Clinical Science (2015) **129**, 515–527 doi: 10.1042/CS20150046

commentary

Original Paper

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

Angela L. Man*[1], Eugenio Bertelli†[1], Silvia Rentini‡, Mari Regoli†, Graham Briars§, Mario Marini‡, Alastair J. M. Watson*‖ and Claudio Nicoletti*

*Gut Health and Food Safety Program, Institute of Food Research, Norwich NR4 7UA, U.K.
†Department of Life Sciences, University of Siena, I-53100 Italy
‡C.O.U. Gastroenterology and Digestive Endoscopy, A.U.O.S. University Hospital, Siena I-53100, Italy
§Department of Paediatric Gastroenterology, Norfolk and Norwich University Hospital, Norwich NR4 7UY, U.K.
‖Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, U.K.

Abstract

The physical and immunological properties of the human intestinal epithelial barrier in aging are largely unknown. Ileal biopsies from young (7–12 years), adult (20–40 years) and aging (67–77 years) individuals not showing symptoms of gastrointestinal (GI) pathologies were used to assess levels of inflammatory cytokines, barrier integrity and cytokine production in response to microbial challenges. Increased expression of interleukin (IL)-6, but not interferon (IFN)γ, tumour necrosis factor (TNF)-α and IL-1β was observed during aging; further analysis showed that cluster of differentiation (CD)11c$^+$ dendritic cells (DCs) are one of the major sources of IL-6 in the aging 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 (ZO-1), occludin and junctional-adhesion molecule-A1 did not vary with age and overall permeability to macromolecules was not affected. Finally, cytokine production in response to different microbial stimuli was assessed in a polarized *in vitro* organ culture (IVOC). IL-8 production in response to flagellin declined progressively with age although the expression and distribution of toll-like receptor (TLR)-5 on intestinal epithelial cells (IECs) remained unchanged. Also, flagellin-induced production of IL-6 was less pronounced in aging individuals. 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 aging affects properties of the intestinal barrier likely to impact on age-associated disturbances, both locally and systemically.

Key words: aging, infection, innate immunity, interleukin 6, intestinal permeability, intestine.

INTRODUCTION

A hallmark feature of aging 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 the suggestion 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 of the effects of aging on the physical and immunological properties of the intestinal epithelial barrier

Abbreviations: CD, cluster of differentiation; CM, conditioned medium; CM_{AD}, CM from culture of adult biopsies; CM_{AG}, CM from culture of aged biopsies; CM_Y, CM from culture of young biopsies; ctk, cytokeratin; DC, dendritic cell; GI, gastrointestinal; HRP, horseradish peroxidase; HSD, honest significance difference; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; IVOC, *in vitro* organ cultures; JAMA-1, junctional adhesion molecule A 1; np, non polarized; p, polarized; RT, real-time; TEER, transepithelial electric resistance; TJ, tight junction; TLR, toll-like receptor; TNF, tumour necrosis factor; ZO-1, zonula occludens-1.

[1]These authors equally contributed to this work.

Correspondence: Dr Claudio Nicoletti (email claudio.nicoletti@ifr.ac.uk).

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 homoeostasis 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 homoeostasis 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 interleukin (IL)-8 (CXCL-8), monocyte chemoattractant protein (MCP)-1 (CCL2) and macrophage inflammatory protein (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 the present 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 aging on intestinal epithelial immunity to different types of microbial stimuli.

MATERIALS AND METHODS

Subjects and biopsies

Terminal ileum biopsies (up to eight 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 aging (67–77 years) and 19 young (7–12 years) individuals were considered to be healthy when not showing symptoms suggesting GI inflammatory disease/cancer during endoscopy and histology endoscopic inspection and later confirmed when routine histology excluded inflammation and neoplasia. Work has been carried out according to the Declaration of Helsinki (2013) and has been approved by the appropriate Ethics Committee (Comitato Etico Area Vasta Sud-Est, Italy and HRGC, Norwich, U.K.). All patients provided written informed consent (parental consent for children). Subjects had not been under medication for at least 3 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 and 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% CO_2) and maintained at 37°C. 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 Krebs' solution containing horseradish peroxidase (HRP type II; Sigma–Aldrich) and transmucosal transport was carried out as described [16]. Samples were collected at 20-min intervals for 100 min; 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 450 nm. Transepithelial electric resistance (TEER) was also assessed in Caco-2 cells in the presence or absence of conditioned medium (CM) from cultures of biopsies from the three donor groups. The cells were seeded on to the upper face of transwell inserts (6.5 mm diameter, 3.0 μm pore size, Corning, Costar) and grown on the filters for 14 days at 37°C, 5% CO_2, until fully differentiated. TEER was monitored at various intervals (Millicell-ERS, Millipore) in the presence or absence of CM obtained from biopsy cultures.

In vitro organ cultures

Non polarized (np)-*in vitro* organ culture (IVOC) biopsies were mounted on foam support, the foam was saturated with a bicarbonate-buffered culture medium consisting of Dulbecco's minimum essential medium and NCTC-135 medium (1:1) with 10% newborn calf serum plus 0.5% (w/v) 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 and continuously gassed with 95% O_2–5% CO_2, at 37°C. 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 (3 μm pore) and this was sandwiched between two 12-mm diameter acrylic glass [or polyvinyl chloride (PVC)] disks; with the upper disk provided with a 2-mm opening prepared in house by workshops at both University of Siena and Norwich Bioscience Institute. This was then accommodated into

a modified Snapwell chamber (Corning). 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 min and permeabilized with 0.5% Tween-20 for 10 min. After rinsing sections were incubated overnight at 4 °C with rabbit anti-TLR5 (toll-like receptor); (Invitrogen; gift from S. Schueller), quenched with 10% donkey serum and incubated with donkey Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch) for 60 min. Alternatively, sections were stained with rabbit anti-claudin 2 antibody (Life Technologies) followed by incubation with FITC-labelled anti rabbit IgG antibody (Sigma) and counter-stained with mouse anti-pan cytokeratin (ctk) 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 microscope.

