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Acoustic stress induces long term severe intestinal inflammation in the mouse

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ABSTRACT

The influence of noise on the presentation and progression of inflammatory bowel diseases has been poorly analyzed. We designed this study to investigate immediate and late effects of acoustic stress (AS) on small intestine. To this aim, CBA/J, BALB/c and DBA/2 mice were divided into AS and control groups. AS mice were exposed to noise (300 Hz–70 dB) during 24hs and randomized into: A) Acute effects group: mice were killed after AS; L) Late effects group: mice were killed 3 weeks after AS and O) Over-exposed effects group: mice were submitted to AS once a week during a month and killed. Small intestine sections were histologically examined. The expression of cytokines (IL-17, IL-22, TNF-α, INF-ɣ and TGF-β), CCL-25 and Ki67 was studied by immunohistochemistry and immunofluorescence techniques. "A" group displayed short and fragmented villi, diminished number of lamina propria cells, leucocyte infiltration, higher number of goblet cells and predominance of IL-17 expression. "L" group showed epithelial proliferative foci (CCL25 + Ki67 +) and increased TNFα/TGF-β expression. Tissue damage was aggravated in "O" group. In conclusion, AS is able to trigger a severe intestinal inflammatory process in healthy mice, which spontaneously amplifies and perpetuates. Noise might be harmful to humans by aggravating inflammatory bowel diseases.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing disorder which comprises two main forms: Crohn's disease (CD) and ulcerative colitis (UC). The inflammation in CD may affect any part of the gastrointestinal tract whereas UC is restricted to the mucosa of the colon. IBD has long been associated with an increased risk for colon cancer (Baumgart and Sandborn, 2012; Danese and Fiocchi, 2011). Although the etiology of IBD is largely unknown, it involves a complex interaction between genetic, luminal, and environmental factors that trigger an inappropriate mucosal immune response (Kaser et al., 2010; Maloy and Powrie, 2011). In the last years, a rising trend in the incidence and prevalence of IBD has been recognized. It has been postulated that this phenomenon may be related to the westernization of lifestyles, including dietary habits and industrialization (Thia et al., 2008). Moreover, there is a growing body of evidence demonstrating the role of psychological stress, anxiety and depression in IBD presentation and progression (Häuser et al., 2014). Rodent models of IBD have also revealed significant effects of stress on inflammation in the bowel affecting intestinal permeability, cytokine expression, cell adhesion molecules, IgA secretion and T cell response (Campos-Rodríguez et al., 2013; Israeli et al., 2008; Reber et al., 2011; Schultz et al., 2008). However, the influence of stressful environmental factors such as noise on the course of these pathologies has been poorly studied.

Occupational and environmental noise levels may cause auditory and non-auditory deleterious effects on health including disturbances in metabolism, reproduction, cardiovascular and neuroendocrine systems, cognition and sleep (Kight and Swaddle, 2011; Basner et al., 2014). In particular, gut lesions have been associated with the non-auditory effects of noise. It has been reported that noise is able to increase intestinal permeability (Bijlsma et al., 2001), disrupt the epithelium, induce degranulation of mucosal mast cells and edema of intestinal villi (Baldwin et al., 2006) and provoke superficial erosions and destruction of villi in laboratory rats (Fonsecaa et al., 2012). The aim of this work was to determine rapid, late or chronic acoustic stress (AS)-induced changes in the morphology and pattern expression of inflammatory cytokines/chemokines and the cell proliferation marker Ki-67 in the mouse small intestine. The influence of gender and genetic background on the AS response was also analyzed.

The sound stress model employed in this work was originally used by Arck et al. (1995) in two H-2d × H-2k mouse crossbreedings: a high fetal loss model (CBA/J × DBA/2) and a low fetal loss one (CBA/

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J × BALB/c) where DBA/2J-mated CBA/J female mice exposed to AS showed a significant increase in the abortion rate. It was shown that AS induces an inflammatory response in the decidua mediated by an increase of pro-inflammatory cytokines, recruitment of inflammatory cells (Blois et al., 2005; Prados et al., 2011) together with reduction in progesterone levels (Joachim et al., 2003), T regulatory cell activity and galectin-1 expression (Blois et al., 2007) that finally caused abortion.

The present work reports that a single 24hs AS exposure is enough to trigger an intestinal inflammatory picture with immunohistological alterations and tissue damage that spontaneously perpetuates and amplifies. Immediate response is characterized by leucocyte infiltration, high expression of IL-17 and IL-22 and low TNF-α while in a late stage, TNF-α predominates with abundant epithelial proliferative foci and increased goblet cells that invade the short villi. Chronic exposure to AS aggravates deeply gut damage. The AS-induced effects were mildly influenced by gender and strain. These results indicate that sound stress has to be considered as a risk factor for human IBD.

2. Materials and methods

2.1. Animals

Two-month-old CBA/J (H-2d), BALB/c (H-2k) and DBA/2 (H-2k) female and male mice were purchased from Comisión Nacional de Energía Atómica (Argentina). Animals were housed in our institutional facilities at least for two weeks until being used in any experiment (acclimatization phase). Animals were maintained on a 12-h light/dark cycle in groups of 2 and had free access to standard rodent chow pellets and water. The procedures employed in these studies were approved by the Institutional Committee for the use and care of laboratory animals (CICUAL, Facultad de Medicina, Universidad de Buenos Aires) which follows the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Experimental design

The procedure for AS induction was performed as described by Arck et al. (1995). Mice were exposed to a sound stress during 24hs using a rodent repellent device (Conrad Electronics, Germany) that emitted at a frequency of 300 Hz and an intensity of 70 dB. The sound lasted for 1 s and was emitted four times per minute. Light/dark cycles were maintained during stress exposure. In all experiments, two animals were placed in one standard cage. The device was placed into the cage to guarantee sound perception. During the AS, animals did not receive food and were provided with water *ad libitum*.

