue, irrespective of the age of the donor. Finally, immunohistochemistry analysis showed absence of age-associated changes in the distribution of mature TLR5 (E, F). Statistical analysis was done using Tukey HSD test for multiple samples comparison (A) and Student's unpaired t-test was used in (C).

Figure 7- VSL#3 probiotics induced normal production of TNFα in ageing but did not down-regulated expression of Claudin 2. Challenge of biopsies with live probiotics for 10 hours in p-IVOC induced the production of similar levels of TNF-α irresepctive of the donor's age. Levels of TNF-α are expressed relative to amounts of total protein. Furthermore, we observed a trend (not statistically significant) towards increased expression of Claudin 2 in ageing tissue following challenge with VSL#3 probiotic mixture (B), thus suggesting that age-associated Claudin 2-mediated up-regulation of intestinal permeability was not reduced by probiotics challenge, at least under these experimental conditions. Statistical analysis was done using Tukey HSD test for multiple samples comparison (A) and Student's unpaired t-test was used in (B).

Figures

Figure 1

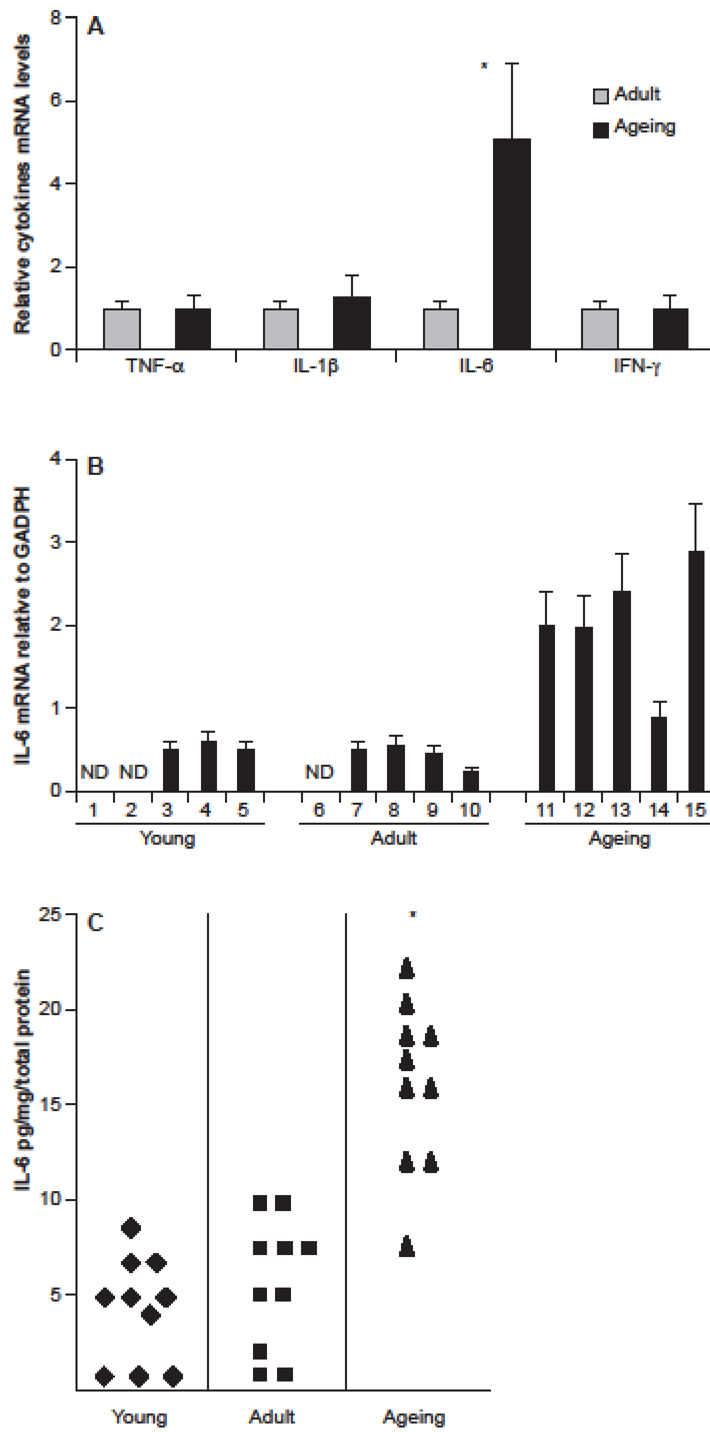


Figure 2

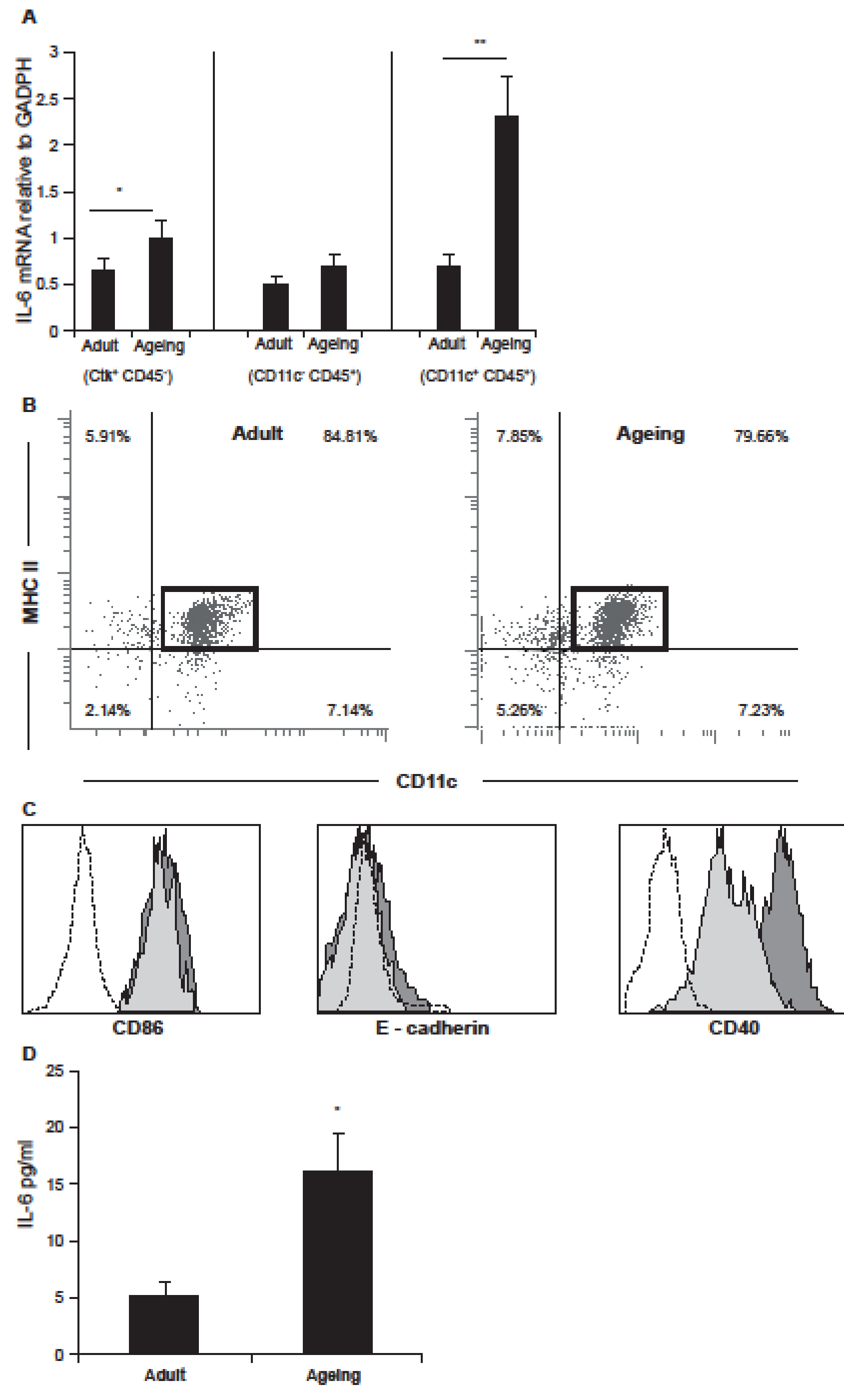


Figure 3

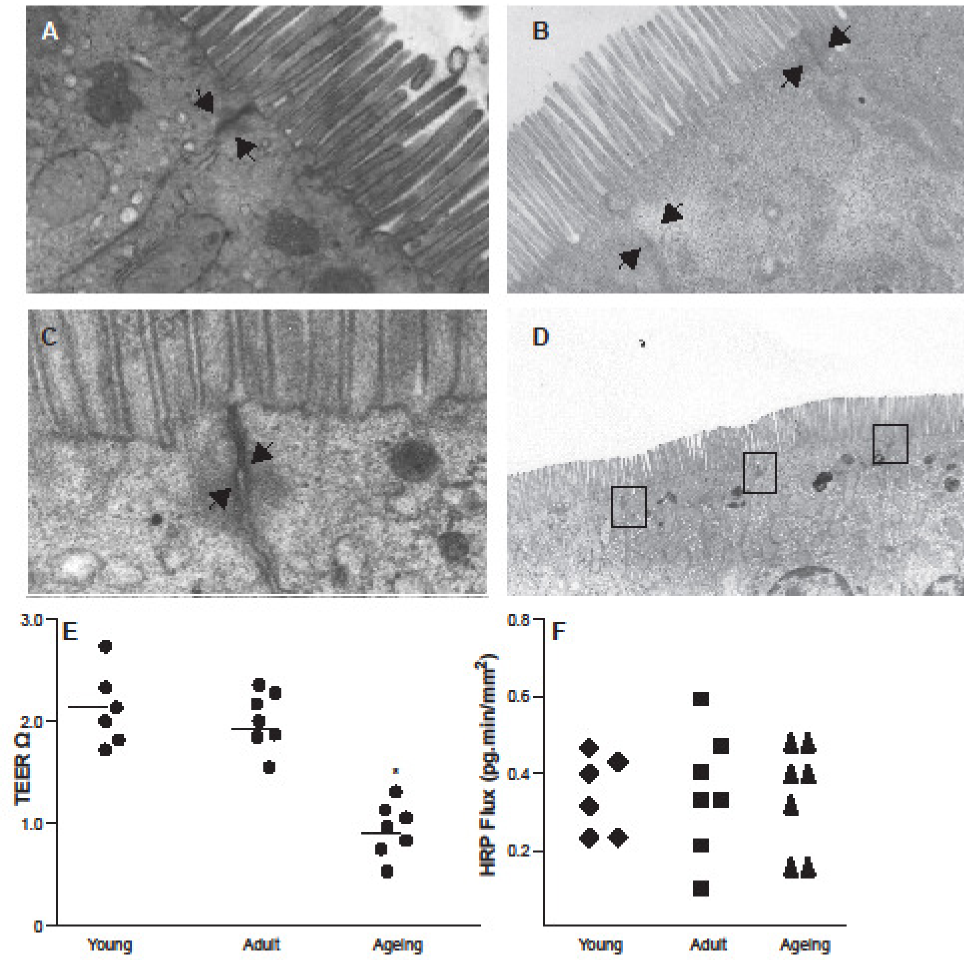
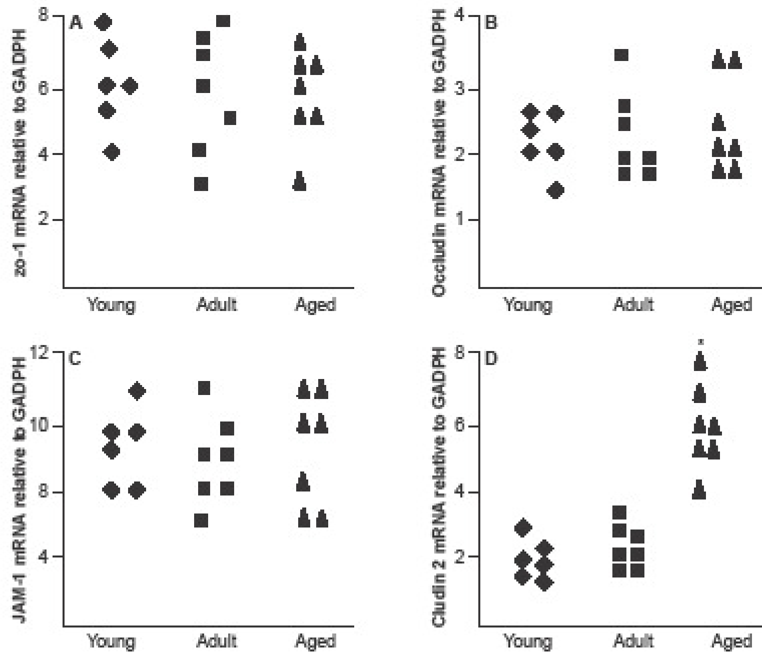