Preparation of conditioned medium from biopsy culture

Immediately after removal, biopsies were placed in a plastic tube containing 1 ml of IVOC medium and kept in 95% O_2–5% CO_2 atmosphere at 37 °C for 5–6 h. Following centrifugation (250 ***g*** for 15 min) the CM was filtered sterile with centrifuge tube filters (0.22 μm), aliquoted and stored for no more than 10 days at $-80C^{\circ}$ until used.

Effects of CM on biopsies and cell culture

The effects of mucosa-derived soluble mediators present in the CM on tight junction (TJ) expression and intestinal permeability were then assessed in both np-IVOC of biopsies and Caco-2 cells. Biopsies were cultured in np-IVOC for 10–12 h in the presence of CM and the medium was replaced with fresh solution every 3–4 h. Caco-2 cells were cultured in the presence of CM for up to 36 h and TEER monitored at various intervals. CM was added to both compartments of the trans-well culture and its volume normalized to the weight of biopsies. In some case, CM was pre-incubated with various concentrations of either anti-IL-6, anti-IL-1β or anti-TNF (tumour necrosis factor)-α antibodies for 3 h at 4 °C. At the end of the culture, both tissues and Caco-2 cells were then used for RNA preparation

Gene expression

Total RNA was extracted from human terminal ileum biopsies or Caco-2 IECs TRIzol reagent (Invitrogen). Following evaluation of RNA integrity by gel electrophoresis RNA reverse transcription was carried out by iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The real-time (RT)-cDNA reaction products were subjected to quantitative RT PCR using the MyiQ single-colour RT 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 RT PCR reactions were performed in triplicate. Primers used are described in Supplementary Materials 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 are expressed as mean $\pm$ S.D. and analysed using Student's unpaired *t* test or Tukey HSD (honest significance difference) test for multiple samples comparison. A *P*-value < 0.05 was considered significant.

RESULTS

Intestinal levels of IL-6 increased in the aging human ileum

Regulatory cytokines produced by IECs and gut-resident immune cells contribute to establish and maintain the intestinal immune homoeostasis; however at this time, very little is known about the effects of age on levels of regulatory cytokines in the human gut. Initially, two matched biopsies from eight individuals from the adult (20–40 years) and aging (67–77 years) groups were used to monitor the expression of cytokine IL-6, interferon (IFN)-γ, IL-1β and TNF-α some of which have been observed to increase significantly at systemic levels in aging 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 aging group showing significantly higher levels (Figure 1A; $P < 0.01$). Levels of IL-6 mRNA were then further assessed in an additional five individuals per age group that also included individuals between 7 and 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 with aging 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 of total protein in the aging donors compared with both young (5.1 ± 3.0 pg/mg of total protein) and adult (5.4 ± 2.2 pg/mg/total protein) individuals. Furthermore, gene expression analysis of cytokeratin $(ctk)^+$ cluster of differentiation $(CD)45^-$ IECs, $CD11c^+$ DCs and the remaining $CD11c^-$ immune cell population showed that $CD11c^+$ cell population displayed a significant increase in IL-6 expression in aging donors ($P < 0.05$; 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 focused 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 express similar levels of CD86 and did not expressed E-cadherin, a marker for inflammation-associated gut-DCs [20]; in contrast, expression

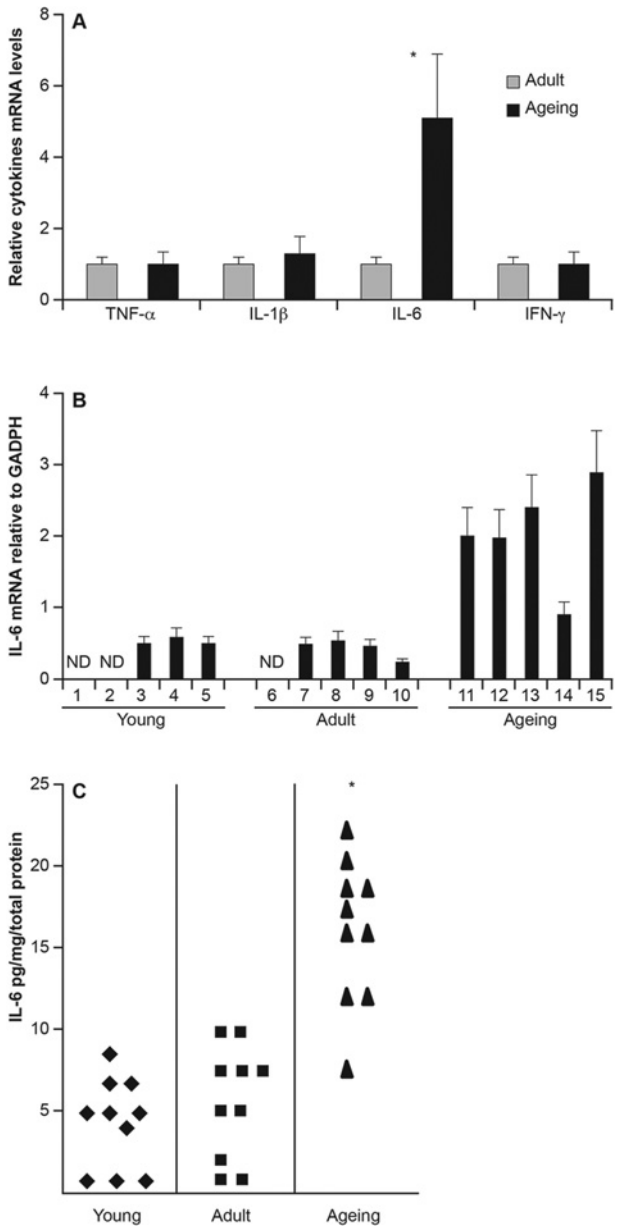

Figure 1 Levels of regulatory cytokines in the small intestine of individuals of different ages

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

of CD40 was higher in CD11c$^+$ cells from aging donors (Figure 2C). Also, isolated CD11c$^+$ DCs from aging donors produced a significant higher levels of IL-6 after 48 h in culture (Figure 2D). Furthermore the percentage of IL-6-producing CD11c$^+$ cells in the isolated fraction increased in aging individuals (Supplementary Figures S1A and S1B). It has to be stressed that given the difficulties in recruiting patients in the young group (7–12 y), we used, for this experiment, biopsies from adult and aging individuals only. These data demonstrated an age-associated increased production of IL-6; they 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 aging in the small intestine