Mice were randomized into three studied groups (each one, n = 10) as follows: 1) Acute effects group (“A”): mice received a single AS and were sacrificed immediately afterwards; 2) Late effects group (“L”): mice received the same AS protocol and then returned to their usual habitat where they stayed during 3 weeks until being sacrificed. This group was used to evaluate the effects of AS after a recovering period or later time; 3) Over-exposed effects group (“O”): mice were submitted to AS once a week during a month and afterwards they were sacrificed. This group was used to study the chronic exposure to the sound stimulus. For each described experiment, a control animal group (“C”) was included, receiving equal treatment with the exception of AS exposure.

Mice of the “L” and “O” groups as well as their respective controls were weighed throughout the experiment. The amount of drinking water per day was also registered.

2.3. Small intestine isolation and processing

Mice were exsanguinated and the small intestine was removed, bathed and washed by flushing with cold PBS. Paraffin embedded small intestine sections were prepared as follow: tissues were fixed in 95% ethanol pre-cooled at 4 °C, dehydrated in pre-cooled absolute ethanol, clarified with pre-cooled xylene and embedded in paraffin at 56 °C. Tissue sections (4–5 μm thickness) were cut and placed on glass slides. Sections were used for different stainings: Hematoxylin-Eosin (H & E), for examining the general histology; May Grunewald-Giemsa, for distinguishing blood cells and PAS (Periodic Acid Schiff)/Hematoxylin, for the identification and counting of goblet cells due to their content in acidic and neutral mucins. Results were expressed as the mean value and the standard deviation of the number of goblet cells counted in 4 fields at 250× magnification. Paraffin embedded sections were also employed for immunohistochemical and immunofluorescence studies.

2.4. Immunohistochemistry

The expression of Ki67, TNF-α and IFN-ɣ was studied by immunohistochemistry on paraffin-fixed tissue sections. Tissue sections were rinsed in PBS and then incubated for 30 min at room temperature with 0.3% H2O2 in methanol. Sections were blocked with 10% normal serum from the same species of the secondary antiserum for 15 min at 37 °C and then incubated overnight at 4 °C with the primary antibodies: rabbit anti-Ki67 (Abcam), goat anti-TNFα or goat anti INF-ɣ (both Santa Cruz Biotech.). The corresponding biotin-conjugated secondary antibody (anti-rabbit IgG Biotin-conjugated or anti-goat IgG Biotin-conjugated, both Santa Cruz Biotech.) was then added for 30 min at 37 °C. Color was developed using the Vectastain® ABC peroxidase kit (Vector Laboratories) and Diaminobenzidine Substrate kit (Vector Laboratories). The sections were counterstained with Mayer’s hematoxylin (Biopur) and mounted employing Canadax® (Biopur). As negative controls, sections were incubated with PBS instead of the primary antibody. The histochemical labelling was analyzed by two independent observers.

2.5. Immunofluorescence studies

The expression of IL-17, IL-22 and the chemokine CCL-25 was performed by immunofluorescence technique on paraffin-fixed gut sections. The primary antibodies used were: rabbit IgG anti IL-17, rabbit IgG anti CCL-25 (both Peprotech) or goat IgG anti IL-22 (Santa Cruz Biotechnology) and the secondary ones were respectively: anti-rabbit F(ab)2 fragment and anti-goat IgG, both Alexa 546 conjugated (Invitrogen). Tissue sections were observed in an Olympus confocal microscope (FV300/BX61) by two blinded investigators. Images were analyzed with the Fluoview 5.0 Software.

2.6. Statistics

Immunohistochemical and immunofluorescent pictures were analyzed with the ImageJ 1.42q software (National Institutes of Health, USA). Results were expressed as mean ± standard deviation (SD) for each group. One-way analysis of variance (ANOVA) and the Newman-Keuls Multiple Comparison Test were performed to compare mean differences among the groups using GraphPad Prism 5-Graphics Software. p-Values < 0.05 were considered significant. Representative microscopic pictures were registered for each experimental group.

3. Results

3.1. Mice clinical status

Mice belonging to the three AS experimental groups (A, L and O) showed no clinical alterations such as gastrointestinal manifestations, diarrhea or other clinical disorders. Even though no signs of aggressive behavior were observed between the members of a cage in any group, AS-treated mice appeared with increased excitability in comparison to controls. No change in the body weight or in daily water consumption was registered after AS exposure (groups L and O).

