Necrotising enterocolitis

ORIGINAL ARTICLE

Amniotic fluid stem cells improve survival and enhance repair of damaged intestine in necrotising enterocolitis via a COX-2 dependent mechanism


ABSTRACT

Objective Necrotising enterocolitis (NEC) remains one of the primary causes of morbidity and mortality in neonates and alternative strategies are needed. Stem cells have become a therapeutic option for other intestinal diseases, which share some features with NEC. We tested the hypothesis that amniotic fluid stem (AFS) cells exerted a beneficial effect in a neonatal rat model of NEC.

Design Rats intraperitoneally injected with AFS cells and their controls (bone marrow mesenchymal stem cells, myoblast) were analysed for survival, behaviour, bowel imaging (MRI scan), histology, bowel absorption and motility, immunofluorescence for AFS cell detection, degree of gut inflammation (myeloperoxidase and malondialdehyde), and enterocyte apoptosis and proliferation.

Results AFS cells integrated in the bowel wall and improved rat survival and clinical conditions, decreased NEC incidence and macroscopic gut damage, improved intestinal function, decreased bowel inflammation, increased enterocyte proliferation and reduced apoptosis. The beneficial effect was achieved via modulation of stromal cells expressing COX-2 in the lamina propria, as shown by survival studies using selective and non-selective cyclooxygenase 2 inhibitors. Interestingly, AFS cells differentially expressed genes of the Wnt/β-catenin pathway, which regulate intestinal epithelial stem cell function and cell migration and growth factors known to maintain gut epithelial integrity and reduce mucosal injury.

Conclusions We demonstrated here for the first time that AFS cells injected in an established model of NEC improve survival, clinical status, gut structure and function. Understanding the mechanism of this effect may help us to develop new cellular or pharmacological therapies for infants with NEC.

INTRODUCTION

Necrotising enterocolitis (NEC) represents up to 10% of admissions to neonatal intensive care unit, and remains a major cause of neonatal morbidity and mortality despite changes in medical and surgical treatment. Although administration of breast milk, arginine or probiotics may reduce the incidence of the disease, there are no specific medical therapies which are of clinical benefit in infants with NEC. Surgical resection of affected segments

Significance of this study

What is already known on this subject?

- Necrotising enterocolitis (NEC) is the most common gastrointestinal surgical emergency occurring in neonates, with high mortality rates ranging from 15% to 30%.

- Despite extensive research in this field, there are still no medical therapies that have proven to be of clinical benefit for the cure of affected neonates. Surgery is still the treatment of choice in case of necrotic bowel.

- There is a growing body of evidence that stem cells could play a therapeutic role in inflammatory bowel diseases and other intestinal pathologies.

What are the new findings?

- In a well-established neonatal rat model of NEC, amniotic fluid stem (AFS) cells improve rat survival, decrease morbidity, and reduce NEC incidence.

- In this model, AFS cells are able to improve intestinal function, decrease bowel inflammation, increase enterocyte proliferation and reduce apoptosis.

- AFS cell beneficial effect is achieved via modulation of stromal cells expressing COX-2 in the lamina propria.

How might it impact on clinical practice in the foreseeable future?

- Stem cell therapy may represent a new therapeutic option for children with NEC. Moreover, understanding the mechanism of action of AFS cells in the experimental NEC may help us develop new cellular or pharmacological therapies for infants with NEC.
leads to intestinal failure and/or short bowel syndrome, with subsequent long-term dependence on parenteral nutrition or need for intestinal transplantation.\textsuperscript{1–3}