The notion that aging 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 TJs, 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 out post-IVOC structural analysis of the terminal ileum by TEM and light microscopy (LM). These did not reveal major age-associated alterations of the overall morphology/structure of the TJs and intestinal mucosa and showed that tissue retained good morphology after 10 h culture in p-IVOC (Figures 3A–3D). In contrast, subsequent functional and molecular analysis of TJ mRNA expression showed the presence of age-associated alterations. First, TEER, a measure of the ionic gradient across freshly collected ileal biopsies was determined in an Ussing chamber. We observed that TEER was significantly reduced in biopsies from aging individuals ($P < 0.01$) whereas no difference was observed between the two younger groups (Figure 3E). Increase in permeability in the aging small intestine appeared to be restricted to solutes, indeed flux of HRP (approximately 44 kDa) did not vary between the different age groups (Figure 3F) showing that permeability to macromolecules was not affected by aging. Permeability assay was paralleled by the analysis of mRNA levels of TJs. 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 with what has been observed previously in colonic biopsies of non-human primates [24], we did not observe any significant age-related effect on the expression of ZO-1, occludin and JAMA-1 (Figures 4A–4C). 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 aging group compared with adult individuals (Figure 4D). Further immunohistochemistry analysis showed the absence of age-associated changes in the distribution of claudin-2 in aged tissues (Figure 4E–4H).

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 CM from cultures of biopsies of different age on the TEER and expression of claudin-2 in human intestinal epithelial Caco-2 cells and biopsies from adult donors. Treatment of Caco-2 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 (CM_{AD})

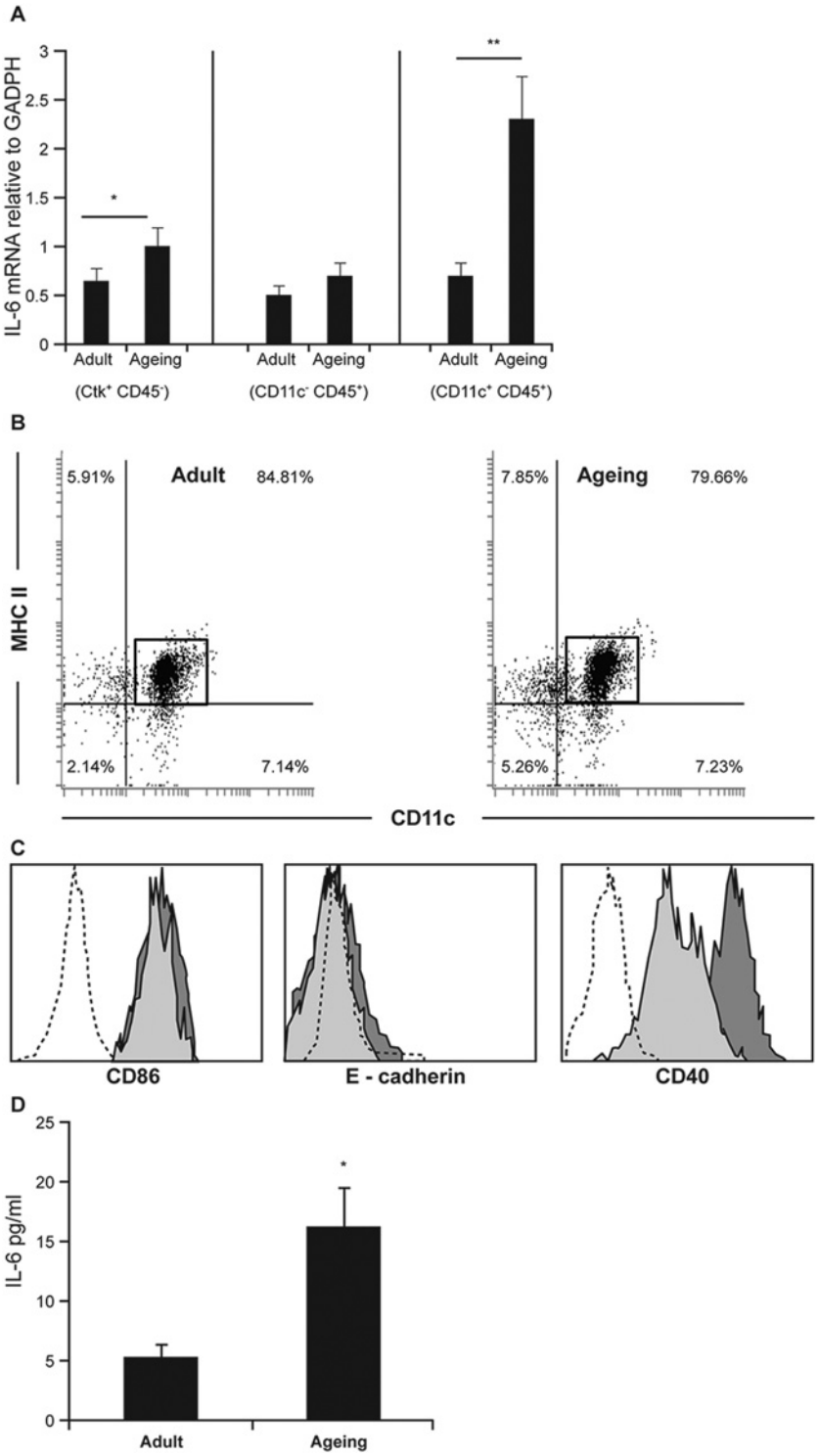

Figure 2 Expression of IL-6 in isolated intestinal cell populations

Three cell populations, IEC (Ctk^+CD45^-), $CD11c^-CD45^+$ and $CD11c^+CD45^+$ were isolated from biopsies (6–8 biopsies/individual) of donors (five individuals/group) of different age and assessed for IL-6 expression (**A**). A trend towards increase in 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 whereas higher expression of CD40 was observed in the aging group. Increased production of IL-6 by $CD11c^+$ cells from aging donors was further confirmed by ELISA using culture (48 h) supernatants (**D**). This set of experiments was performed using only biopsies from adult (n=5/group; 20–40 y) and aging (67–77 y) individuals due to difficulties in recruiting donors in the young group. Statistical analysis was carried out using Student's unpaired t test.

