ORIGINAL ARTICLE

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

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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 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.

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.

INTRODUCTION

Necrotising enterocolitis (NEC) represents up to 10% of admissions to neonatal intensive care unit,[1] and remains a major cause of neonatal morbidity and mortality despite changes in medical and surgical treatment.[1 2] 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.[3] 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 mice[5] 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)-β1 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 three germ layers in vitro and in vivo.[11 12] 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.[11] 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 $2\times10^3/cm^2$, 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[10] 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-TujI 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 ZeissAxiophot microscope 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-cecum) 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_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, Tgfa, Tgfb1 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.

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.[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 µg in 40 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 2D,E) and cytokeratin 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

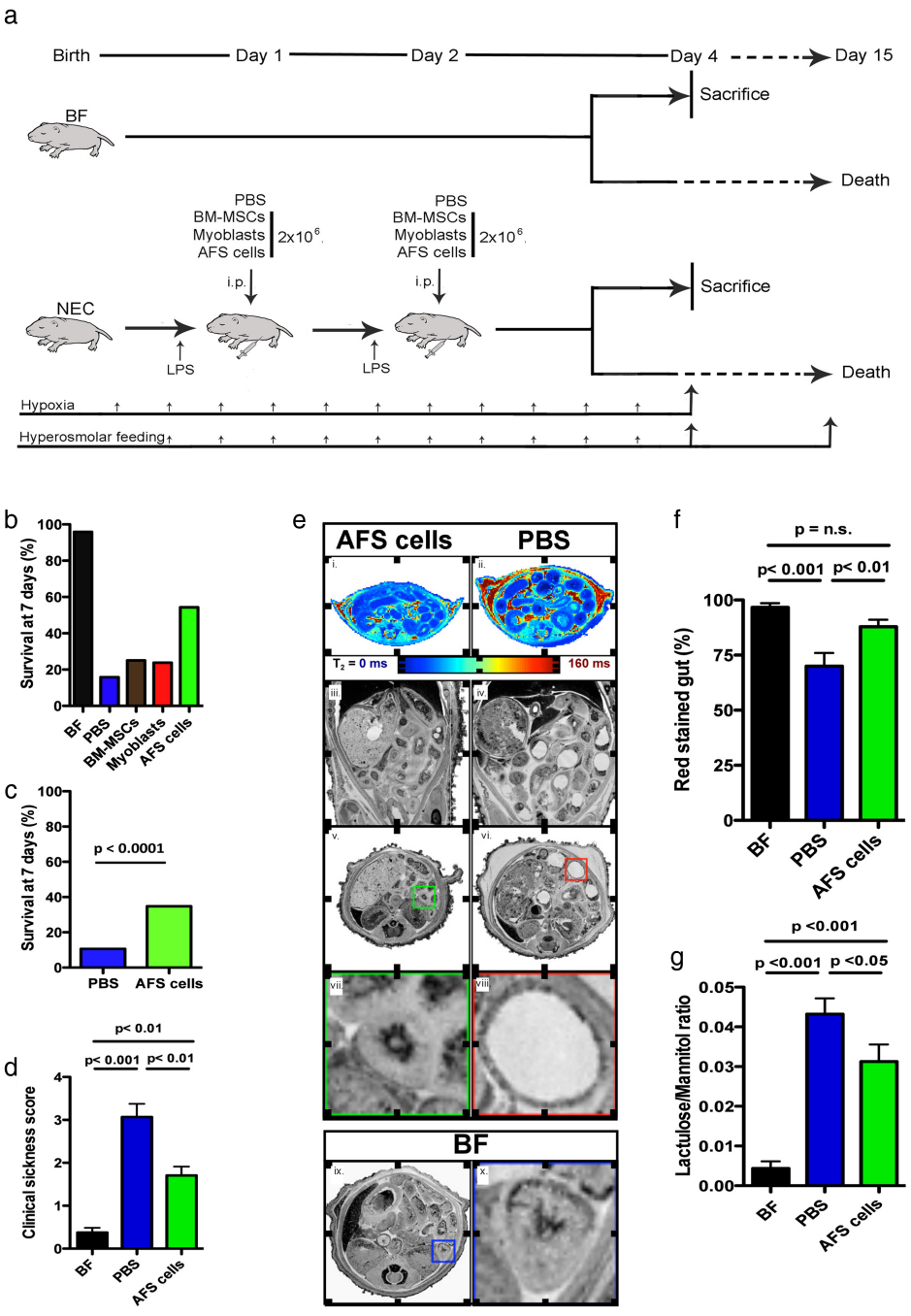

Figure 1 Amniotic Fluid Stem (AFS) cells lengthen survival and decrease morbidity in rats with Necrotising enterocolitis (NEC) by preserving gut function. (A) Experimental design. (B) Bone marrow mesenchymal stem cells (BM-MSCs)-treated NEC rats had a similar survival rate at 7 days of life as control 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=n.s.) 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).