Stem cells have become a therapeutic option for other intestinal diseases, which share some features with NEC, such as inflammatory bowel diseases (IBD).\textsuperscript{4} Following the first report in 1993, in which autologous stem cell transplantation was used for haematopoietic malignancy caused regression of Crohn's disease,\textsuperscript{4} stem cell therapy has become available for refractory IBD.\textsuperscript{4} It remains however unclear whether bone marrow (BM) cells act by immunoregulatory mechanisms, and/or by intestinal regeneration. BM cells have an anti-inflammatory effect in interleukin (IL) 10 knockout mice\textsuperscript{5} and experimental colitis.\textsuperscript{6} They may also differentiate into epithelial cells of the gastrointestinal (GI) tract, in animals and humans, in which repopulation of the GI tract epithelia by donor cells is related to the degree of epithelial damage.\textsuperscript{7} BM cells also integrate in the mucosa in experimental colitis where they are involved in repair and formation of blood vessels, contributing to endothelial cells, vascular smooth muscle cells and pericytes.\textsuperscript{8} These two mechanisms, namely anti-inflammatory and regenerative, could also operate together, as BM mesenchymal stem cells (BM-MSCs) topically implanted in inflamed areas not only differentiates into colonic interstitial cells, but can also provide various factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β to the injured area, which are responsible for fibroblast activation, angiogenesis and tissue repair.\textsuperscript{9} Given these data and the limited clinical management options in human NEC, we investigated the potential use of stem cells in experimental NEC. A well-established neonatal rat model of NEC, based on gavage-feeding with hyperosmolar formula, experimental NEC.\textsuperscript{10} Given these data and the limited clinical management options in human NEC, we investigated the potential use of stem cells in experimental NEC. A well-established neonatal rat model of NEC, based on gavage-feeding with hyperosmolar formula, hypoxia and oral administration of lipopolysaccharide (LPS), NEC rats were randomised to receive either: cells (2×10\textsuperscript{6} AFS cells, BM-MSCs or myoblasts in 50 μl of phosphate buffered saline, PBS) 50 μl of PBS alone or 50 μl of conditioned or non-conditioned media at 24 h and 48 h of life via intraperitoneal injection.\textsuperscript{11} Further control group consisted of breastfed (BF) animals. Survival curves were compared by the logrank test.

**Magnetic resonance imaging**

MRI studies were performed using a Varian 9.4T VNMRS 20 cm horizontal-bore system (Varian Inc. Palo Alto, California, USA), using 100 G/cm imaging gradients. A 26 mm quadrature birdcage coil (RAPID Biomedical GmbH, Wurzburg, Germany) was used for volume transmit and receive. Details are reported in the online supplementary information.

**Immunohistochemistry**

Three micrometre thick tissue sections from formalin-fixed paraﬃn embedded samples were immunostained using the following antibodies: anti-GFP (either mouse or rabbit; Invitrogen); 1:200 and rabbit anti-Cytokeratin wide spectrum 1:100 (Dako); mouse anti-smooth muscle actin 1:200 Dako); mouse anti-Tuj1 1:500 (Covance); anti-cyclooxygenase (COX) antibody (mouse; BD Biosciences); cleaved caspase 3; 488 Alexa Fluor; 568 Alexa Fluor (Molecular Probes). Slides were mounted in Vectashield with 4’, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Sections were viewed with a Zeiss Axiohot microscope attached to a Leica DC500 digital colour camera employing the Leica Firecam software. Images were compiled using Adobe Photoshop C54.

**Gut motility and gut permeability**

To assess motility, 0.1 ml carmine red solution (10 mg/ml in water) was administered by gavage at 92 h of life and GI transit (stomach to rectum) tested blindly by two independent scorers after 4 h.\textsuperscript{12} Permeability was assessed as previously described.\textsuperscript{13}

**Molecular biology**

**RNA extraction.** After sacriﬁce of the animals at 96 h, the entire intestine (jejunum-ecum) was isolated from six NEC rats, (two PBS rats and four AFS cell rats). A third group of BF rats was used as reference (n=5). Samples were snap-frozen in liquid N\textsubscript{2} immediately after collection using RNase-free vials without other protective solutions. After tissue homogenisation with a rotor-stator homogeniser with disposable probe tips (Ultra-Turrax, Ika), total RNA was extracted with TRIzol Reagent (Invitrogen) and quantified with a ND-1000 spectrophotometer (Nanodrop). RNA was extracted with TRIzol Reagent and retrotranscribed in cDNA (Invitrogen). Vegfa, Fgf2, Tgfα, Tgfb1 and PDGFB expression was assessed in duplicates through real-time PCR (Syr Green method). Transcript levels were normalised on the geometrical mean of three different housekeeping genes (Actb, Gadph, B2m) using Genorm software. A microarray-based gene expression analysis was performed as previously reported.\textsuperscript{14} Briefly, cDNA microarrays were employed to interrogate expression of 3734 rodent genes, selected on the basis of their relevance to processes such as inflammation, apoptosis, cell cycle regulation and others.