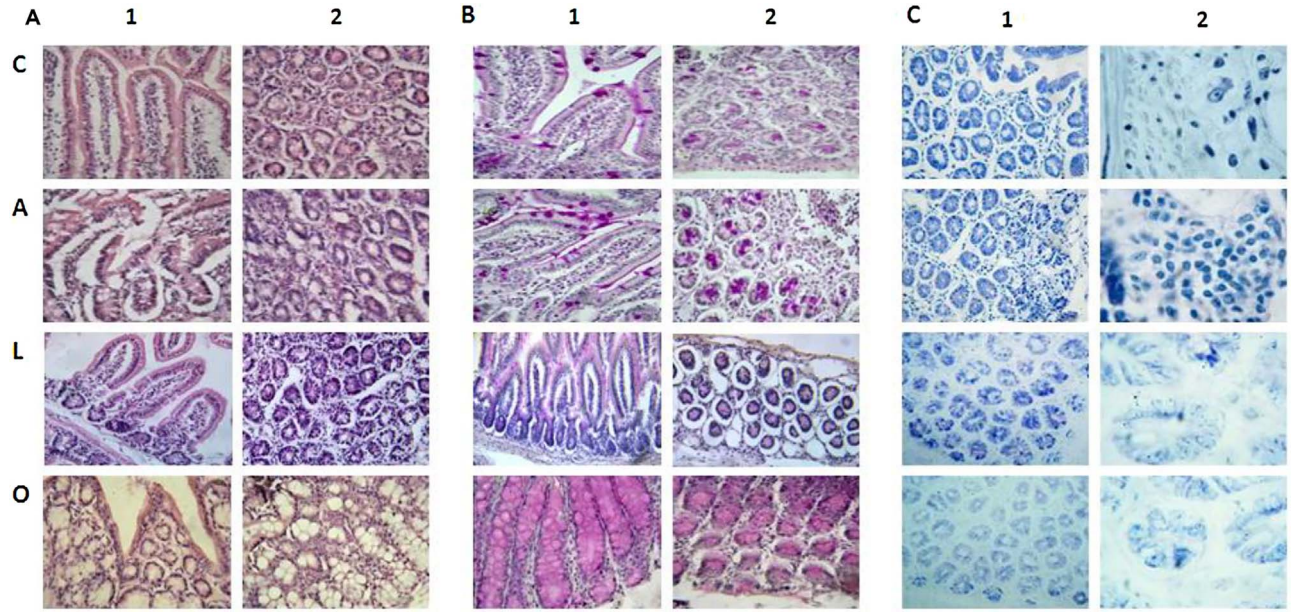

Fig. 1. Histological alterations in mouse small intestine after AS exposure.
The photographs show small intestine sections from CBA/J female mice of the following experimental groups: (C) control (no AS treatment); (A) acute effects observed immediately after AS; (L) late effects observed three weeks after AS; (O) effects after four weekly AS, n = 10 per group. A) H & E staining; B) PAS-Hematoxylin staining. For A and B: column 1 represents intestinal villi (250×); column 2 represents the crypt zone (250×). C shows the crypt zone stained with May Grünwald-Giemsa: column 1 at 250× and column 2 at 1000× magnification.

3.2. Histological alterations

Following the published works of Arck et al., we first evaluated the effects of AS on CBA/J female mice. H & E staining of gut sections from mice killed immediately after sound exposure (A group) showed disorganized, shorter and fragmented intestinal villi and a decreased number of lamina propria cells in comparison to the control group. Interestingly, female CBA/J mice killed 3 weeks after the single AS (L group) exacerbated the histological alterations showing broken and deformed villi with fewer lamina propria cells. Additionally, A and L groups showed altered crypts containing H & E unstained cells. The group exposed to repeat AS (O group) showed more severe histological alterations, massive intestinal villi disorganization, broken and very short villi and loss of lamina propria cells. Notably, crypts in these samples were completely full of unstained cells which invaded lamina propria of villi. (Fig. 1A)

PAS-H stain allows the identification of mucins, characteristic of the presence of goblet cells which are stained purple. Goblet cells were counted (4 fields/section at 250× magnification). Mean results showed an increase immediately after AS (C Group: 19 ± 3; A Group: 40 ± 3, C *vs* A $p < 0.001$), which was higher three weeks later (L Group: 57 ± 5, C *vs* L $p < 0.001$; A *vs* L $p < 0.05$). The content of mucins in crypts also increased in both A and L groups in comparison to control animals. This effect was aberrant in mice subjected to repeated AS (O group), where crypts filled of mucins (which were previously looked colorless with H & E staining) completely invaded intestinal villi. (Fig. 1B).

May Grünwald-Giemsa staining showed that small intestine sections from mice exposed to AS and sacrificed (A group) presented a massive leucocyte infiltration with a high number of neutrophils. In contrast, no leucocyte infiltration was detected in the other groups (L and O) (Fig. 1C).

These results indicate that small intestine of female CBA/J mice exposed to AS showed important histological alterations which varied with the experimental AS protocol used. To evaluate whether gender or genetic background modify the intestine reaction to AS, we next repeated the three AS protocols (A, L and O groups and their controls) employing CBA/J male mice, DBA/2 and BALB/c male and female mice (each group, n = 5) and compared the histological alterations in the small intestine employing the same staining. Results showed that gut from DBA/2 and BALB/c male and female mice respond to AS with similar histological alterations to those seen in CBA/J female mice. Small intestine sections from AS-treated CBA/J male maintained the increase in the number of goblet cells but showed less histological damage than the others even though with clear differences when compared to the control group (data not shown). These results indicate that mice exposed to AS develop profound histological alterations in the small intestine and this response can be mildly influenced by the genetic and hormone backgrounds.

3.3. CCL25 and Ki67 expression

The chemokine CCL25 is selectively and constitutively expressed in the small intestinal epithelium and plays an important role in mediating recruitment of CCR9+ gut tropic B and T lymphocytes being responsible for the pool of intra-epithelial lymphocytes and resident memory cells in lamina propria (Svensson et al., 2002). Moreover, CCL25 was also related with the intestinal generation of murine small-intestinal CD8αα intraepithelial lymphocyte compartment (Marsal et al., 2002). In this work, CCL25 expression was investigated in small intestine sections of control and AS mice by an immunofluorescence assay (Fig. 2A). Results showed that mice sacrificed after AS (A group) increased the expression of CCL25 in villi and crypts epithelium and in lamina propria cells. This increase was higher three weeks after the original AS (L group), Fig. 2B. Interestingly, different villi zones presented frequently CCL25 multilayers suggesting the existence of proliferative zones pointed in the photograph. Chronic exposure to AS diminished the expression of this chemokine (O group).

To evaluate a proliferative response of epithelial cells to AS exposure, the cell proliferation marker Ki67 was investigated in small intestine sections by immunohistochemistry (Fig. 3A). In animals sacrificed after AS (A group), Ki67 increased in lining and crypt epithelium. Interestingly, three weeks after the first exposure (L group), multiple thick foci of KI67+ cells were observed over the short villi. This aberrant pattern was exacerbated in the overexposed group (O group) while the expression of this marker in the whole tissue