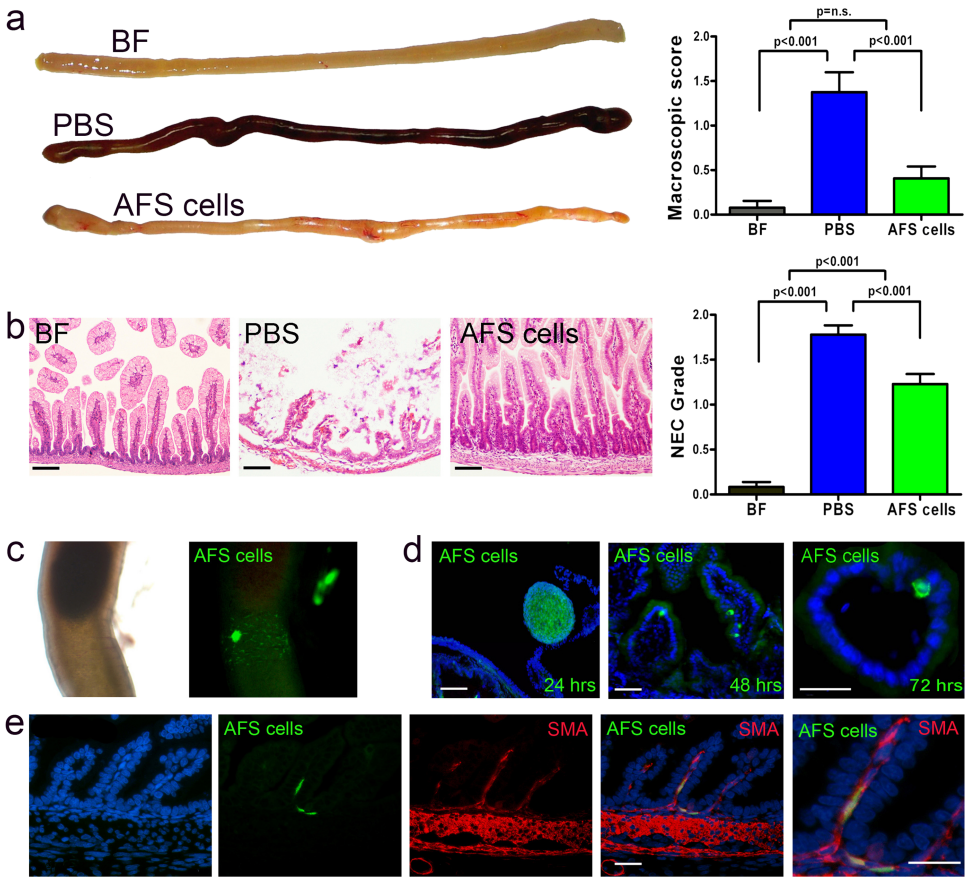

Figure 2 Amniotic Fluid Stem (AFS) cells decrease macroscopic and microscopic intestinal damage, localise to the damaged gut and migrate systemically. (A) Examples of the macroscopic gut appearance of breastfed (BF) rats and necrotising enterocolitis (NEC) rats injected with phosphate buffered saline (PBS) or AFS cells. Gut damage of NEC rats injected with AFS cells (0.4±0.7, n=27) was significantly smaller than in animals injected with PBS (1.4±1.1 n=24, p<0.001), but showed no difference with BF rats (0.1±0.3; n=12, p=n.s.). (B) NEC rats treated with AFS cells revealed significantly less histological damage (0.23±0.8, n=48, p<0.001) in comparison with NEC rats treated with PBS (1.78±0.7, n=50); no damage was observed in BF rats (0.08±0.2, n=12). (C) Macroscopic appearance of bundles of AFS cells injected intraperitoneally on the mesentery and on the gut wall. (D) GFP epifluorescence from AFS cells 24 h after injection was detected in the mesentery and adherent to the serosa of the intestine. At 48 h and 72 h rare AFS cells were found within the villus structure in close junction to the epithelial layer. (E) Sections of rat ileum stained with anti-GFP antibodies to localise AFS cells (green) and anti-smooth muscle actin (SMA) antibodies to label smooth muscle cells (red) (scale bar 20μm). AFS cells coexpressing GFP and SMA were found integrated in the mucosal layer.

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 human[18] 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[22] 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 inflammation[32] 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:

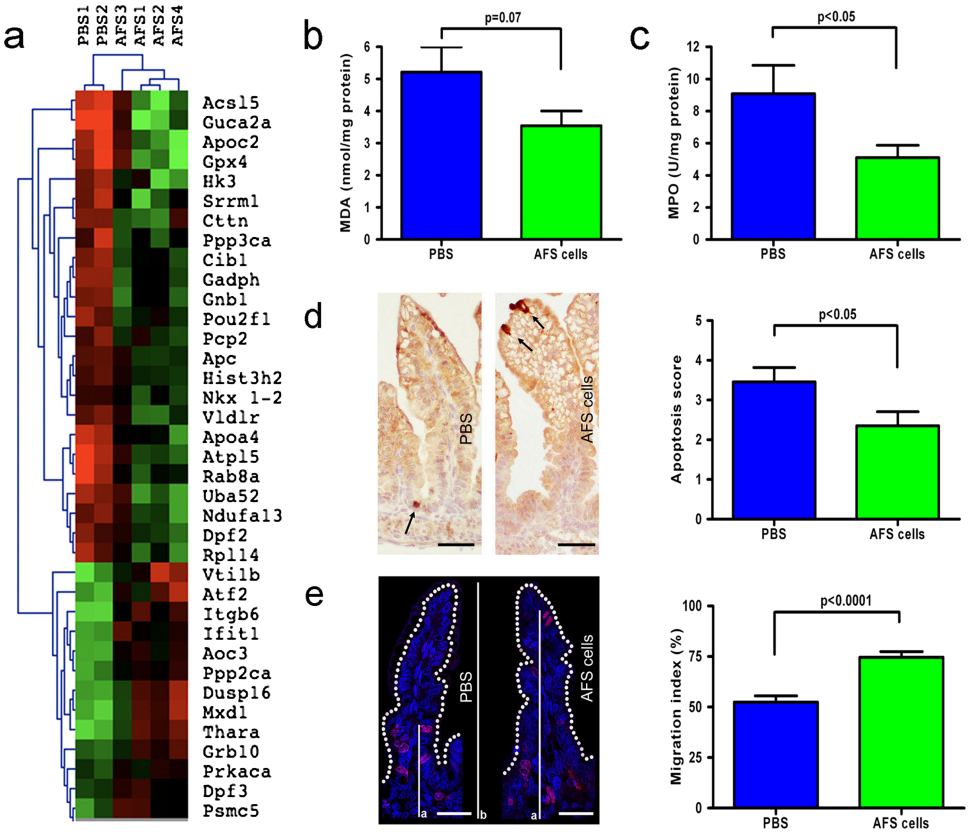

Figure 3 Amniotic Fluid Stem (AFS) cells decrease gut inflammation and enterocyte apoptosis and promote enterocyte proliferation/migration in rats with necrotising enterocolitis (NEC). (A) Cluster analysis of cDNA microarray data. The intestinal gene expression profile of NEC rats receiving phosphate buffered saline (PBS) was significantly different from that of NEC rats treated with AFS cells through a two dimensional hierarchical clustering by the Euclidean distance. Rows correspond to genes, and columns to NEC rats treated groups (PBS: PBS1, PBS2; AFS cells: AFS1, AFS2, AFS3, AFS4); red and green respectively indicate gene upregulation and downregulation relative to a reference sample consisting of a pool of intestinal RNA from five different breastfed rats. Thirty-seven genes, mainly involving inflammation, tissue repair, cell differentiation and cell cycle regulation, resulted able to differentiate the two groups of animals at a molecular level. (B) NEC rats injected with AFS cells showed lower degree of intestinal lipid peroxidation (3.6±2.7 nmol/mg protein), measured as malondialdehyde (MDA) level, when compared with NEC rats injected with PBS (5.2±4.6; p=n.s.). (C) Gut neutrophil infiltration, measured as myeloperoxidase (MPO) activity, was significantly lower in NEC rats treated with AFS cells than in those injected with PBS (AFS cells 5.1±0.8 U/mg protein; PBS 9.1±1.7; p<0.05). (D) Cleaved caspase 3 immunostaining of terminal ileum revealed apoptotic cells (arrows) in the villus crypts of NEC rats injected with PBS and at the villus tips of NEC rats injected with AFS cells. The apoptotic index, scored blindly as expression of cleaved caspase 3, was significantly lower in NEC rats injected with AFS cells than in those injected with PBS (p=0.04). (E) Fluorescence detection of dividing cells stained with EdU for cell migration/proliferation (EdU, purple, counterstained with DAPI, blue) of terminal ileum of NEC rats injected with PBS or AFS cells. Enterocyte migration/proliferation was calculated as percentage of the foremost EdU-labelled enterocyte (A) over the total mucosal thickness (B). Enterocyte migration/proliferation index assessed by EdU was significantly increased in NEC rats treated with AFS cells than in those treated with PBS (75% vs 53%, p<0.0001).

(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 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 CTNNA1) 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.[1] 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.[34] The specificity of this effect to AFS cells is in contrast with other animal models of bowel disease, in which BM-MSCs are effective.[7 35] This may be due to differences in pathogenesis;