**Animals**

This study was approved under the UK Home Office regulations for Animals (Scientiﬁc Procedures) Act 1986 (Licence N 6723). NEC was induced using a well-established protocol\textsuperscript{10} based on gavage-feeding with hypersomolar formula, hypoxia and oral administration of LPS. After 24 h of life, NEC rats were randomised to receive either: cells (2×10\textsuperscript{6} AFS cells, BM-MSCs or myoblasts in 50 μl of phosphate buffered saline, PBS) 50 μl of PBS alone or 50 μl of conditioned or non-conditioned media at 24 h and 48 h of life via intraperitoneal injection.\textsuperscript{11} Further control group consisted of breastfed (BF) animals. Survival curves were compared by the logrank test.

**Methods**

**Cells**

Clonal AFS cells lines were generated from green fluorescent protein (GFP)+ transgenic Sprague-Dawley rats at E14 as previously described.\textsuperscript{15} Clones E8, E9 and E11 were characterised and used for the experiments. BM-MSCs were obtained from the femurs of adult Sprague-Dawley rats as previously reported.\textsuperscript{15} Adherent cells were characterised by flow cytometric analysis and were used up to a maximum of nine passages. Rat skeletal muscle myoblasts were used as control. Conditioned medium collected from supernatant of AFS cells seeded at 2×10\textsuperscript{4}/cm\textsuperscript{2}, and cultured in α-minimum essential medium (MEM) for 30 h, was ﬁltered using a 0.22 μm hydrophilic Durapore Membrane Filter (Millipore).
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A GE Healthcare microarray platform was used to deposit DNA probes onto aminosilane-coated mirrored slides (AmphiSlide, GeneWave). Labelled cDNA was obtained from total RNA by reverse transcription by using a Genisphere Array50 kit. Data analysis was performed with GeneSpring GX Software (Agilent).

**Gut inflammation**

At sacrifice, the intestine (from the jejunum to the proximal colon) was removed from NEC rats receiving either PBS or AFS cells, and malondialdehyde (MDA; lipid peroxidation marker) and myeloperoxidase (MPO; measure of neutrophil infiltration) were measured as described previously. $^{20}$ MDA and MPO were normalised to protein.

**Apoptotic index**

Groups were compared by two blinded investigators using a modified apoptotic index (0=no apoptosis; 1=scattered apoptotic cells at the villus tip; 2= numerous apoptotic cells at the villus tip; 3= scattered apoptotic cells in the villus axis; 4= numerous apoptotic cells in the villus axis; 5= apoptotic cells in the crypts). $^{21}$

**Enterocyte migration/proliferation**

PBS and AFS cell-injected NEC rats received an intraperitoneal injection of 5-ethynyl-2’-deoxyuridine (EdU) at 72 h of life (Click-iT EdU Cell Proliferation Assays, Invitrogen, UK; 100 μl of PBS). $^{22}$

**Treatment with COX-2 inhibitors-survival study**

At 24 h of life NEC and BF animals, were randomly divided in four subgroups receiving by gavage: (1) vehicle (1% dimethyl sulfoxide (DMSO), tid); (2) COX-1 inhibitor (sc-560, 20 mg/kg, bid); (3) COX-1+2 inhibitor (ibuprofen, 120 mg/kg, tid); (4) COX-2 inhibitor (celecoxib, 60 mg/kg, bid).

**Statistical analysis**

Continuous data (mean±SEM) were compared using t test or Mann-Whitney tests as appropriate (where two groups were compared), parametric or non-parametric analysis of variance (ANOVA), with Tukey or Dunn’s post-test, as appropriate (where more than two groups were compared). Dichotomous data were compared using Fisher’s exact test. Survival curves were compared using the logrank test; p<0.05 was considered statistically significant.