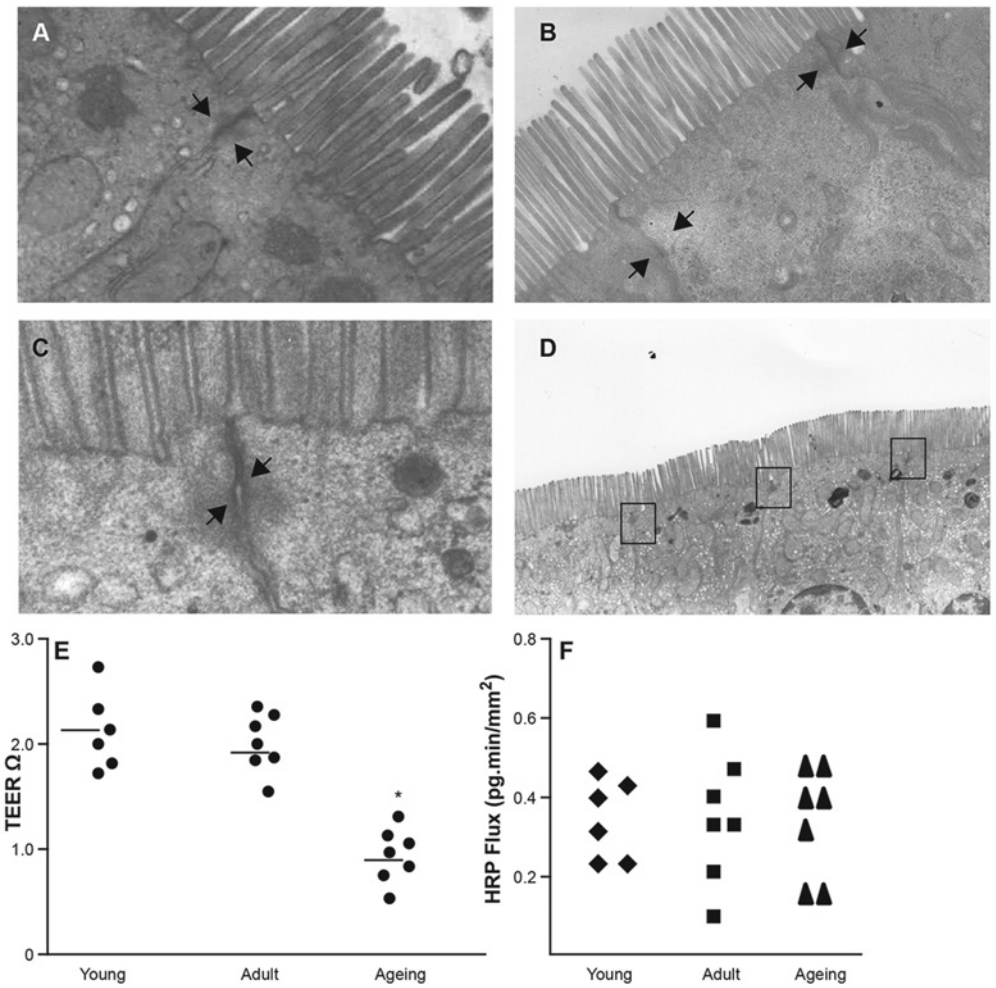

Figure 3 Intestinal permeability to solutes and macromolecules in aging

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

biopsies 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. Caco-2 cells treated with CM_{AG} showed a significant increase in the expression of claudin-2 compared with untreated Caco-2 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 Caco-2 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 claudin-2 when cultured in the presence of CM_{AG} compared with 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 aging 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 cytokines, 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 aging. Terminal ileum biopsies were challenged for 10–12 h with flagellin using a p-IVOC culture, a model that allows reproducing faithfully real-life host–pathogen interaction by restricting the antigenic challenge to the mucosal apical side