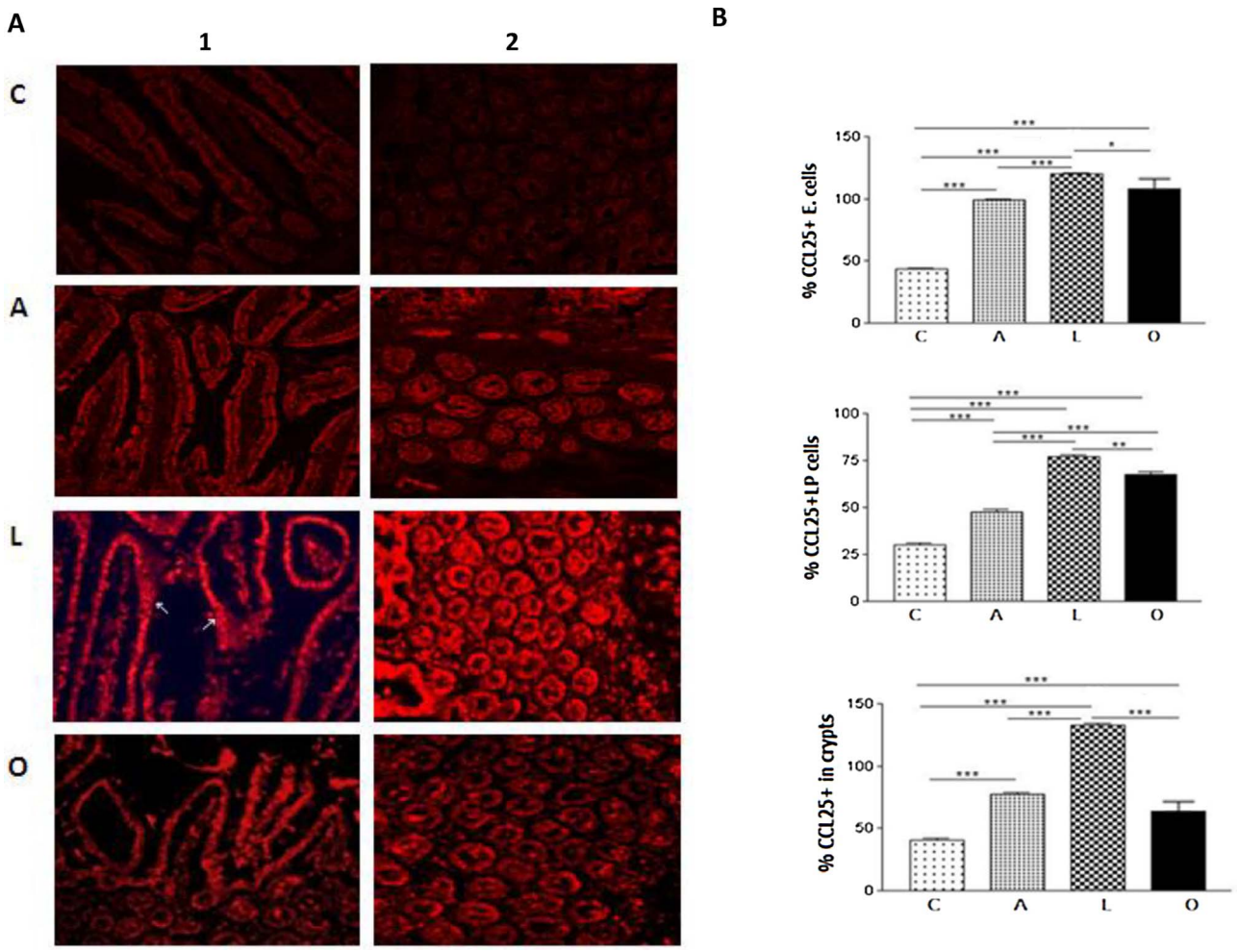

Fig. 2. CCL25 expression in small intestine after AS exposure.
A) The photographs show representative small intestine sections from the following experimental groups: (C) control (no AS treatment); (A) acute effects observed immediately after AS; (L) late effects observed three weeks after AS; (O) effects after weekly AS over-exposure, n = 10 per group. CCL25+ cells (in red) were detected by immunofluorescence and visualized by confocal microscopy. Column 1 represents intestinal villi; column 2 represents the crypt zone (both at 400× magnification). In L group, column 1: arrows indicate multilayers of CCL25+ cells that suggest proliferative zones. B) Statistical analysis. The bars of the graphs represent the mean and the standard deviation of % CCL25+ cells in the epithelial lining (E. cells), in lamina propria (LP) and in the crypt zone (*P < 0.5; **P < 0.01; ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diminished (Fig. 3B).

3.4. IL-17 and IL-22 expression

Expression of IL-17 and IL-22 in small intestine was evaluated by immunofluorescence. Results of IL-17 tissue expression are shown in Fig. 4A. The intestinal expression of IL-17 peaked in mice sacrificed immediately after AS (A group) where IL-17 was expressed by epithelial cells of villi and crypts. Moreover, abundant IL-17 positive cells were detected between the interface of epithelium and lamina propria (Fig. 4B). IL-17 expression diminished to control levels three weeks post-AS (L group) while repeated exposures to AS did not induce its expression (Fig. 4C). These data indicated that IL-17 expression constitutes an important acute response of small intestine to AS.

Results of IL-22 expression are shown in Fig. 4D. Mice sacrificed after AS (A group) showed crypts with many IL-22 positive cells. Epithelial lining increased its expression and lamina propria looked moderately positive. Mice sacrificed three weeks after AS (L group) diminished the expression in crypts and epithelium while isolated positive cells appeared in lamina propria which increased in the over-exposed (O) group (Fig. 4E and F).

3.5. TNF-α, INF-γ and TGF-β expression

The expression of these cytokines was investigated by immunohistochemistry in small intestine sections. The results of TNF-α tissue expression are represented in Fig. 5A and B and show that this cytokine was only mildly increased in lamina propria cells after AS (A group) but increased mainly in the whole tissue three weeks after AS (L group) and in the over-exposed group (O). In contrast, INF-γ was poorly detected in control and the three AS-treated groups (data not shown).

The expression of TGF-β diminished scarcely immediately after AS (A group). However, it significantly increased especially in epithelial lining three weeks after AS (L group) and after re-exposures to AS (O group) (Fig. 5C and D).