**RESULTS**

**AFS cells improve mortality and morbidity in rats with NEC by preserving gut function**

We first observed that NEC rats injected intraperitoneally with BM-MSCs (see online supplementary figure S1) at 24 h and 48 h of life did not show improved survival compared with animals injected with PBS (figure 2SA). Age-matched BF rats not subjected to NEC induction had 92% survival (see online supplementary figure S2A). Considering this lack of effect, NEC rats were injected with AFS cells, BM-MSCs, myoblasts (as a committed negative control), PBS and compared with BF rats as normal controls (figure 1B, see online supplementary figure S2B). NEC rats injected with AFS cells showed significantly higher survival at 7 days when compared with all the other groups (figure 1B, see online supplementary figure S2B), even when tested on a large number of animals (figure 1C, see online supplementary figure S2C). At 96 h, AFS cells improved the clinical status$^{10}$ of NEC rats (figure 1D). Peritoneal fluid accumulation (assessed using MRI imaging (figure 1E) as voxels with T2>160 ms), was significantly greater in the NEC rats injected with PBS, than those treated with AFS or the BF rats (see online supplementary figure S3A). In addition, using high-resolution μMRI after gadolinium fixation, $^{23}$ PBS pups, similarly to human infants with NEC, but not AFS or BF animals, displayed dilated bowel loops, with significantly thinned gut walls (row 4, vii. and viii.; figure 1E, see online supplementary figure 3B). The superior clinical status and MRI appearance due to AFS cells was also reflected in gut function; intestinal motility, measured with carmine red transit, was severely decreased in NEC rats injected with PBS in comparison with BF rats, but it was preserved in the AFS cell treated rats (figure 1F). Although gut weight and length did not differ among the groups (see online supplementary table S1), carmine red completed gut transit in 75% of BF rats, 19% of PBS rats and 47% of those treated with AFS cells. This was confirmed by organ bath studies in which only intestine from AFS rats showed spontaneous contractions, resembling peristalsis (see online supplementary Video S1). Similarly, while intestinal permeability (plasma lactulose/mannitol ratio) was significantly higher in PBS rats compared with BF rats, AFS cell injections in NEC rats partially prevented this increase (figure 1G).

AFS cells decrease intestinal damage, localise to the damaged gut and migrate systemically.

At 96 h, macroscopic gut appearance of AFS rats was similar to BF rats, with significantly less damage and necrosis than PBS rats (figure 2A). Gut damage by NEC, evidenced by the histological presence of villus sloughing, core separation and venous congestion, $^{10}$ was less in AFS rats than PBS rats (figure 2B). This corroborates previous studies in which stem cells reversed colonic damage in an IBD model. $^{8}$ Since AFS rats had improved survival, clinical status, intestinal function and histology, we hypothesised that AFS cells had migrated and integrated in the damaged intestine. Indeed, AFS cells exhibited various degrees of distribution, sometimes forming a characteristic ring around the intestine (figure 2C). After 48 h, cell bundles were adherent to the mesentery (figure 2D); at 72 h, AFS cells were in the serosa and in the muscularis (figure 2D); whereas at 96 h a few AFS cells were found in the villi as smooth muscle positive (figure 2DE) and cytokertatin and antineuron-specific class III β-tubulin negative (data not shown). Newborns with NEC may develop multiorgan failure over time. Interestingly, DNA extracted from various organs of 32 NEC rats injected with AFS cells showed that the intestine was always positive for GFP while liver was positive in 32% of animals, kidneys in 21%, spleen in 20%, heart in 17% and lungs in 15% while no GFP signal was detected in brain and BM. Half of AFS rats were GFP+ exclusively in the intestine; 23% were positive in the intestine plus one other organ, 17% in intestine and two other organs and 10% in intestine and three or more organs (10%).

AFS cells decrease gut inflammation and enterocyte apoptosis and promote enterocyte proliferation/migration in rats with NEC