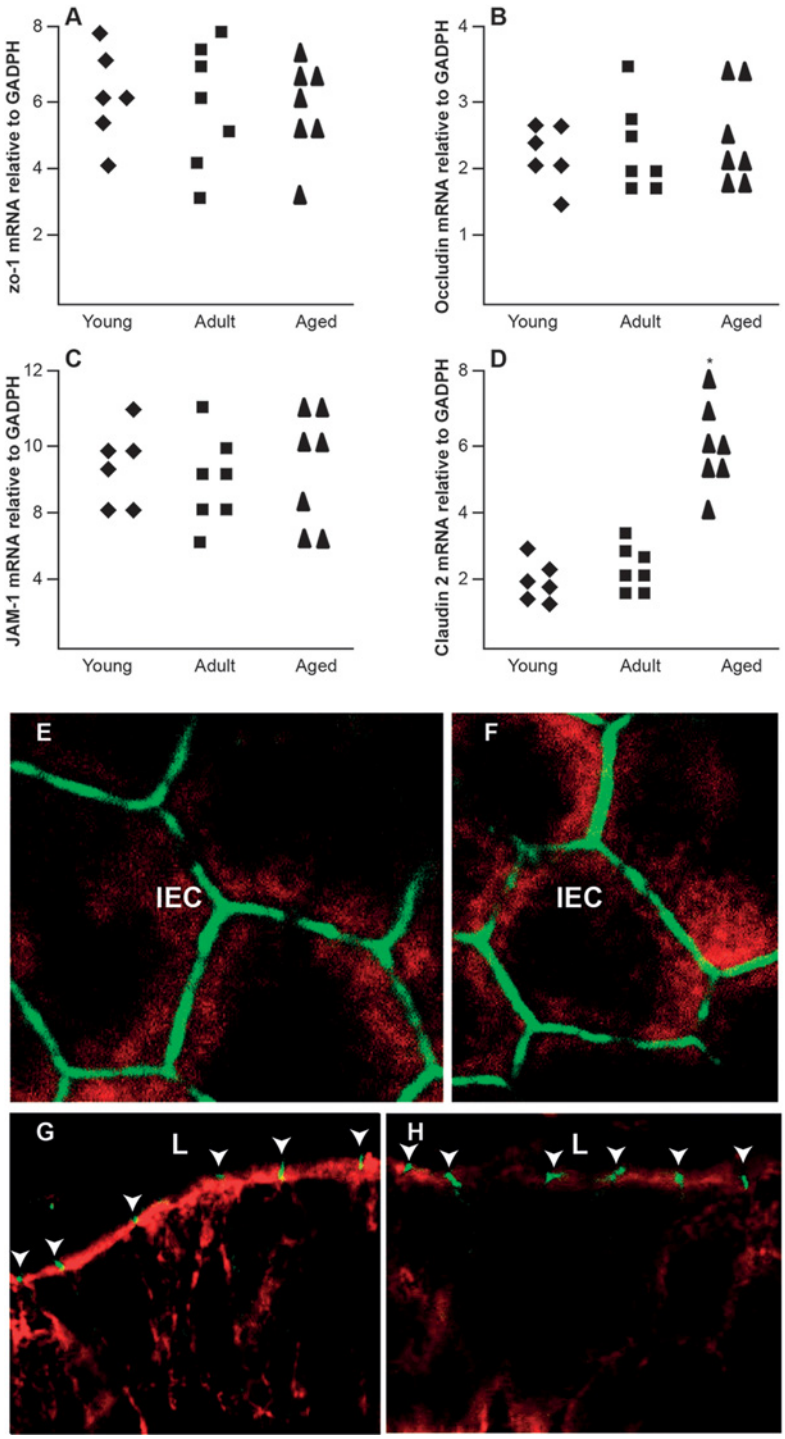

Figure 4 **Expression of TJs in the aging gut**

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 aging individuals (**D**) ($P < 0.01$) compared with younger individuals. Each symbol represents the average from two biopsies/individual (6–8 individuals/group). Statistical analysis was carried out using Tukey HSD test for multiple samples comparison; asterisk (*) indicates significant difference. Also, distribution of claudin-2 did not change with age (**E**–**H**). Cross-section of biopsies (apical area) from adult (**E**) and aging (**F**) individuals showed that claudin-2 (green) is restricted to the TJ complex between adjacent epithelial cells (IEC). Similar distribution was seen in longitudinal section of biopsies from adult (**G**) and aging (**H**) individuals; also in this case claudin-2 (green indicated by arrow heads) was restricted at the apical domain (L, lumen) as part of the TJ complex. Sections were counterstained with anti-pan ctk antibody (red). As average, 3–5 sections from three biopsies/donor (three donors/group) were examined.

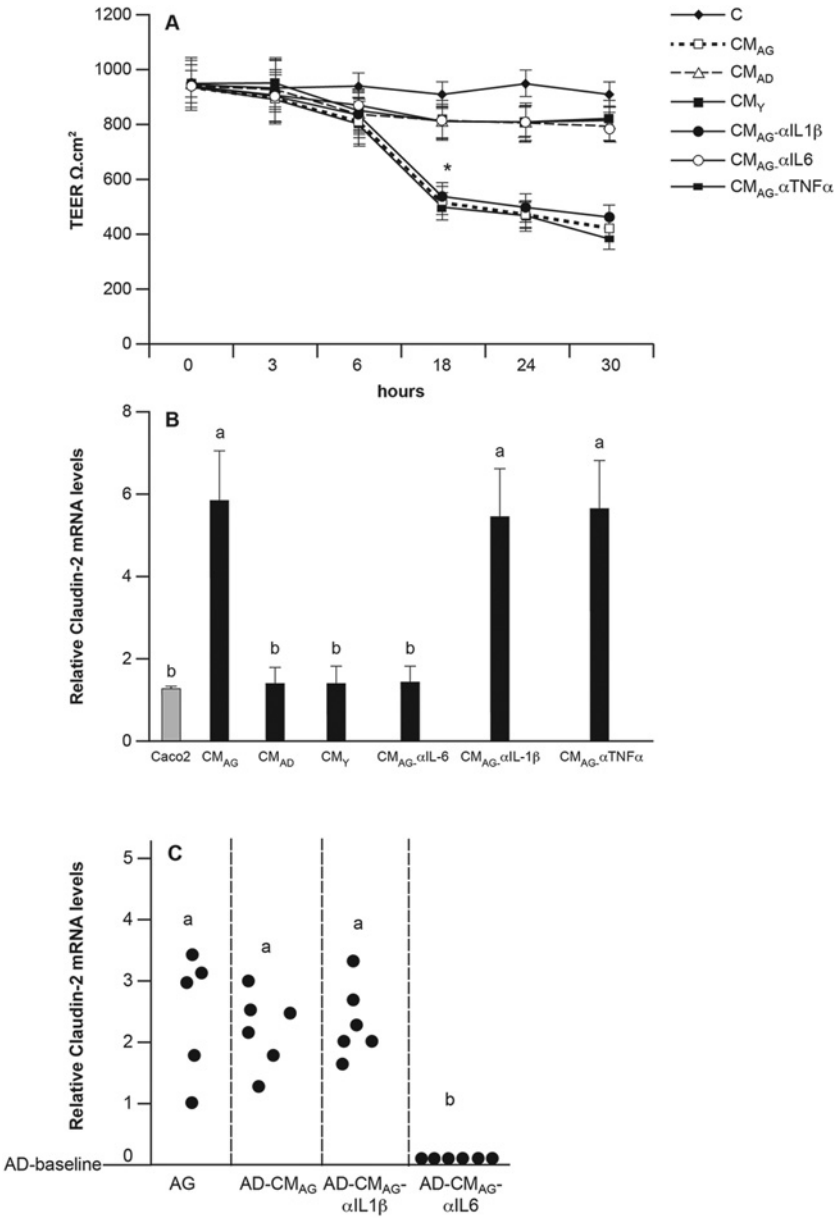

Figure 5 CM from cultures of aging biopsies affected intestinal permeability and expression of claudin-2