4. Discussion

In the present study, results showed that after AS exposure, mouse small intestine displayed tissue disorganization, short and fragmented intestinal villi, diminished number of lamina propria cells, massive leucocyte (mainly neutrophils) infiltration and increased number of goblet cells. During the acute response to AS, CCL25 was upregulated while IL-17 represented the main cytokine, which was detected in the epithelial cell lining, in cells located at the interface with lamina

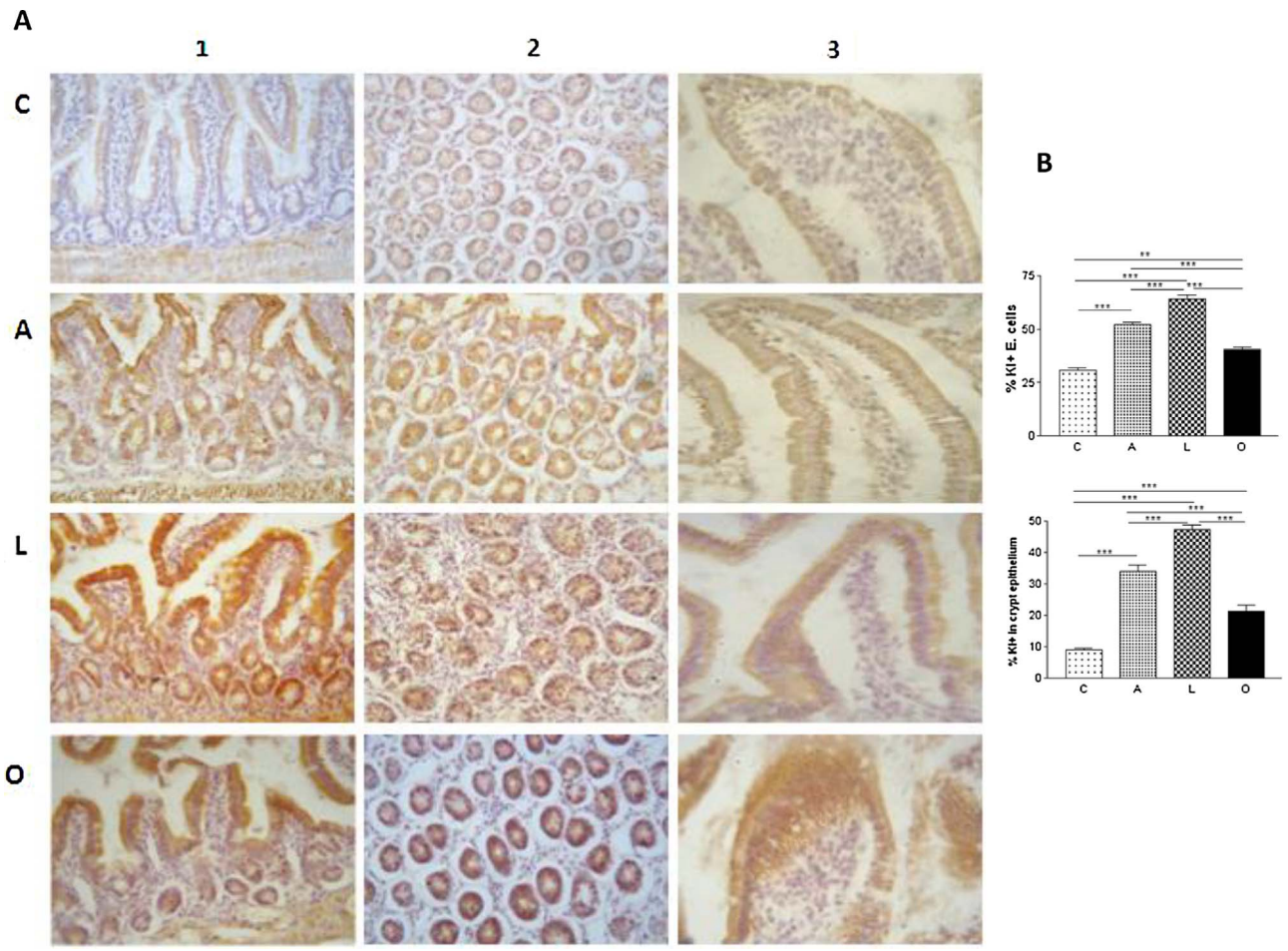

Fig. 3. Ki67 expression in small intestine after AS exposure.
A) The photographs show representative small intestine sections from the following experimental groups: (C) control (no AS treatment); (A) acute effects observed immediately after AS; (L) late effects observed three weeks after AS; (O) effects after weekly AS over-exposure, n = 10 per group. Ki+ cells were stained brown by immunohistochemical detection and visualized by optical microscopy. Column 1 represents intestinal villi; column 2 represents the crypt zone (both at 250× magnification). Column 3 shows intestinal villi at 400× where multiple thick foci of KI67+ cells were observed in the L and O groups. B) Statistical analysis. The bars of the graphs represent the mean and the standard deviation of% Ki+ cells in the epithelial lining and crypt (**P < 0.01; ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

propria and in crypts. It would likely be the main factor responsible for the recruitment of neutrophils detected at this moment. The IL-17 family consists of six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F (Iwakura et al., 2011). All IL-17 family members share the C-terminal cysteine-knot motif. At this early innate response, mainly IL-17A and IL-17F can be secreted by many cell types: activated monocytes, neutrophils, γdT cells and innate lymphoid cells (ILC) such a NK, NKT, ILC3 (Björkström et al., 2013; Sabat et al., 2013) and bound to the IL-17 receptor (IL-17R) in epithelial cells. However, epithelial cells may directly contribute to IL-17F production under certain inflammatory conditions (Ishigame, 2009). In addition, Ramirez-Carrozzi (2011) reported that intestine epithelial cells are able to early produce IL-17C in an autocrine manner in response to inflammatory stimulus favoring the synthesis of cytokines, chemokines and antimicrobial peptides, as do IL-17A and IL-1 7F. As a consequence, the strong detection of this cytokine in the whole epithelial lining in mice sacrificed after AS would be also due to its own epithelial production of IL-17C and IL-17F recognized by the antibody used.