Figure 4



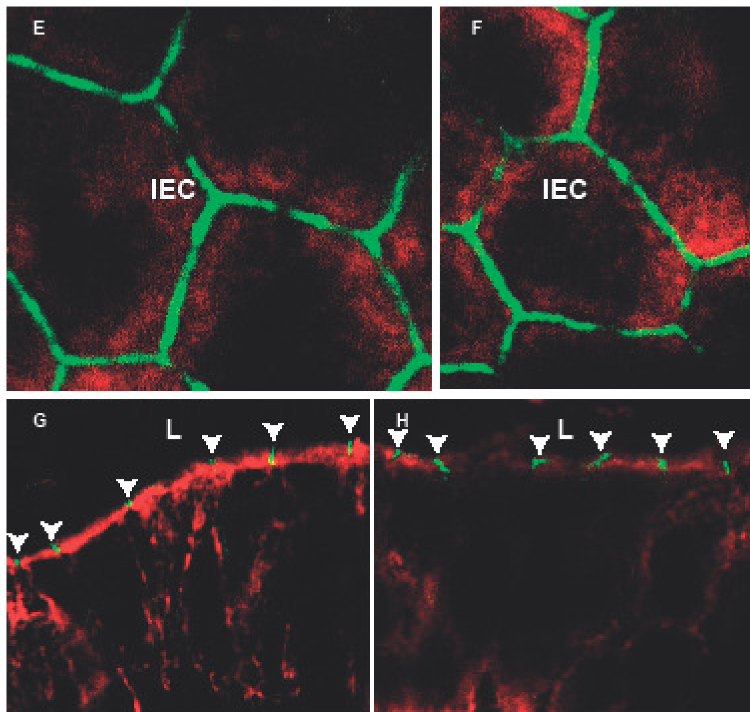


Figure 5