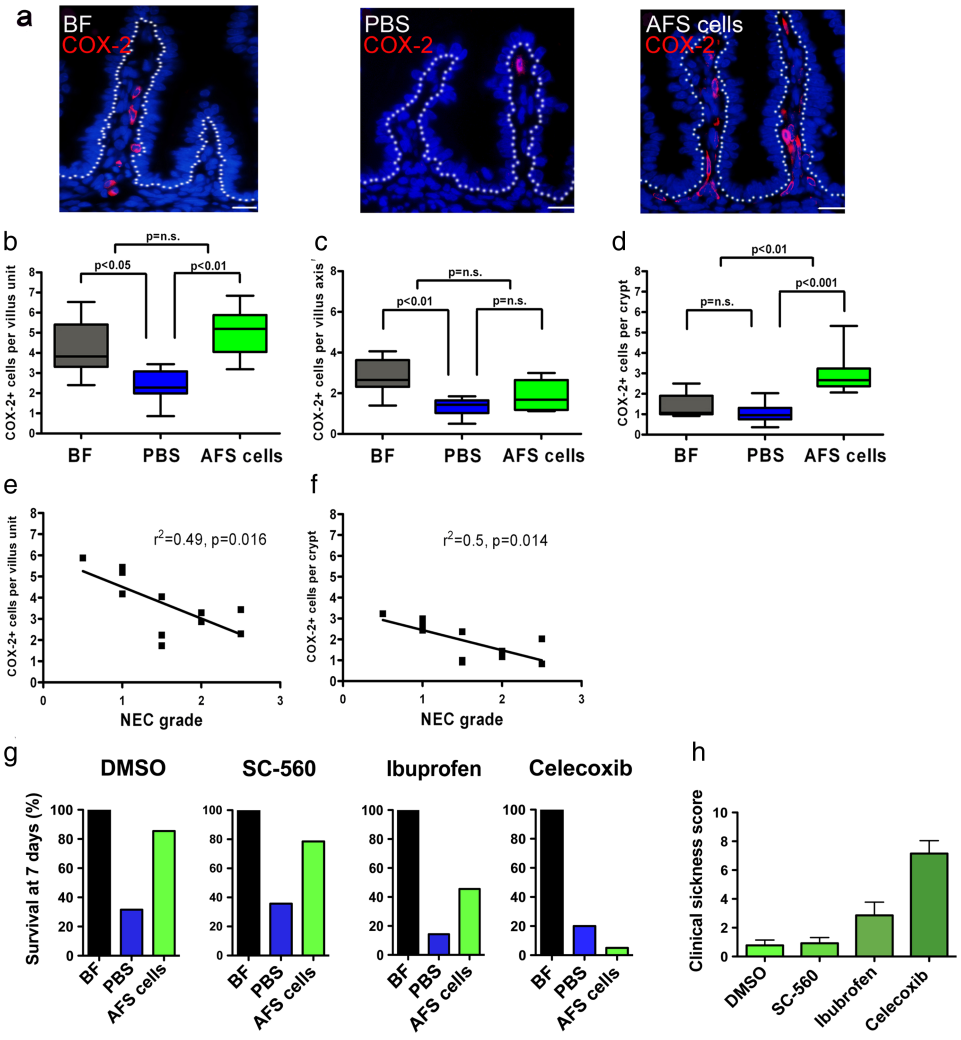

Figure 4 Amniotic fluid stem (AFS) cells modulate stromal cells expressing COX-2 in the lamina propria. (A) Representative cryosections of the terminal ileum from breastfed (BF) and necrotising enterocolitis (NEC) rats receiving phosphate buffered saline (PBS) and AFS cells stained with anti-COX2 Ig (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=ns) rats, but by their number underlying the cryptae 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 cryptae 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<0.0001; 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.).

NEC is associated with ischaemia and bowel immaturity, whereas in IBD, the pathological changes are primarily related to immune dysregulation.[7] 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[6] or intramural injection of peptidoglycan-polysaccharide, whereas the NEC model comprises several pathogenic factors that are also directly implicated in the human disease.[36]

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.[10] While human NEC can be suspected radiologically, confirmed at surgery and graded histologically,[2 3] 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