Caco-2 cells (**A**) were cultured in the presence of CM from CM_Y, CM_{AD} and CM_{AG} individuals and TEER monitored for at least 36 h. Both CM_{AD} and CM_{AG} did not have a significant effect on intestinal permeability, in contrast, addition of CM_{AG} to the culture had a significant impact on permeability that became evident after 18 h. Pre-incubation of CM_{AG} with anti-IL-6 antibody but not anti-IL-1β or anti-TNF-α prevented CM_{AG}-mediated increased permeability. Asterisk (*) indicates significant difference. RT-PCR analysis carried out after 24 h 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 with untreated biopsies from adults (AD-baseline) and comparable to what was observed in biopsies from aging donors (AG). Addition of anti-IL6 antibody (AD-CM_{AG}-αIL6) but not anti-IL1β (AD-CM_{AG}-αIL1β) prevented up-regulation 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.

and that has been already utilized to assess colonic tissue response to soluble flagellin [19]. We observed that a significant variation in IL-8 production in response to flagellin occurred across the course of life (Figure 6A). Tissue levels of IL-8 reached their 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 with 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 (Figures 6B and 6D). Furthermore, although a

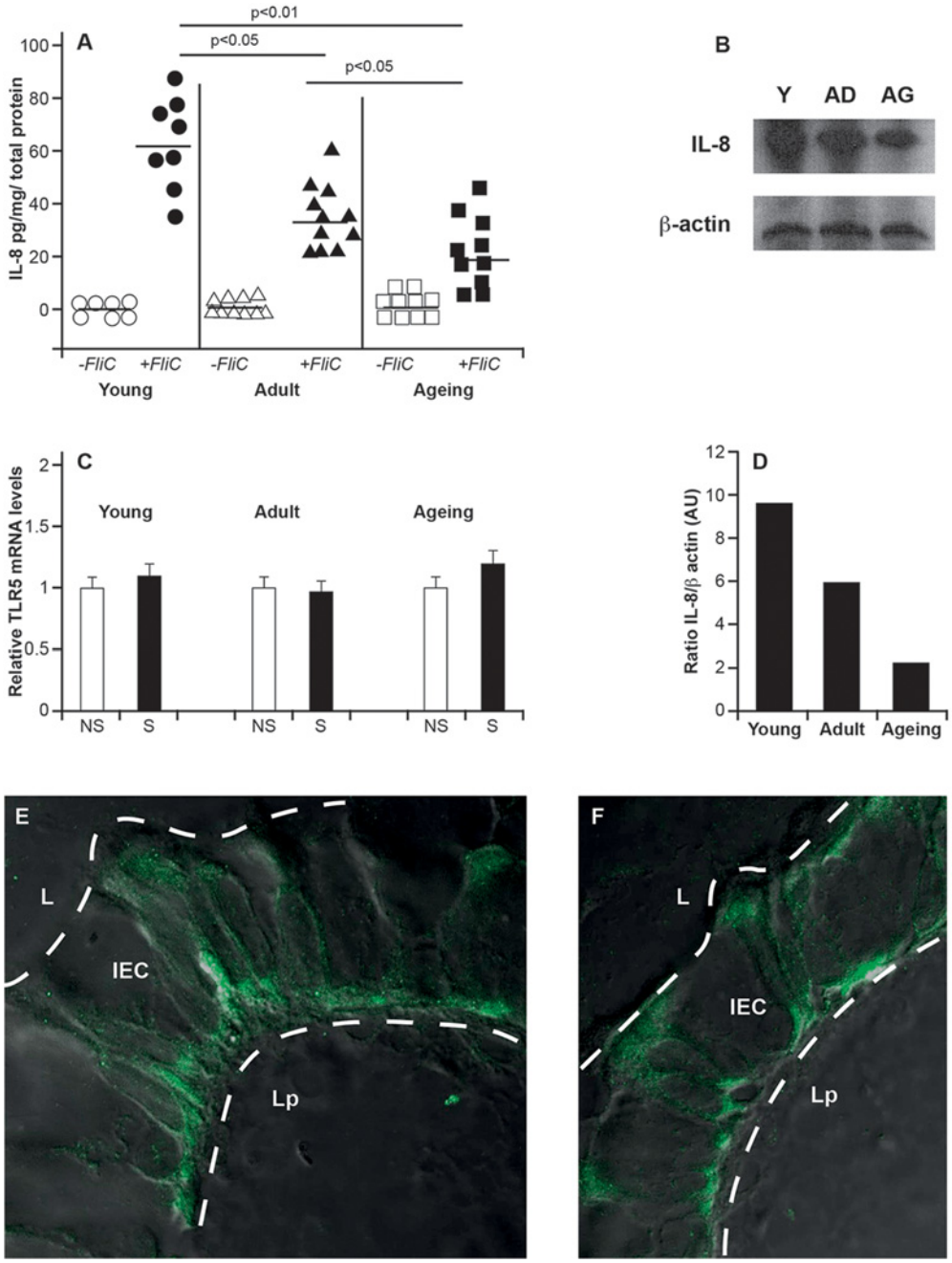

Figure 6 **Intestinal response to flagellin**

Tissue levels of IL-8 in response to flagellin were assessed after 10 h p-IVOC. Levels of IL-8 progressively declined from young individuals to adult and continued to decline further in aging subjects (**A**) as seen by ELISA. IL-8 tissue levels are expressed relative to amounts of total protein. Age-dependent variation of tissue levels of 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 with 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** and **F**). Statistical analysis was done using Tukey HSD test for multiple samples comparison (**A**) and Student's unpaired *t* test was used in (**C**).

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 aging; pre-treatment of biopsies with neutralizing anti-IL-6 antibody (IgA isotype) did not restore the ability to produce IL-8 in aging subjects (result 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 the absence of age-associated changes in the distribution of mature TLR5 (Figures 6E and 6F). 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 aging (Supplementary Figure S3A)

Aging 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 TNF-α rather than its suppression. Indeed, production of TNF-α 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 aging did affect the ability of the gut epithelium to produce TNF-α in response to VSL#3. The pattern appeared to be different compared with 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 TNF-α 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 TNF-α (Figure 7B); this suggested that at least in this experimental setting VSL#3 did not restore the compromised intestinal permeability.