Although the benefits of AFS cells appeared to be related to their gut presence, the low degree of engraftment suggested a paracrine action. To test this hypothesis, 155 neonatal NEC rats were randomised on day 1 of life to intraperitoneal injection of either PBS (n=42), AFS cells (n=46), α-MEM (n=23) or conditioned medium (CM, n=44). Rats injected with CM had a significantly longer survival than rats injected with PBS (p<0.01) or with α-MEM (p<0.0001; see online supplementary data analysis were compared using Fisher’s exact test. Survival curves were compared using the logrank test; p<0.05 was considered statistically significant.
**Figure 1** Amniotic Fluid Stem (AFS) cells lengthen survival and decrease morbidity in rats with Necrotising enterocolitis (NEC) by preserving gut function. (A) Experimental design. (B) Bone marrow mesenchymal stem cells (BM-MSCs)-treated NEC rats had a similar survival rate at 7 days of life as control NEC rats injected with phosphate buffered saline (PBS) (p=ns), while breastfed (BF) rats survived significantly longer than both groups (p<0.0001). AFS cells-treated NEC rats (n=40) had a significantly higher survival rate at 7 days of life than NEC rats treated with BM-MSCs (n=17; p=0.024), PBS (n=24; p<0.0001) or myoblasts (p<0.0001). (C) This effect of AFS cells was extremely reproducible, as cumulative results of several experiments showed a consistent survival benefit (AFS cells n=121 vs PBS n=120, p<0.0001). (D) Morbidity analysis, evaluated using a validated clinical sickness score, confirmed a significant benefit of AFS cell treatment in comparison with PBS (AFS cells 2.0±1.6 vs PBS 3.7±2.1, p<0.01), although AFS cell-treated NEC rats showed a worse outcome compared with BF rats (BF 0.2±0.39, p<0.01 vs AFS cell rats, p<0.001 vs PBS rats). (E) MRI of AFS cells-treated NEC rats (left column of images) and untreated rats (right column of images). Row 1 (i. and ii.): degree of ascites measured using T2 maps: the total number of voxels with T2>160 ms identified as dark red regions which indicates areas of fluid accumulation were different between the PBS (1682±453) and AFS (224±135, p<0.05) groups which did not differ from the BF (278±27). Row 2 (iii. and iv.): bowel wall thickness using MRI images: marked structural changes were observed in the untreated rats. Row 3 (v. and vi.): representative axial slices demonstrate a similar pattern. Row 4 (vii. and viii.): magnified images of bowel loops from the respective axial slices highlight the loss of bowel wall integrity in the untreated rats. Row 5 (ix. and x.): representative axial slices of BF rats and magnified image of bowel loop showing normal intestinal architecture. (F) Carmine red solution administration revealed that motility was decreased in NEC rats injected with PBS (p<0.001) but it was normal in rats injected with AFS cells (p=ns) when compared with BF. (G) Intestinal permeability, measured as plasma lactulose/mannitol ratio: in comparison with BF rats (0.004±0.002, n=9), PBS rats also had a significant increase in intestinal permeability (0.043±0.004; n=21, p<0.001) which was restored in AFS rats (0.031±0.004; n=25, p<0.05).

Figure S4). Similarly, rats injected with AFS cells survived significantly longer than rats which received PBS (p<0.001) or α-MEM (p<0.0001). No differences were noted between AFS cell group and CM group (p=n.s.), thus supporting a paracrine mechanism of action (see online supplementary figure S4).

Hierarchical cluster analysis of cDNA arrays identified 37 genes, which distinguished the two groups of animals (figure 3A). Unsurprisingly, genes with the largest expression differences were involved in inflammation and tissue repair (eg, Aoc3, Itgb6), cell cycle regulation (eg, Atf2, Dusp16, Gpx4, Mxd1) and enterocyte differentiation (eg, Acsl5, Rab8a, Thra).24 In human18 and experimental NEC,25 26 therapies have been aimed at the inflammatory cascade. In NEC rats, AFS cells reduced lipid peroxidation (MDA level), (figure 3B) and significantly decreased neutrophil infiltration (MPO activity; figure 3C). Villus apoptosis is another key factor in gut barrier failure, in human and experimental NEC.27 As was recently shown in myocardial infarction,28 AFS cells reduced apoptosis (cleaved caspase 3) in NEC rats, particularly in the crypts (positive cells in 45% of PBS rats with NEC vs 12% of AFS rats, p<0.05; figure 3D). These results have parallel findings in IBD models, where BM-MSCs decrease apoptosis.29 Finally, in AFS rats, EdU positive enterocytes22 migrated significantly further from the villus crypt than in PBS rats (figure 3E), indicating that AFS cells stimulate proliferation, similar to the reported effects of HB-epidermal growth factor (EGF).30 AFS cells modulate stromal cells expressing COX-2 in the lamina propria

Hence, AFS cells diminish apoptosis and inflammation, and promote enterocyte proliferation. Intriguingly, inducible COX-2, normally at low levels in intestine also decreases enterocyte apoptosis,31 diminishes inflammation32 and promotes epithelial proliferation.33 We therefore questioned whether AFS cells acted via a COX-2 related mechanism. COX-2+ cells, present in the lamina propria of BF rats and AFS rats, were markedly diminished in PBS rats (figure 4A,B). While the number of COX-2+ cells in the villus axis was similar in AFS rats or BF rats (figure 4C), cryptal COX-2+ cells were increased in AFS rats compared with BF rats and PBS rats (figure 4D). Moreover, the number of COX-2+ cells per villus unit (figure 4E) and the number of cryptal COX-2+ cells (figure 4F) inversely correlated with the degree of intestinal damage.

