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 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 cyclooxygenase 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...
leads to intestinal failure and/or short bowel syndrome, with subsequent long-term dependence on parenteral nutrition or need for intestinal transplantation.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).4 Following the first report in 1993, in which autologous stem cell transplantation used for haematopoietic malignancy caused regression of Crohn’s disease,4 stem cell therapy has become available for refractory IBD.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 mice5 and experimental colitis.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.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.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 differentiate 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.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, hypoxia and oral administration of lipopolysaccharide (LPS), factors known to be implicated in the pathogenesis of human NEC, was used.10

In this model we first attempted to use BM-MSCs, but a lack of effect on survival prompted us to focus on cells from amniotic fluid, which may have higher regeneration potential, due to their fetal origin. Amniotic fluid stem (AFS) cells are immunoselected by the stem cell factor receptor c-kit (CD117) and are able to give rise to lineages representing the tri germ layers in vitro and in vivo.11 Moreover, they can also exert a beneficial paracrine action in model of bladder, heart, kidney and lung disease, but have not been tested before in a model of bowel disease.13 Interestingly, their use could be supported by a recent paper from Good et al.,14 where amniotic fluid was shown to attenuate the severity of intestinal damage in experimental NEC via inhibition of Toll-like receptor 4 signalling.14 Herein we present the first demonstration that a similar effect can be achieved using AFS cells in a neonatal rat model of NEC.

METHODS

Cells

Clonal AFS cells lines were generated from green fluorescent protein (GFP)+ transgenic Sprague-Dawley rats at E14 as previously described.13 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.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 2×10^6/cm^2, and cultured in α-minimum essential medium (MEM) for 30 h, was filtered using a 0.22 μm hydrophilic Durapore Membrane Filter (Millipore).

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 based on gavage-feeding with hyperosmolar formula, hypoxia and oral administration of LPS. After 24 h of life, NEC rats were randomised to receive either: cells (2×10^6 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.16 A 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-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 ZeissAxiopt photomicroscope attached to a Leica DC500 digital colour camera employing the LeicaFirecam software. Images were compiled using Adobe Photoshop CS4.

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.17 Permeability was assessed as previously described.18

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 N₂ 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, Tgfβ1 and PDGFβ expression was assessed in duplicates through real-time PCR (Sybr 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.19 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.
Necrotising enterocolitis

A GE Healthcare microarray platform was used to deposit DNA probes onto aminosilane-coated mirrored slides (AmpliSlide, 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. 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=numerous apoptotic cells in the villus axis; 4=numerous apoptotic cells in the villus axis; 5=apoptotic cells in the crypts).

**Enterocyte migration/proliferation**

PBS and AFS cell-injected NEC rats received an intraperitoneal injection of 5-ethylthi-2'-deoxyuridine (EdU) at 72 h of life (Click-iT EdU Cell Proliferation Assays, Invitrogen, UK; 100 μg in 40 μl of PBS).

**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 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 figure S1).
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 μMRI 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).
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 enterocytes 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: [Graphical data]
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 than NEC rats treated with PBS+vehicle (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 AFS rats was annulled by COX-2 inhibition, reduced by COX-1+2 inhibition and 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 CTNNAA1) 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)β, 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. Moreover, when cultured in the presence of LPS, AFS cells increased expression of VEGFα, FGF-2, TGFβ1, TGFα and platelet-derived growth factor (PDGF)β, compared with BM cells and myoblasts which could also justify their unique therapeutic effect in this model of disease (Figure 5B–F).
NEC is associated with ischaemia and bowel immaturity, whereas in IBD, the pathological changes are primarily related to immune dysregulation. Furthermore, 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 intramural 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) The difference was not determined by the number 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 crypts 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 crypts 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=32; 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+DMSO: p<0.001; AFS cells+celecoxib vs PBS+DMSO: p=n.s.), but conserved in rats receiving COX-1 inhibitor (AFS cells+ibuprofen vs AFS cells+DMSO: p=n.s.; AFS cells+ibuprofen 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 localise 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).

Severe intestinal inflammation leading to intestinal damage is the main pathological event that characterises NEC.18 24 In the attempt to reduce incidence and severity of NEC, studies have attempted to directly influence the inflammatory cascade in human24 and experimental NEC.25 26 38 We observed that AFS cell injection reduced gut lipid peroxidation and neutrophil sequestration in NEC rats. Stem cells are well known to have anti-inflammatory effects, which are exerted in different ways on different organs.39–41 When injected in a model of endotoxin-induced lung inflammation, BM-MSCs downregulate the proinflammatory response while increasing production of anti-inflammatory IL10.41 Interestingly, in a similar model of lung injury, BM-MSCs decrease the systemic and local inflammatory responses induced by endotoxin.41 These effects do not require either lung engraftment or differentiation of the stem cells and are due at least in part to the production of stem cell growth factors.

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), Fgf2 (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).
chemoattracants 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, Lactobacillus bulgaricus. Herein, we demonstrated for the first time that AFS cells also reduce apoptosis in NEC rats, particularly in the crypts. These results also parallel findings in animal models of IBD, in which administration of BM-MSCs decreases 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 lamina propria. Administration of BM-MSCs decreases cell migration and proliferation. Similarly, we found that proliferation and migration of Edu positive enterocytes along the whole villus length was observed in NEC rats treated with AFS cells, whereas proliferation and migration was markedly decreased in untreated NEC rats. This is in keeping with results obtained in a model of radiation-induced intestinal injury, where human MSCs transplanted into immunotolerant non-obese diabetic/severe combined immunodeficiency mice increase small intestinal villus height and increase gut self-renewal. Similarly, enteral administration of amniotic fluid in neonatal mice with NEC restored enterocyte proliferation to levels similar to those of untreated mice. Epithelial barrier integrity is paramount for intestinal recovery in NEC, but it is still unclear which factors could influence its restoration and/or maintenance.

Hence, AFS cells diminish apoptosis and inflammation, and promote enterocyte proliferation. Intriguingly, COX-2, normally expressed at very low levels in intestine, has been reported to have similar effects: it decreases enterocyte apoptosis, diminishes granulocyte infiltration and promotes epithelial proliferation. COX-2 has also been suggested to have a dual role in NEC, with high activities being proinflammatory, and lower levels protective; systemic COX-2 inhibitors worsen intestinal inflammation and increase mortality in an NEC model. Moreover, perinatal and postnatal exposure to glucocorticoids and non-steroidal anti-inflammatory drugs, which inhibit COX-2 expression and activity, respectively, are risk factors for NEC development. In previous studies of COX-2 in NEC, no correlation was found between COX-2 expression and intestinal injury severity. This apparent discrepancy can be reconciled when the localisation of COX-2+ cells is taken into account, as in these reports expression was evaluated throughout the whole intestinal wall or 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+ /haematopoietic lineage-negative/myofibroblast lineage-negative cells and their activation could involve 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 (PGE2) 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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