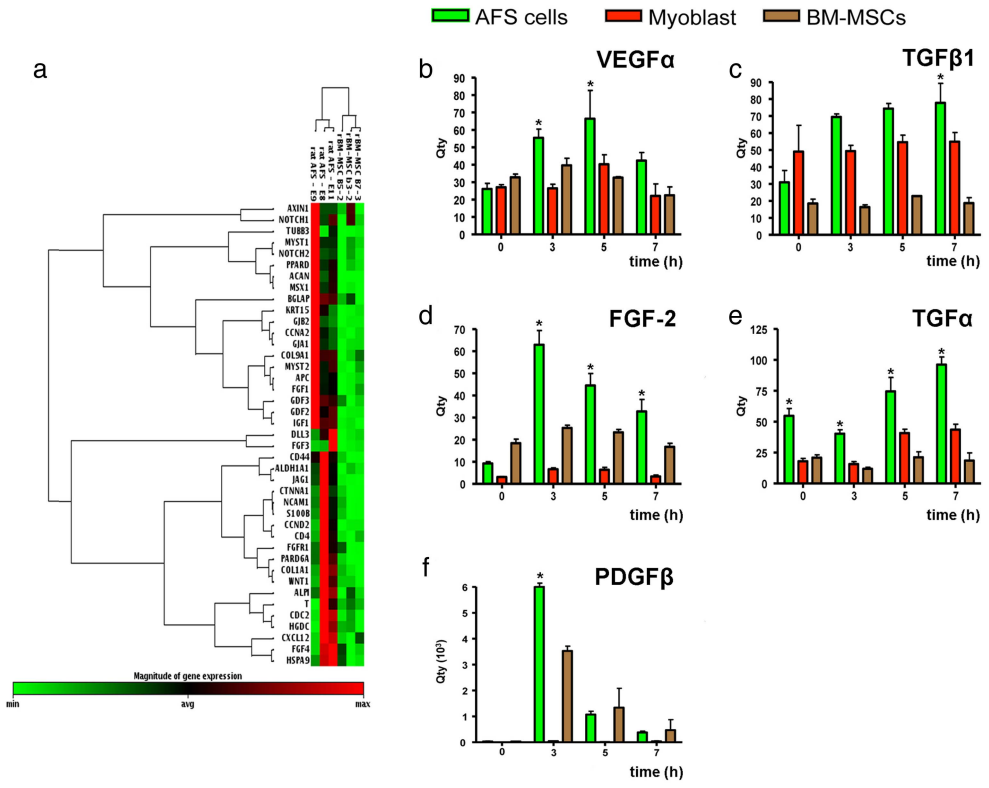

Figure 5 Amniotic Fluid Stem (AFS) cells express growth factors in response to lipopolysaccharide (LPS) exposure. (A) RT2 profiler PCR array system were performed comparing the three clonal cell lines of AFS cells (E8, E9, E11) with three from bone marrow mesenchymal stem cells (BM-MSCs) preparations (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).

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 fluid[14] 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 human[24] 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.[39] 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

chemoattractants by the lungs and to humoral and physical interactions between stem cells and lung cells.[41] Adult progenitor cells can also improve postischaemic myocardial function when used as a preventive measure, by inducing a 50% reduction in proinflammatory cytokine production,[42] or when used as a therapy after myocardial infarction.[40] 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.[40]

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.[27] It usually precedes widespread tissue damage[43] and its reduction in experimental NEC has been achieved using various agents such as epidermal growth factor,[44] anti-TNF-α,[21] HB-EGF,[45] IGF-1,[46] *Lactobacillus* GG,[47] *Lactobacillus bulgaricus*.[48] 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.[29] 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 covering villi,[49] and is commonly observed in human[50] and experimental NEC . Administration of HB-EGF protects the intestine from NEC via preservation of enterocyte migration and proliferation.[30] 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.[51 52] Similarly, enteral administration of amniotic fluid in neonatal mice with NEC restored enterocyte proliferation to levels similar to those of untreated mice.[14] 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[52] has been reported to have similar effects: it decreases enterocyte apoptosis,[31 53] diminishes granulocyte infiltration[32 54] and promotes epithelial proliferation.[55–57] COX-2 has also been suggested to have a dual role in NEC, with high activities being proinflammatory, and lower levels protective[58]; systemic COX-2 inhibitors worsen intestinal inflammation and increase mortality in an NEC model.[59] 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.[60] In previous studies of COX-2 in NEC, no correlation was found between COX-2 expression and intestinal injury severity.[59] 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.[33] 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 involved different pathways.[33]

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.[61] 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.[61]

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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Contributors AZ, MC, AA, AP, SE, PDC: study concept, and manuscript drafting. AZ, MC, FFL, GL: animal experiments. VVS, NJS: histology review. SB, MPo, MPi: cell isolation and characterisation. JW, BS, MFL: MRI. ADA, AL: microarray-based gene expression analysis. MC, MG: Molecular biology. AH, CB: cDNA microarrays.

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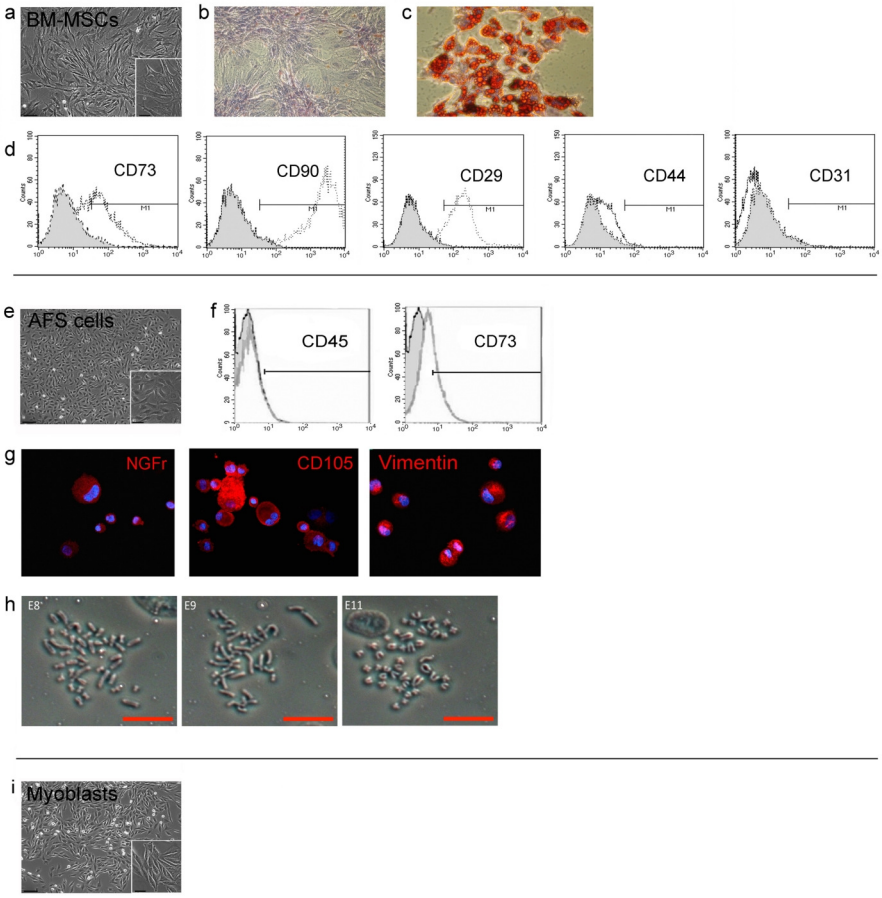
Supplementary figure 1.