To further investigate whether the beneficial effects of AFS cells were COX-2-dependent, we performed a survival study using COX-1 and COX-2 inhibitors. BF rats and NEC rats receiving PBS or AFS cells were randomised to receive:
(1) vehicle; (2) sc-560 (COX-1 inhibitor); (3) ibuprofen (COX-1+2 inhibitor) and (4) celecoxib (COX-2 inhibitor). As expected, NEC rats treated with AFS cells+vehicle survived significantly longer (Figure 4G) and had a better clinical score (PBS: 0.77±0.36 vs 3.09 ±1.10, p<0.05). The survival effect of AFS cells was abolished by the selective COX-2 and the non-selective COX-1+2 inhibitors, but unaffected by the selective COX-1 inhibitor (Figure 4G). Similarly, the improved clinical status in NEC rats was annulled by COX-2 inhibition, reduced by COX-1+2 inhibitors, but unaffected by COX-1 inhibition (Figure 4H). None of the COX inhibitors modified survival (Figure 4G) or clinical status (data not shown) of PBS or BF rats. In comparison with BM cells, AFS cells differentially expressed genes in the wnt-β-catenin pathway which regulate intestinal epithelial stem cell function (eg, AXIN, APC and CTNNB1) and cell migration (CXCL12), and growth factors known to maintain gut epithelial integrity and reduce mucosal injury in experimental IBD (eg, insulin-like growth factor (IGF)-1, fibroblast growth factor (FGF)-1, FGF-3 and FGF-4, fibroblast growth receptor1; figure 5A). Moreover, when cultured in the presence of LPS, AFS cells increased expression of VEGFα, FGF-2, TGFβ1, TGFα and platelet-derived growth factor (PDGF), compared with BM cells and myoblasts which could also justify their unique therapeutic effect in this model of disease (figure 5B–F).

**DISCUSSION**

NEC remains a major cause of neonatal morbidity and mortality. We demonstrated for the first time that AFS cells significantly improve survival of rats with NEC. The ability of AFS cells to lengthen survival is particularly important, as intensive care support cannot be given to pup rats and this model is not compatible with long-term survival. The specificity of this effect to AFS cells is in contrast with other animal models of bowel disease, in which BM-MSCs are effective. This may be due to differences in pathogenesis;
NEC is associated with ischaemia and bowel immaturity, whereas in IBD, the pathological changes are primarily related to immune dysregulation. Hence, while IBD can be rescued by MSCs, beneficial effects from cell therapy in NEC appear to require a different mechanism. Rat models of IBD are usually obtained using either dextran sodium sulfate or intra-mural injection of peptidoglycan-polysaccharide, whereas the NEC model comprises several pathogenic factors that are also directly implicated in the human disease.

In addition to the pronounced and consistent effect on survival, several clinical indicators also demonstrated the beneficial effects of AFS cell treatment. First, AFS rats clinically improved, which is a marker of less severe gut damage (macroscopic and microscopic) in this animal model. While human NEC can be suspected radiologically, confirmed at surgery and graded histologically, only the latter have been used in experimental models. Herein, for the first time, we were able to define bowel appearance using MRI imaging. Similarly to human infants with NEC, we demonstrated that peritoneal fluid collection and dilated bowel loops are features of rats with NEC, while MRI images of animals treated with AFS cells were indistinguishable from BF rats. Moreover, treatment with AFS cells rescued gut motility and partially restored intestinal permeability in NEC rats. Villus sloughing, venous congestion and villus core