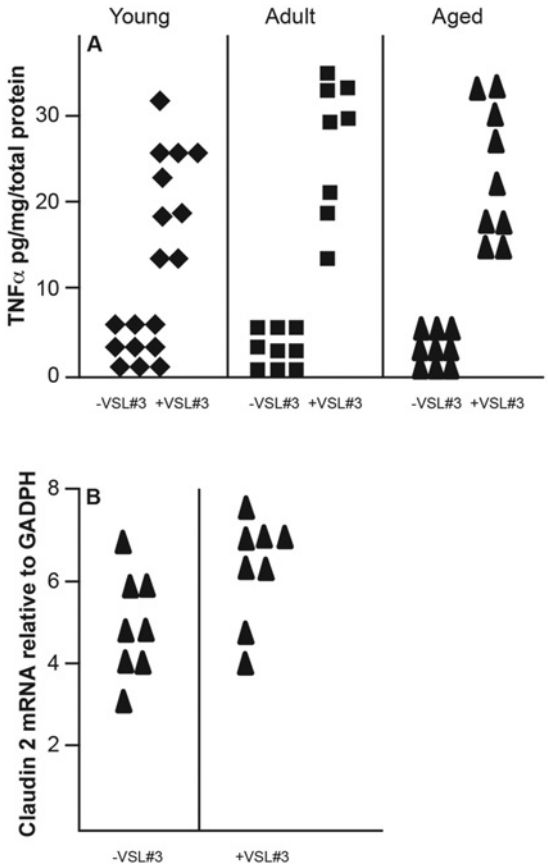

Figure 7 VSL#3 probiotics induced normal production of TNF-α in aging but did not down-regulate expression of claudin-2

Challenge of biopsies with live probiotics for 10 h in p-IVOC induced the production of similar levels of TNF-α irrespective 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 aging 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**).

DISCUSSION

Compared with immunosenescence of systemic immunity, age-associated changes in the mucosal immune system are less well understood. In particular, at the 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 aging 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 aging affects important physical and immunological functions of the intestinal epithelial barrier. Our first objective was to determine the levels of inflammatory cytokine in the aging 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 aging 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 TJs 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 aging 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 whereas terminal ileum DCs are characterized by increased production of IL-6 in aging, 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 TJ 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 (1 Å=0.1 nm) [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 aging biopsies prevented both claudin-2 overexpression and decline of TEER strongly suggested that IL-6 is the main factor underlying claudin-2 up-regulation in aging. Age-associated increase in 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 the systemic level. Indeed, although it has been known for some time that aging in humans is associated with increased levels of circulating IL-6 and that this has a strong association with markers of physical frailty [32–34] 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 bacterial 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 inflammaging [3] at systemic level by contributing to promote the differentiation of pro-inflammatory T helper (TH)17 cells [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 aging 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 antigens of different nature we observed that the production of cytokines in the gut in response to different microbial challenges in aging may or may not decline, possibly depending on the nature of the antigenic stimulus. Indeed, whereas epithelial production of IL-8 in response to flagellin progressively declined across life and it is significantly compromised in aging the production of TNF-α in response to exposure to probiotics did not decline in the elderly. Interestingly, in contrast with a previous report [40], elevated levels of IL-6 did not affect the expression of flagellin-specific TLR5 on IECs that remained unchanged in aging. Thus, it would seem that increased levels of IL-6 alone did not suffice to induce major changes of the expression of TLR5 and dysregulation of the major TJs. 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 TNF-α, 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 TNF-α, 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 affect local and possibly systemic age-associated disorders.

CLINICAL PERSPECTIVES

- The effects of aging on physical and immunological properties of the intestinal epithelial barrier in humans are largely unknown.
- We report that the aging gut is characterized by higher levels of the cytokine IL-6 that in part affects intestinal permeability. Furthermore, aging 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.

- The present 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 in 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, procured 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 Research Council, grant "The Gut Health and Food Safety-ISP" [grant number BB/J004529/1 (to C.N.)] and University of Siena intramural funds (to E.B.).