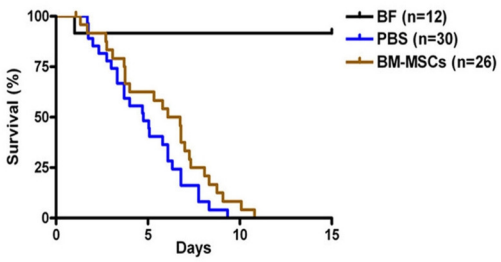
Cell characterization: **a.** BM-MSCs were cultured up to maximum 9 cell passages and cell morphology was assessed at each passage using a phase contrast Zeiss Axiovert 135 microscope (100µm scale bar in the 10x magnification photo; 50µm scale bar in the 32x magnification photo). **b-c.** Mesenchymal differentiation potential. BM-MSCs at the 5th passage were induced to differentiate into adipocytes by defined medium [11]. Adipogenesis was assessed with Oil red O staining on untreated (**b.**) and treated (**c.**) cells (20x). **d.** Immunophenotype. BM-MSCs at passage 5 were harvested and stained for specific cell surface antigens. At flow-cytometric analysis, cells were positive for mesenchymal stem cell markers (CD29, CD44, CD73, CD90) and negative for endothelial markers (CD31). The control isotype is indicated in light grey. **e.** AFS cell lines were cultured on non-tissue culture petri dishes as previously reported . **f.** Immunophenotype. At flow-cytometry and immunofluorescence analyses, AFS cells were negative for hematopoietic (CD45) and positive for mesenchymal (CD73, NFGr, CD105, Vimentin) markers. **h.** Phase contrast image of the three different clones (E8, E9, E11) used in the experiments showed a normal karyotype (scale bar= 20µm, 40x magnification). **i.** A line of committed cells, i.e. rat skeletal muscle myoblasts, were used as a supplementary negative control vs. AFS cells in rat NEC. Cell morphology was assessed using a phase contrast Zeiss Axiovert 135 microscope (100µm scale bar in the 10x magnification photo; 50µm scale bar in the 32x magnification photo).


a
BM-MSCs
b
c
d
CD73
CD90
CD29
CD44
CD31
e
AFS cells
f
CD45
CD73
g
NGFr
CD105
Vimentin
h
E8
E9
E11
i
Myoblasts

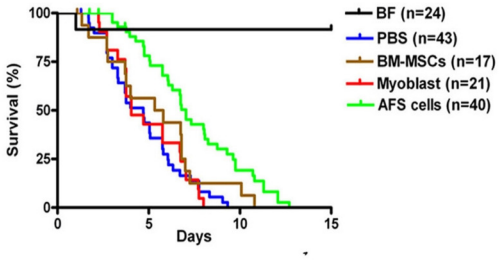
Supplementary figure 2.

NEC rats injected intraperitoneally (i.p.) with BM-MSCs (n=26; Fig. **supplementary 1**) at 24 and 48 hours of life did not show improved survival compared with animals injected with phosphate buffered saline (PBS; n=30) (Fig. 1a). Age-matched breastfed rats (BF=12) not subjected to NEC induction and used as controls, had 92% survival ($p<0.0001$) (**Fig. supplementary 2a**). Considering this lack of effect, NEC rats were injected with AFS cells, BM-MSCs, myoblasts (n=21, as a committed negative control) or PBS (n=43) and compared with BF rats (**Fig. 1b and supplementary 2b**).

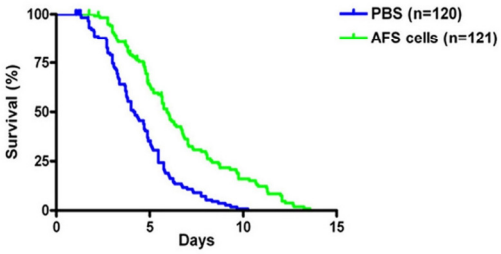
a


BF (n=12)
PBS (n=30)
BM-MSCs (n=26)
Survival (%)
Days
0
25
50
75
100
0
5
10
15

b


BF (n=24)
PBS (n=43)
BM-MSCs (n=17)
Myoblast (n=21)
AFS cells (n=40)
Survival (%)
Days
0
25
50
75
100
0
5
10
15