Figure 4 Amniotic fluid stem (AFS) cells modulate stromal cells expressing COX-2 in the lamina propria. (A) Representative cryosections of the terminal ileum from breastfed (BF) and necrotising enterocolitis (NEC) rats receiving phosphate buffered saline (PBS) and AFS cells stained with anti-COX2 Ig (red) and DAPI (blue). Scale bars: 20 μm. (B) In NEC rats treated with AFS cells, the number of COX-2+ cells per villus unit (4.97±0.46; n=7, p=n.s.) was similar to that of BF rats (4.25±0.52; n=8), but higher compared with NEC rats treated with PBS (2.37±0.29; n=8, p<0.05). (C, D) This difference was not determined by the quantity of COX-2+ cells in the villi, which was similar between the BF and AFS (1.95±0.28 vs 1.32±0.16, p=n.s) rats, but by their number underlying the crypte which was higher in NEC rats treated with AFS cells (3.01±0.41) compared with BF rats (1.41±0.23, p<0.01) and NEC rats injected with PBS (1.05±0.18, p<0.001). (E, F) The number of COX-2+ cells per villus unit and in the crypte inversely correlated with the histological grade of NEC by linear regression. (G) A survival study employing selective and non-selective COX inhibitors showed that COX inhibitors did not modify the high survival rate of BF rats (n=78; BF+DMSO vs BF+sc-560, BF+ibuprofen, BF+celecoxib: p=n.s.) and the low survival rate of PBS-treated NEC rats (n=77; PBS+DMSO vs PBS+sc-560, PBS+ibuprofen, PBS+celecoxib: p=n.s.). However, the improved survival of NEC rats receiving AFS cells (n=78) was annulled by COX-2 (AFS cells+celecoxib vs AFS cells+sc-560 vs AFS cells+ibuprofen, AFS cells+DMSO: p<0.0001; AFS cells+celecoxib vs PBS+DMSO: p=n.s.) and COX-1+2 inhibitors (AFS cells+ibuprofen vs AFS cells+DMSO: p<0.01; AFS cells+ibuprofen vs PBS+DMSO: p=n.s.), but conserved in rats receiving COX-1 inhibitor (AFS cells+sc-560 vs AFS cells+DMSO: p=n.s.; AFS cells+sc-560 vs PBS+DMSO: p=0.001). (H) The clinical sickness score improvement observed in NEC rats treated with AFS cells (0.77±0.36) was abolished by COX-2 inhibitor (7.15±0.89; AFS cells+celecoxib vs AFS cells+DMSO: p<0.001), diminished by COX-1+2 inhibitor (2.86±0.91; AFS cells+ibuprofen vs AFS cells+vehicle: p=n.s.) and unaltered by COX-1 inhibitor (0.92±0.39; AFS cells+sc-560 vs AFS cells+vehicle: p=n.s.).
separation are classic histological features of NEC, and we showed that AFS cell-treated animals had normal intestinal architecture with decreased incidence of all of these hallmarks. These findings corroborate a very recent study in which enteral administration of amniotic fluid per se attenuated the severity of experimental NEC through activation of the epidermal growth factor receptor (EGFR).14 The beneficial role of AFS cells on clinical outcome and survival was closely related to their presence in the gut. While AFS cells administered intravenously home primarily in the lung, and subsequently colonise spleen and liver, with no distribution to the gut,37 we have shown that AFS cells injected intraperitoneally colonise the gut in 80% of BF pup rats.16 Remarkably, AFS cells injected intraperitoneally, localised in 100% of intestines, homing to the mesentery or the gut. In most of the animals, 48 h or 72 h after injection, AFS cells were present, albeit in small numbers, in the smooth muscle, submucosal layers and/or in the villi. As the improvements in morbidity and mortality occurred within hours after injection, at which time relatively small numbers of AFS cells were found in the bowel, their direct contribution to tissue regeneration is unlikely to be the major mechanism for the beneficial effects. This is further confirmed by the improvement obtained with conditioned media or amniotic fluid14 administration, thus supporting a paracrine mechanism of action. We hypothesised that in this environment, AFS cells released specific growth factors that acted on resident progenitor cells. In particular, damage resolution is probably achieved via activation of multiple pathways acting on tissue inflammation, cell apoptosis and proliferation.18 Microarray analyses of NEC guts receiving AFS cells showed modification of the transcriptional profile of genes involved in inflammation and tissue repair (eg, Aoc3, Itgb6), cell cycle regulation (eg, Atf2, Dusp16, Gpx4, Mxd1) and enterocyte differentiation processes (eg, Acsl5, Rab8a, Thra).