REFERENCES

1 Ershler, W.B. (2003) Biological interactions of aging and anemia: a focus on cytokines. J. Am. Geriatr. Soc. **51**, S18–S21 CrossRef PubMed
2 Gardner, I.D. (1980) The effect of aging on susceptibility to infection. Rev. Infect. Dis. **2**, 801–810 CrossRef PubMed
3 Franceschi, C. and Campisi, J. (2014) Chronic inflammation (inflammageing) and its potential contribution to age-associated diseases. J. Gerontol. **69** Suppl1, S4–S9 CrossRef
4 Guigoz, Y., Dore, J. and 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 CrossRef PubMed
5 Man, A.L., Gicheva, N. and Nicoletti, C. (2014) The impact of ageing on the intestinal epithelial barrier and immune system. Cell Immunol. **289**, 112–118 CrossRef PubMed
6 Turner, J.R. (2009) Intestinal mucosal barrier function inhealth and disease. Nat. Rev. Immunol. **9**, 799–809 CrossRef PubMed
7 Bleau, C., Karelis, A.D., St-Pierre, D.H. and 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., doi:10.1002/dmrr.2617
8 Maes, M., Kubera, M. and Leunis, J.C. (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**, 117–124 PubMed
9 Forsyth, C.B., Shannon, K.M., Kordower, J.H., Voigt, R.M., Shaikh, M., Jaglin, J.A., Estes, J.D., Dodiya, H.B. and 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 CrossRef PubMed
10 Vrieze, A., de Groot, P.F., Kootte, R.S., Knaapen, M., van Nood, E. and 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. and Walker, D.W. (2012) Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. **109**, 21528–21533 CrossRef PubMed
12 Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., Viale, G., Allavena, P. and Rescigno, M. (2005) Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat. Immunol. **6**, 507–514 CrossRef PubMed
13 Iliev, I.D., Mileti, E., Matteoli, G., Chieppa, M. and Rescigno, M. (2009) Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal. Immunol. **2**, 340–350 CrossRef PubMed
14 Mowat, A.M. (2003) Anatomical basis of tolerance and immunity to intestinal antigens. Nat. Rev. Immunol. **4**, 331–341 CrossRef
15 Sansonetti, P.J. (2004) War and peace at mucosal surfaces. Nat. Rev. Immunol. **4**, 953–964 CrossRef PubMed
16 Greenwood-Van Meerveld, B., Tyler, K. and Keith, Jr, J.C. (2000) Recombinant human interleukin-1 modulates ion transport and mucosal inflammation in the small intestine and colon. Lab. Invest. **80**, 1269–1280 CrossRef PubMed
17 Hicks, S., Candy, D.C. and Phillips, A.D. (1996) Adhesion of enteroaggregative Escherichia coli to pediatric intestinal mucosa *in vitro*. Infect Immun. **64**, 4751–4760 PubMed
18 El Asmar, R., Panigrahi, P., Bamford, P., Berti, I., Not, T., Coppa, G.V., Catassi, C. and Fasano, A. (2002) A. Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. Gastroenterology **123**, 1607–1615 CrossRef PubMed
19 Schuller, S., Lucas, M., Kaper, J.B., Girón, J.A. and Phillips, A.D. (2009) The *ex vivo* response of human intestinal mucosa to enteropathogenic *E. coli* infection. Cell Microbiol. **11**, 1607–1615 CrossRef
20 Siddiqui, K.R., Laffon, S. and Powrie, F. (2010) E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. Immunity **23**, 557–567 CrossRef
21 Hollander, D. and Tarnawski, H. (1985) Aging-associated increase in intestinal absorption of macromolecules. Gerontology **31**, 133–137 CrossRef PubMed
22 Suzuki, T., Yoshinaga, N. and Tanabe, S. (2011) Interleukin-6 (IL-6) regulates Claudin-2 expression and tight junction permeability in intestinal epithelium. J. Biol. Chem. **286**, 31263–31271 CrossRef PubMed
23 Van Itallie, C.M., Holmes, J., Bridges, A., Gookin, J.L., Coccaro, M.R., Proctor, W., Colegio, O.R. and Anderson, J.M. (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 CrossRef PubMed
24 Tran, L. and Greenwood-Van Meerveld, B. (2013) Age-associated remodeling of the intestinal epithelial barrier. J. Gerontol. A Biol. Sci. Med. Sci. **68**, 1045–1056 CrossRef PubMed
25 Eckmann, L., Kagnoff, M.F. and Fierer, J. (1993) Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun. **61**, 4569–4574 PubMed
26 Pagnini, C., Saeed, R., Bamias, G., Arseneau, K.O., Pizarro, T.T. and Cominelli, F. (2010) Probiotics promote gut health through stimulation of epithelial innate immunity. Proc. Natl. Acad. Sci. U.S.A. **107**, 454–459 CrossRef PubMed

27 Mann, E.R., Bernardo, D., English, N.R., Landy, J., Al-Hassi, H.O., Peake, S.T., Man, R., Elliott, T.R., Spranger, H., Lee, G.H. et al. (2015) Compartment-specific immunity in the human gut: properties and functions of dendritic cells in the colon versus the ileum. Gut, doi: 10.1136/gutjnl-2014-307916

28 Al-Sadi, R., Ye, D., Boivin, M., Guo, S., Hashimi, M., Ereifej, L. and Ma, T.Y. (2014) Interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by JNK pathway activation of claudin-2 gene. PLoS One **9**, e85345 CrossRef PubMed

29 Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A.H., Bürgel, N., Fromm, M. et al. (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 CrossRef

30 Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T. and Fujiyama, Y. (2003) Increased expression of interleukin 17 in inflammatory bowel disease. Gut **52**, 65–70 CrossRef PubMed

31 Mankertz, J., Amasheh, M., Krug, S. M, Fromm, A., Amasheh, S., Hillenbrand, B., Tavalali, S., Fromm, M. and Schulzke, J.D. (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 CrossRef PubMed

32 Ershler, W.B. and Keller, ET. (2000) Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu. Rev. Med. **51**, 245–270 CrossRef PubMed

33 Roubenoff, R. (2014) The cytokine for gerontologist has some company. J. Gerontol. A Biol. Sci. Med. Sci. **69**, 163–164 CrossRef PubMed

34 Sanders, J.L., Ding, V., Arnold, A.M., Kaplan, R.C., Cappola, A.R., Kizer, J.R., Boudreau, R.M., Cushman, M. and Newman, A.B. (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**, 174–181 CrossRef

35 Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V. et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell **139**, 485–498 CrossRef PubMed

36 Claesson, J., Jeffery, IB., Conde, S., Power, S.E., O'Connor, E.M., Cusack, S., Harris, H.M., Coakley, M., Lakshminarayanan, B., O'Sullivan, O. et al. (2012) Gut microbiota composition correlates with diet and health in the elderly. Nature **488**, 178–184 CrossRef PubMed

37 Kimura, A. and Kishimoto, T. (2010) IL-6: regulator of Treg/Th17 balance. Eur. J. Immunol. **40**, 830–835 CrossRef

38 Schmitt, V., Rink, L. and Uciechowski, P. (2013) The Th17/Treg balance is disturbed during aging. Exp. Gerontol. **48**, 1379–1386 CrossRef PubMed

39 Perona-Wright, G., Jenkins, S.J., O'Connor, R.A., Zienkiewicz, D., McSorley, H.J., Maizels, R.M., Anderton, S.M. and MacDonald, AS. (2009) A pivotal role for CD40-mediated IL-6 production by dendritic cells during IL-17 induction *in vivo*. J. Immunol. **182**, 2808–2815 CrossRef PubMed

40 Sánchez-Muñoz, F., Fonseca-Camarillo, G., Villeda-Ramírez, M.A., Miranda-Pérez, E., Mendivil, E.J., Barreto-Zúñiga, R., Uribe, M., Bojalil, R., Domínguez-López, A. and Yamamoto-Furusho, J.K. (2011) Transcript levels of toll-like receptors 5, 8 and 9 correlate with inflammatory activity in ulcerative colitis. BMC Gastroenterol. **11**, 138–142 CrossRef PubMed

Received 13 January 2015/11 March 2015; accepted 31 March 2015

Published as Immediate Publication 7 May 2015, doi: 10.1042/CS20150046