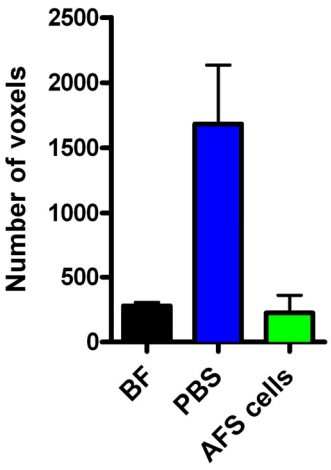
c


PBS (n=120)
AFS cells (n=121)
Survival (%)
Days
0
25
50
75
100
0
5
10
15

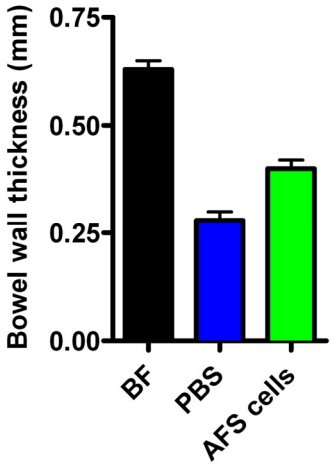
Supplementary figure 3.

Peritoneal fluid accumulation, (assessed using MRI imaging as voxels with T2>160ms) was significantly greater in the NEC rats injected with PBS, than those treated with AFS or the BF rats (**Supplementary Fig. 3a**). 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 (**Supplementary Fig. 3b**).

a

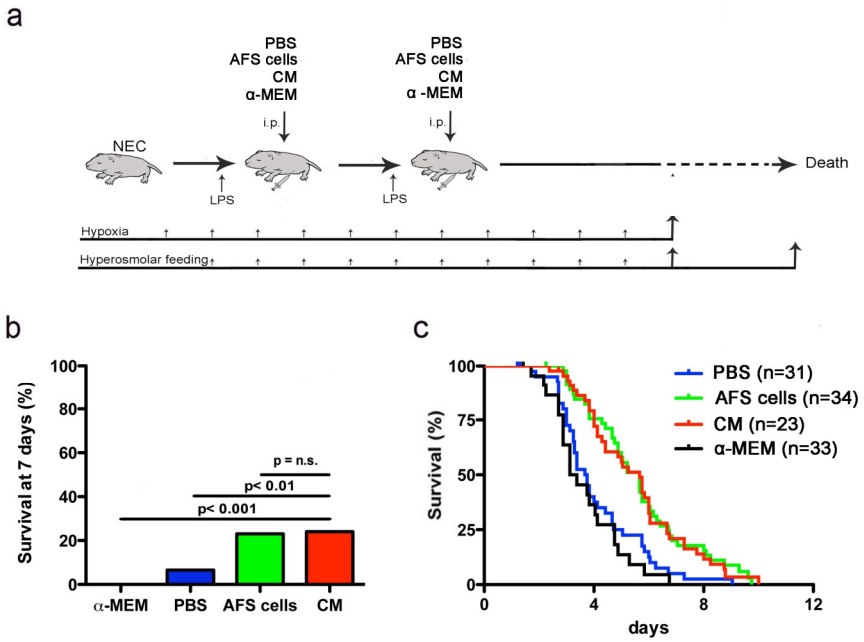

Number of voxels
2500
2000
1500
1000
500
0
BF
PBS
AFS cells

b


Bowel wall thickness (mm)
0.75
0.50
0.25
0.00
BF
PBS
AFS cells

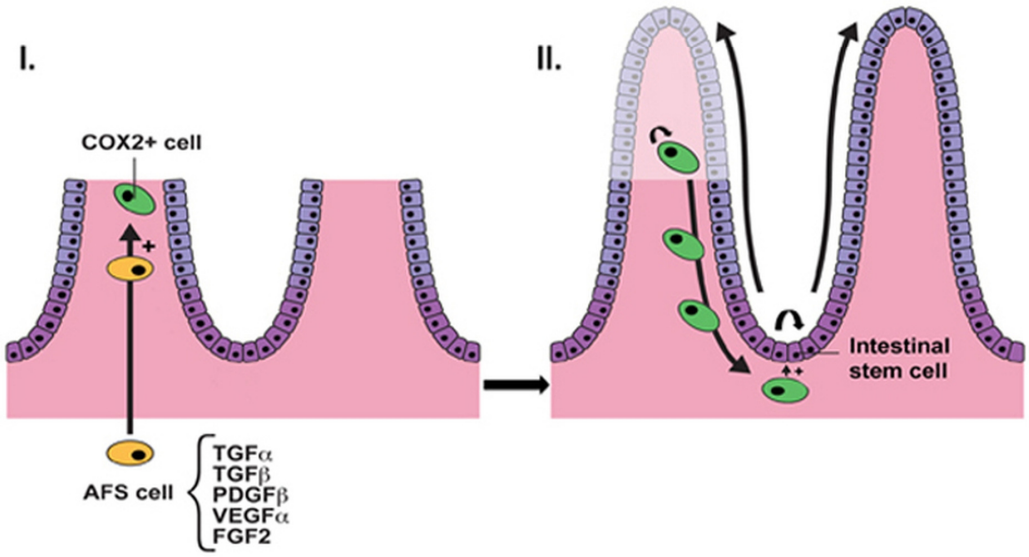
Supplementary figure 4.