Figure 5 Amniotic Fluid Stem (AFS) cells express growth factors in response to lipopolysaccharide (LPS) exposure. (A) RT2 profiler PCR array system were performed comparing the three clonal cell lines of AFS cells (E8, E9, E11) with three from bone marrow mesenchymal stem cells (BM-MSCs) preparations (b3-2, B5-2, B7-3) used in the experiments. There is an upregulation of AFS cells expressed genes involved in the wnt-β catenin pathway, cell migration and several growth factors. (B–F) In order to further evaluate specific factors, which may be induced by the NEC environment, AFS cells, myoblasts and BM-MSCs were cultured in the presence of 1μg/ml LPS. RNA was extracted from the different cells at basal level (time 0) and at 3 h from LPS stimulation and retrotranscribed into cDNA. Levels of transcription factors were measured by real time PCR (Sybr green) after normalisation with three different housekeeping genes (gadph, b2m, bact). AFS cells responded to LPS stimulation progressively increasing the expression of VEGFα (B), Tgfβ1 (C), Fgfl2 (D), TGFα (E) and PDGFβ (F). In the myoblast lines and BM-MSCs, the relative yield of these transcripts remained substantially unchanged (*p<0.05).

chemoattractants by the lungs and to humoral and physical interactions between stem cells and lung cells. Adult progenitor cells can also improve postischaemic myocardial function when used as a preventive measure, by inducing a 50% reduction in proinflammatory cytokine production, or when used as a therapy after myocardial infarction. In the latter scenario, MSCs decreased proinflammatory cytokines, inhibited collagen deposition, decreased expression of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 and attenuated left ventricle cavitary dilation and transmural infarct thinning, thus preventing myocardial remodelling.

In addition to inflammation, intestinal apoptosis has also been shown to be a key factor in gut barrier failure, in human and experimental NEC. Abundant epithelial apoptosis of the villi is observed in histological specimens collected at the time of bowel resection in patients with NEC. It usually precedes widespread tissue damage and its reduction in experimental NEC has been achieved using various agents such as epidermal growth factor, anti-TNF-α, HB-EGF, IGF-1, Lactobacillus GG, and Lactobacillus bulgaricus. Herein, we demonstrated for the first time that AFS cells also reduce apoptosis. Finally, we have shown that AFS cells are able to influence villus cell proliferation. Impairment of cell proliferation and migration, which extends beyond the crypts, is continuous, irregular or spreads into the entire mucosa. Hence COX-2+ cells in the crypts promote epithelial proliferation and migration while preventing apoptosis; this agrees with the observation that repositioning of COX-2+ cells to the crypts is necessary to maintain proliferation of colonic epithelial progenitors after damage. Although their identity remains to be completely established, COX-2+ cells may consist of a population of stromal CD44 (+) hematopoietic lineage-negative/miyoblast lineage-negative cells and their activation could have been involved in different pathways.

The mechanism by which AFS cells specifically activate COX-2+ cells in the lamina propria needs to be further investigated. However, our results show that, after LPS stimulation, AFS cells respond increasing the expression of growth factors able to induce COX-2 directly (ie, VEGFα, FGF-2, TGFβ1) or via activation of the EGFR (ie, TGFα). Not only, in fact, COX-2 products (PG2) activate EGFR but also EGFR plays an important role in the induction of COX-2 expression in enterocytes. Moreover, as well as COX-2, EGFR activation in enterocytes induces repair mechanisms following GI mucosal injury, promotes cell survival, reduces intestinal inflammation and protects against experimental NEC.

In conclusion, AFS cells injected in a model of NEC, improved survival, clinical status, gut structure and function. These beneficial effects were not due to direct repopulation of damaged intestine by AFS cells, but instead were probably related to paracrine effects including decreased inflammation and apoptosis and concomitant increase in enterocyte proliferation and migration, thus aiding epithelial restitution. These effects may be mediated, at least in part, by COX-2+ cells, as their presence in the crypts was enhanced by AFS cell injection, and beneficial effects were abolished by COX-2 inhibitors (see online supplementary figure S5). Future work should focus on the potential clinical use of AFS cells and further elucidation of their mechanism of action in order to develop innovative pharmacological agents suitable for neonates affected by NEC.

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