In the same experimental group, we detected a moderate increase of IL-22 (in epithelial lining and crypts) and TNF-α (in lamina propria cells) with a mild diminishing of the anti-inflammatory cytokine TGF-β. During immune responses, IL-22 and IL-17A/F are often simultaneously present at high levels in inflamed tissues. IL-22 is a member of the IL-10 cytokine family. Compatibly, these mediators can be secreted by numerous identical types of cells from the lymphoid lineage even rarely secreted by exactly the same cell population (Sabat et al., 2013). ILCs are often the source of IL-22 (Björkström et al., 2013; Dumoutier et al., 2011; Spits and Cupedo, 2012). Immune cells do not bear the IL-22 receptor complex. However, in the gut, IL-22R1 is expressed by intestinal epithelial cells and intestinal subepithelial myofibroblasts (Wolk et al., 2010). IL-22 prominently stimulates the production of antimicrobial acting proteins including Reg3c, lipocalin-2 and β-defensins and also mucins and directly contributes to the repair and maintenance of epithelial barriers. In this sense, in agreement with the proliferative effects of IL-22 on epithelial cells, we detected an increase of KI67+ intestine epithelial cells in mice exposed to AS suggesting a protective role of this cytokine during this early immune response to AS, as was previously proposed (Sugimoto et al., 2008; Zenewicz et al., 2008).

Surprisingly, three weeks after the single AS (L group), histological alterations exacerbated. IL-17 was not detected, which likely could explain the absence of leukocyte infiltration. IL-22 expression diminished while TNF-α increased in all tissue, probably participating in the perpetuation of inflammation. At the same moment, TGF-β expression increased in epithelial cells and lamina propria. However, considering the deterioration of the small intestine tissue with respect to the conditions observed immediately after AS (A group), this increase failed to suppress the inflammatory circuit triggered by the sound stimulus. Besides, several epithelial proliferative foci of CCL25+Ki67+ cells were detected along the intestinal villi. This fact would indicate that

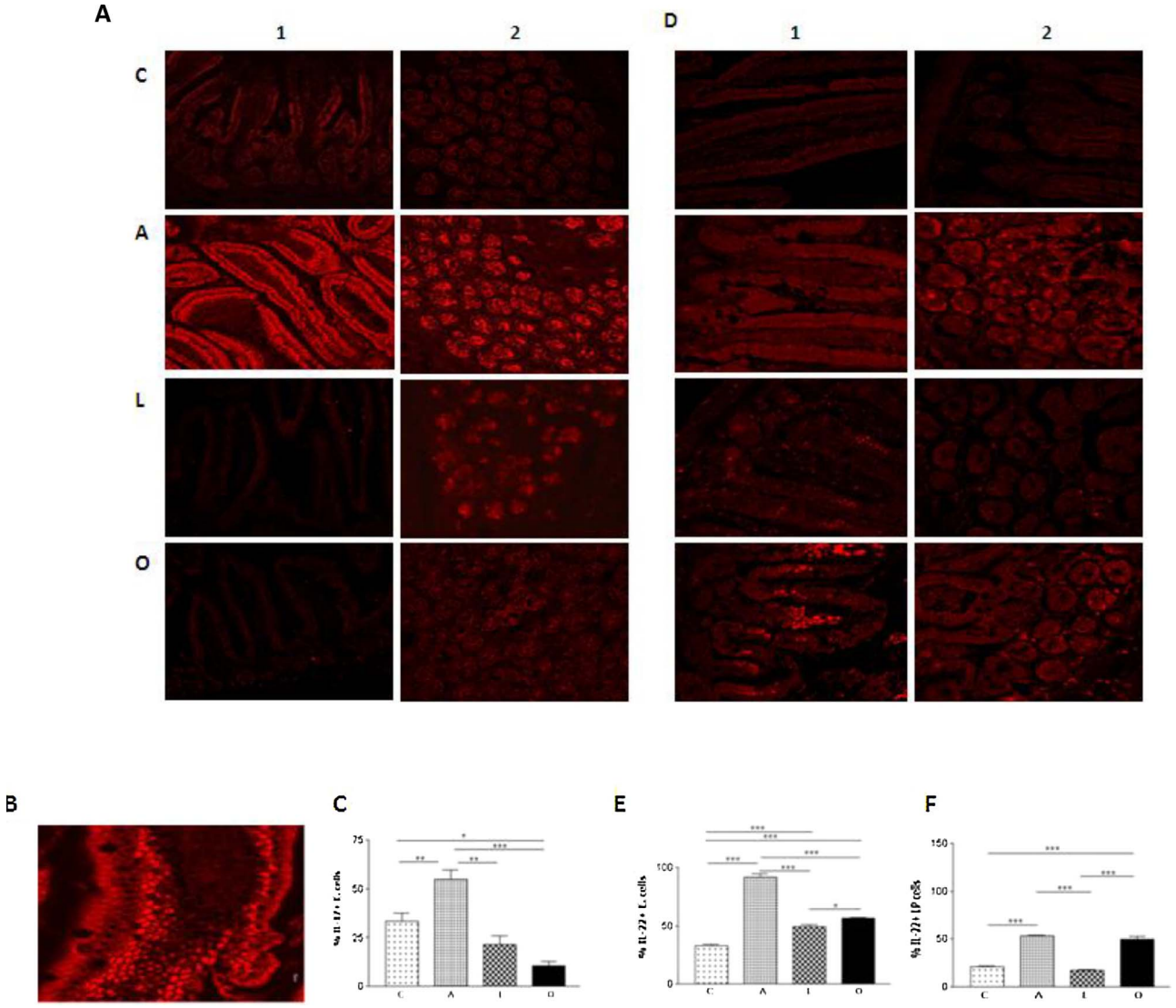

Fig. 4. IL-17 and IL-22 expression in small intestine after AS exposure.
A, B and C: IL-17 expression; D, E and F: IL-22 expression. Positive cells (in red) were respectively detected by immunofluorescence and visualized by confocal microscopy. The photographs show representative small intestine sections from the following experimental groups: (C) control (no AS treatment); (A) acute effects observed immediately after AS; (L) late effects observed three weeks after AS; (O) effects after weekly AS over-exposure, n = 10 per group. Sections A and D: Column 1 represents intestinal villi; column 2 represents the crypt zone (both at 400× magnification). In B: abundant IL-17 positive cells were detected between the interface of epithelium and lamina propria in the A group (600×). Statistical analysis. The bars of the graphs represent the mean and the standard deviation of% IL-17+ cells in the epithelial lining (C) and of% IL-22+ cells in epithelial cells (E) and lamina propria (F) (*P < 0.5; **P < 0.01; ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

under inflammatory conditions, other factors different from IL-22 are responsible for this effect.