a. NEC rats received an i.p. injection of either PBS, AFS cells, CM or α-MEM at 24 and 48 hours of life and were followed until natural death occurred. **b**. At 7 days of life, NEC rats treated with CM had a similar survival rate of those treated with AFS cells (p=n.s.), but had a higher survival than those treated with PBS ($p< 0.01$) or with α-MEM ($p< 0.001$). **c.** The overall survival study confirmed that CM injected rats had a significant longer survival than rats injected with PBS ($p< 0.01$) or with α-MEM ($p<0.0001$). Similarly AFS cells injected rats had a significant longer survival than rats injected with PBS ($p< 0.001$) or with α-MEM ($p<0.0001$). No differences were noted between AFS cell group and CM group (p=n.s.).


a
PBS
AFS cells
CM
α-MEM
i.p.
PBS
AFS cells
CM
α -MEM
i.p.
NEC
LPS
LPS
Death
Hypoxia
Hyperosmolar feeding
b
Survival at 7 days (%)
100
80
60
40
20
0
p = n.s.
p< 0.01
p< 0.001
α-MEM
PBS
AFS cells
CM
c
Survival (%)
100
75
50
25
0
0
4
8
12
days
PBS (n=31)
AFS cells (n=34)
CM (n=23)
α-MEM (n=33)

Supplementary figure 5.

This cartoon explains the beneficial action mechanism of AFS cells on damaged gut epithelium: (I) i.p. injected AFS cells activate resident COX-2+ cells via a paracrine effect; (II) as a result, COX-2+ cells migrate from the villus axis down to the crypt, where they stimulate resident stem cells to repopulate the sloughed epithelium.



Supplementary methods

Magnetic Resonance Imaging. Images were acquired at two stages. In stage one, pups were imaged immediately after sacrifice (AFS[n=3], PBS [n=3], BF [n=2]). Multi-slice spin-echo axial images were acquired at echo times of 15, 20, 30 and 45ms to generate T2 maps for visualisation of abdominal fluid. Other parameters were: no of slices = 40; in plane matrix size = 128 × 128; field of view = 19.2mm × 19.2mm; slice thickness = 0.5mm; slice separation = 0mm; repetition time = 3.5s. T2 maps were generated by fitting a mono-exponential decay model to the images acquired at increasing echo times. In stage 2, 1 pup from each group was imaged using μMRI methods [23]. Directly after stage 1 imaging, pups were immersed in 4% formaldehyde solution (from paraformaldehyde), doped with 8mM gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA, [Magnevist], Bayer–Schering, Newbury, UK) and placed on a rotator for 3 weeks. Pups (1 from each group) were scanned using a 3D gradient echo sequence. Parameters were: matrix size 512×512×512; imaging volume = 27 × 27 × 27mm; echo time =6ms; repetition time = 20ms; flip angle =60^{o}; number of averages = 8; 10 dummy scans. From the high resolution images, bowel wall thicknesses were measured in 5 axial slices in the same plane using the following anatomical landmarks: the top, bottom and midpoint of the right kidney, and the midpoint and bottom of the left kidney. Within these slices, only small bowel sections running perpendicular to the axial slice were considered to minimise partial volume effects. The thicknesses of bowel walls were evaluated by measuring the shortest distance between the inner diameter to the outer diameter at 4 points:

the most dorsal, ventral, lateral (sinister and dexter). The extent of fluid accumulation outside the bowels was estimated by counting the number of voxels with a T2 greater than 160ms in the same 5 slices. Voxels with a T2> 160ms within the bowel, bladder and spinal cord and kidney were excluded by manual segmentation based on visual inspection of the images. The threshold of 160ms was chosen based on the lower limit of the 95% confidence interval of the estimated T2 values within a region of fluid in the abdomen. A Student's T-test was used to test for possible differences between the wall thickness measurements as well as between the T2 voxel counts (see **Fig. supplementary 3a and b**).

Supplementary videos. Segment of rat intestine was immersed in oxygenated Krebs solution and maintained at constant length. A video camera linked to a computer recorded intestinal wall movement under isometric conditions. Gut movements of NEC rats (**video1**) are compared to gut derived from AFS cell treated NEC rats (**video 2**).

Supplementary table1.

Gut weight, length, weight/length index, and mass index (weight/length*100g of body weight at sacrifice) of breastfed rats and of rats injected with either PBS or AFS cells.

	F (mean ± SD	PBS	AFS cells	p*
Gut weight (g)	0.99 ± 0.2	0.31 ± 0.1	0.30 ± 0.1	>0.05
Gut length (cm)	36.5 ± 4.4	17.0 ± 2.3	15.8 ± 3.7	>0.05
Gut w/l (g/cm)	0.03 ± 0.004	0.02 ± 0.005	0.02 ± 0.007	>0.05
Gut mass (mg/cm*100g	238.2	368.9	412.8	>0.05

* (PBS vs. AFS cells)