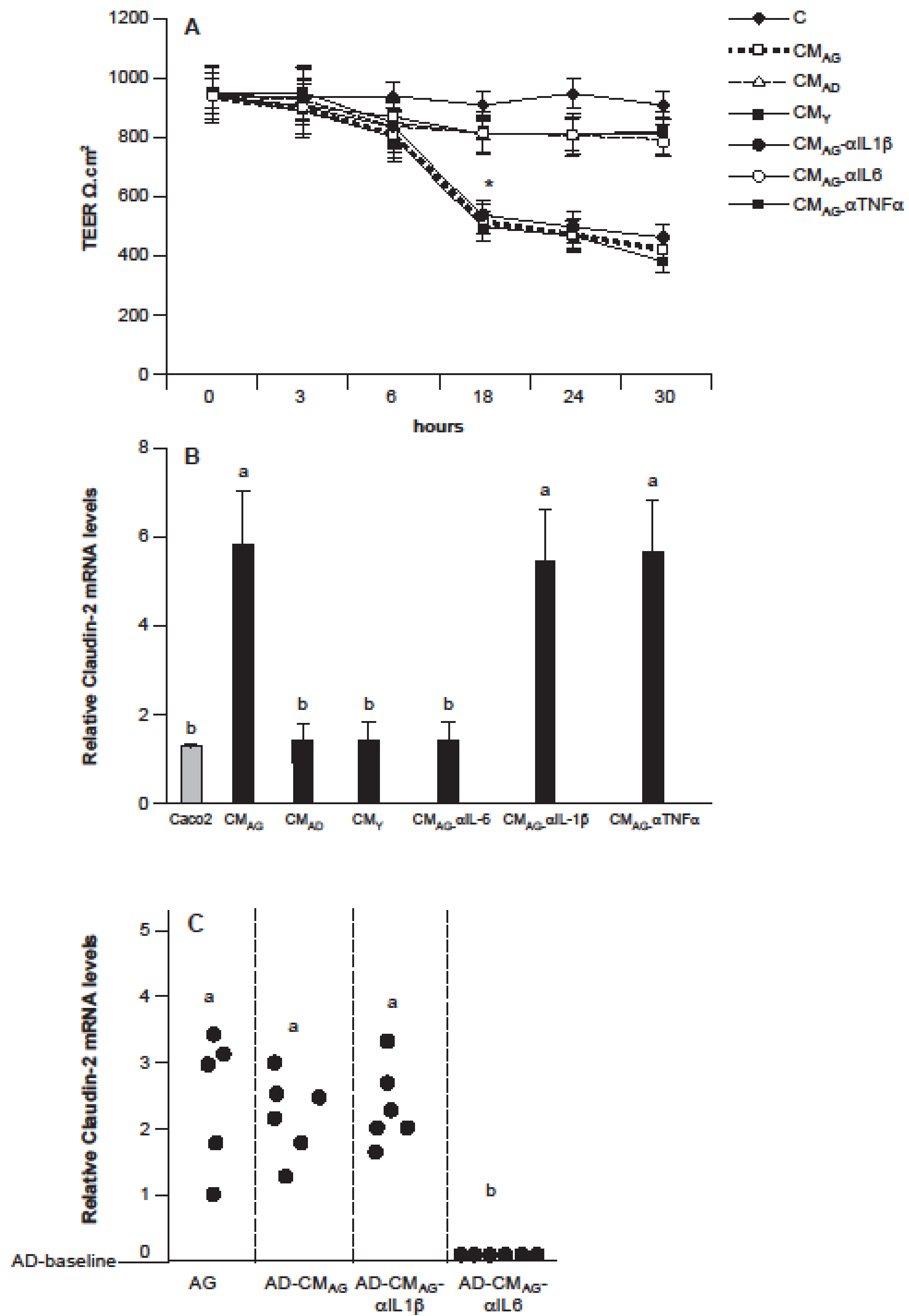


Figure 6

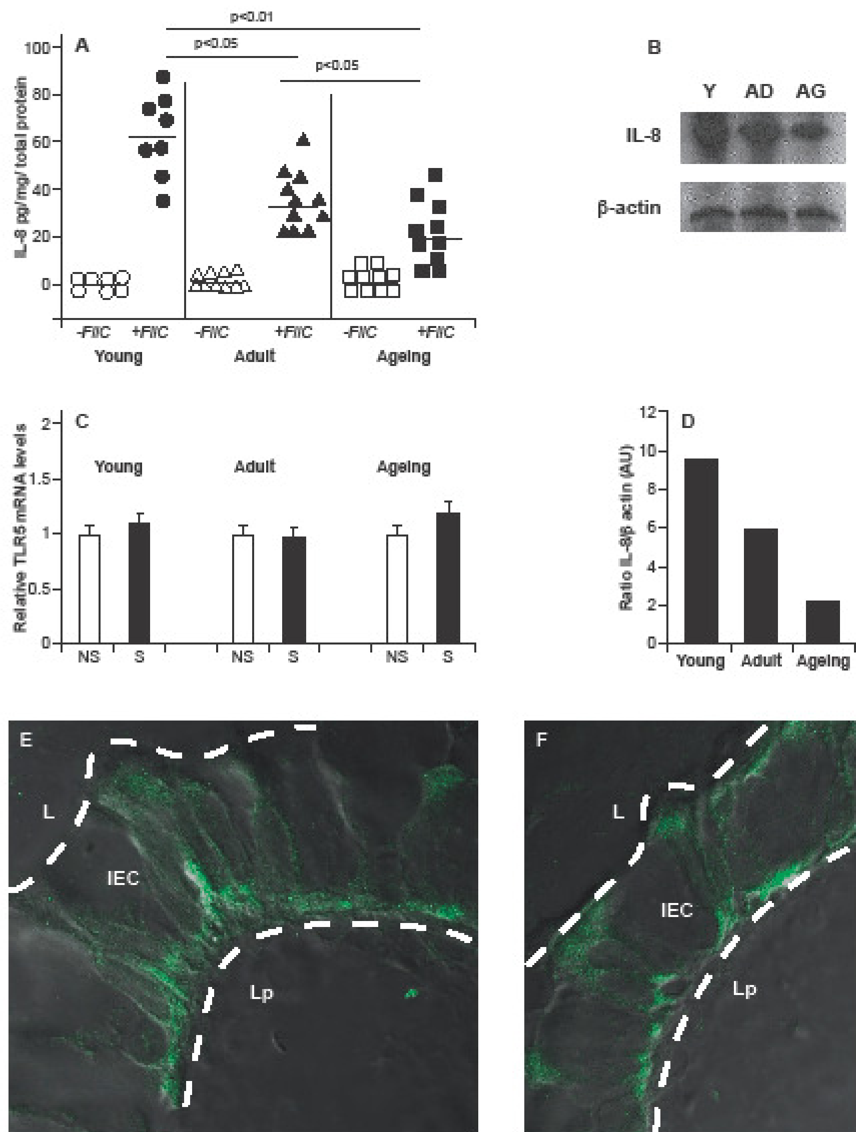


Figure 7