Re-exposure to AS (O group) induced severe histological alterations: massive tissue disorganization, very short and broken villi frequently invaded by crypts full of mucins and abundant foci of proliferative epithelial cells. The expression of TNF-α increased in the whole tissue suggesting that this cytokine could play a main role in the maintenance of the pro-inflammatory local circuit. Despite the poor histological conditions, epithelial cells and lamina propria cells maintained a moderate capacity to synthesize TGF-β. As in L group, a high expression of CCL25 was observed along the epithelium lining.

In the late (L) and overexposed (O) groups, it is expected that activated T cells added to the inflammatory scenario. Naive CD4T cells can differentiate into the Th1 or Th17 lineage depending on the exogenous cytokine environment. IL-12/IFN-ɣ induce the expression of the transcription factor t-bet and drive the development of Th1 lineage cells which mainly produces IFN-ɣ, IL-2 and TNF-α. The initial development of Th17 cells is induced by a TGF-β and IL-6 synergism in the presence of IL-1β followed by their expansion through IL-21 and a stabilization phase driven by the pro-inflammatory cytokine IL-23 (Siakavellas and Bamias, 2012). The differentiation of Th17 cells requires a distinct set of transcription factors including signal transducer and activator of transcription 3 (Stat3), retinoic acid receptor related orphan receptor ɣ (RORɣ) and retinoic acid receptor-related orphan receptor α (RORα) (Dong, 2011). Th17 lineage mainly produces IL-17/IL-22 cytokines. However, Th17 lymphocytes displayed an extraordinary plasticity due to their ability to upregulate t-bet (transcription factor) generating Th1/Th17 double positive cells (Lee et al., 2009). Moreover, Th1- and Th17-mediated signaling may act in synergy. Such Th1/Th17 population was found in the inflamed gut mucosa (Annunziato et al., 2007) and may differentiate into specific Th1 cells, thus excluding the Th17 population (Cătană et al., 2015; Sallusto et al., 2012).

Our results showed a predominance of TNFα expression in response to AS during the chronic phase of gut inflammation. In agreement with our results, Th1-effects are more prominent during Crohńs disease, as compared to the Th17 signaling pathway (Strober and Fuss, 2011).

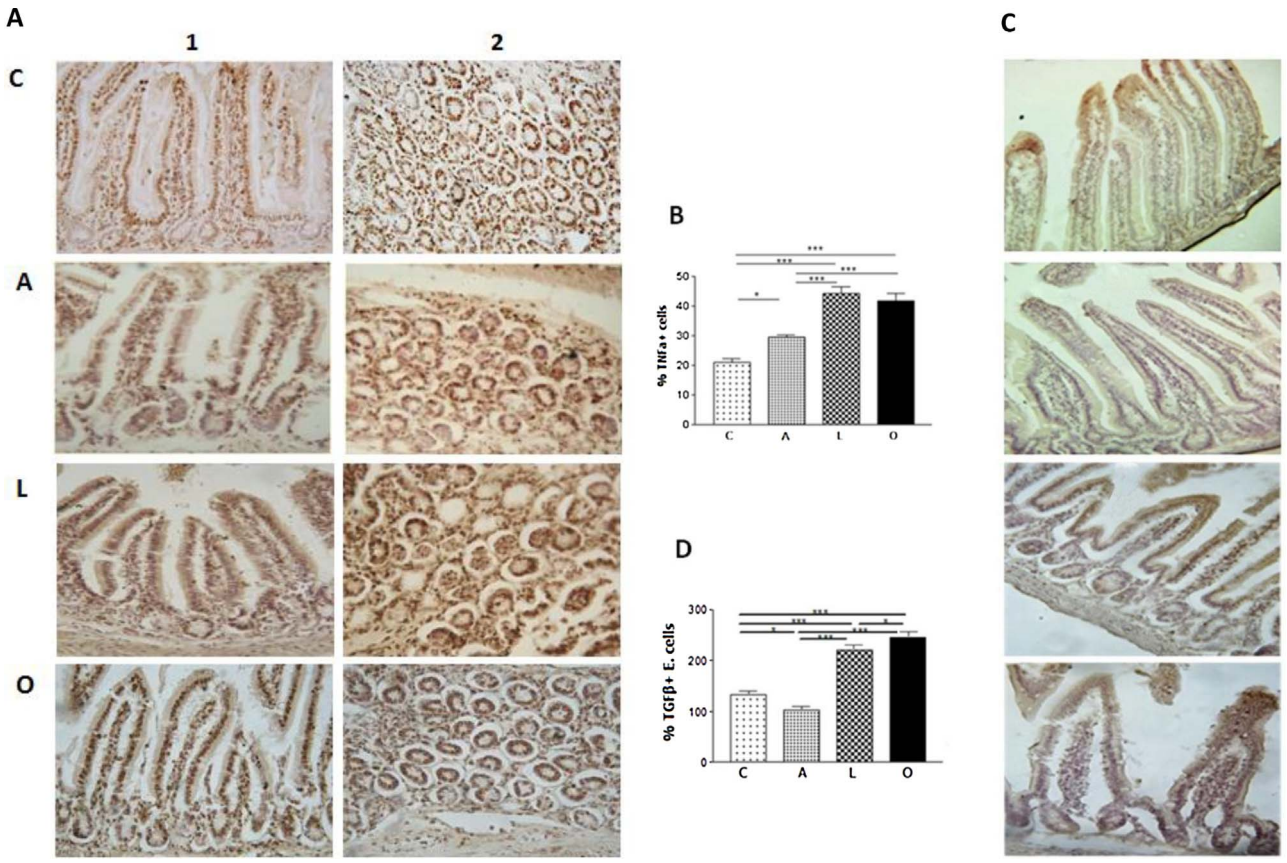

Fig. 5. TNFα and TGFβ expression in small intestine after AS exposure.
The photographs show representative small intestine sections from the following experimental groups: (C) control (no AS treatment); (A) acute effects observed immediately after AS; (L) late effects observed three weeks after AS; (O) effects after weekly AS over-exposure, n = 10 per group. A) TNFα+ cells (in brown) were detected by immunohistochemistry and visualized by optical microscopy. Column 1 represents intestinal villi; column 2 represents the crypt zone (both at 250× magnification). B) Statistical analysis. The bars of the graphs represent the mean and the standard deviation of% TNFα+ cells in the tissue (*P < 0.5; ***P < 0.001). C) TGFβ+ cells (in brown) were detected by immunohistochemistry and visualized by optical microscopy (intestinal villi, 250× magnification). D) Statistical analysis. The bars of the graphs represent the mean and the standard deviation of% TGFβ+ cells in the tissue (*P < 0.5; ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, TNF-α is considered a major mediator of intestinal inflammation in Crohn's disease. Neutralization of TNF-α has been shown to decrease recruitment of inflammatory cells and granuloma formation in several animal models and humans (van Dullemen, 1995) whereas anti-TNF-α therapy has been consistently shown to be effective in numerous clinical trials (Nguyen et al., 2015). In agreement with our results, a predominance of TNF-α producing lamina propria cells and a relative infrequency of cells secreting IFN-γ in Crohn's disease was reported by several groups (MacDonald et al., 1990; van Deventer, 1997). However, an increased mucosal production of IFN-γ by Th1 cells was also reported in some cases of Crohńs disease and in experimental models (Yamashita et al., 2013).

Despite the importance of CCL25/CCR9 in small intestinal immunity, the mechanisms underlying the selective and constitutive expression of CCL25 in the small intestine are not fully understood. In our experimental model, we detected an increase in the expression of this chemokine, especially in the L and O groups in the context of a severe inflammatory scenario meaning that lymphocyte homing but also CD8αα T cells *in situ* generation would be enhanced as has been demonstrated by us (manuscript in preparation). Interestingly, in contrast to most chemokines, CCL25 intestinal epithelial expression is independent of the presence of intestinal microflora or interactions with mature intra-epithelial lymphocytes as well as of signaling through the lymphotoxin β receptor, LPS and TNFα (Ericsson et al., 2006). Instead, CCL25 transcription depends on the intestinal environment including a local complex network of transcription factors not totally defined that appeared to be activated in the AS mice.

Noise may be harmful through a large range of mechanisms. First, loud noise may be very stressful. Short term exposure to noise increases corticosterone levels in mice serum (Guha et al., 1976; van Raaij et al., 1997) suggesting that noise induces a classical neural stress response in rodents. As reported by Friebe et al. (2011), employing a 24 h AS equivalent to this work, we found no effect on serum corticosterone levels in the experimental groups (data not shown). However, it was reported increased corticotrophin releasing hormone and brain vasopressin mRNA expression (Friebe et al., 2011) as well as locally produced neuropeptides, such as substance P (Joachim et al., 2001) and nerve growth factor (Tometten et al., 2006) which are able to activate mast cells, phagocytes, lymphocytes and vasculature inducing a neurogenic reaction.

Interestingly, noise may be also harmful through non-auditory pathways by means of a direct mechanical action of vibration. Nakamura et al. (1992) found that vibration induced stomach lesions in truncal vagotomized rats. In agreement with our present results, in a model of rats exposed to loud frequent noise, Fonseca et al. reported damage of the duodenal epithelial layer, villi with variable length and thickness and fragmentation as well as alterations in structures of the cytoskeleton which are proposed to be the primary target of vibration injury (Fonsecaa et al., 2012).

Once disrupted the intestinal epithelium, lumen resident bacteria as well as noxious agents such as fungi, parasites, toxins, undigested protein, fat, and waste that are normally not absorbed into the bloodstream in the healthy state, pass through a damaged gut and enter the bloodstream. In consequence, excess reactive oxygen species (ROS)

produced by activated phagocytes could contribute to the auto perpetuation of local inflammation. Additionally, epithelial cells are particularly sensible to perturbations of their homeostasis and responds generating an accumulation of misfolding proteins in the endoplasmic reticulum (ER), state known as ER stress. Protein misfolding in the ER of intestinal epithelial cells has increasingly been suggested to directly contribute to IBD and can be triggered due to an impaired integrated stress response to situations such as ER stress, oxidative stress, infections, inflammation, and others (Kaser et al., 2010; Luo and Cao, 2015; Maloy and Powrie, 2011). Strikingly, accumulation of misfolded MUC2 precursors in the ER of goblet cells with reduced mucin secretion, impaired mucus layers and inflammation involving both innate and adaptive immunity were detected in ulcerative colitis patients and in mice expressing a mutant Muc2 gene suggesting that the protein folding defect is relevant to goblet cell pathology in ulcerative colitis (Heazlewood et al., 2008; Eri et al., 2011). The generation of ER stress by exposure to AS is currently under study in our group and could explain the aberrant accumulation of mucins in crypts invading gut villi in AS overexposed mice (O Group) reported in this study.

5. Conclusions

The present study reports that an acoustic stimuli exposure is able to trigger a severe intestinal inflammatory process in healthy mice characterized by several immunohistological alterations and tissue damage. Small bowel effects amplify and perpetuate spontaneously, being practically independent on gender and mouse strain. This work sheds light on the local inflammatory circuit by which occupational and environmental noise might be harmful to humans by generating or aggravating inflammatory bowel diseases and hence it would be considered as a risk factor for this disease.

Conflict of interest

The authors declare that they have no conflicts of interests.

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