CD24 is expressed in gastric parietal cells and regulates apoptosis and the response to *Helicobacter felis* infection in the murine stomach

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Duckworth CA, Clyde D, Pritchard DM. CD24 is expressed in gastric parietal cells and regulates apoptosis and the response to *Helicobacter felis* infection and γ-irradiation using CD24-null mice. Gastric CD24 expression was determined by immunohistochemistry in C57BL/6 mice. Female CD24-null and C57BL/6 mice were infected with *H. felis* for 6 wk, and inflammation, proliferation, apoptosis, and parietal cell numbers were assessed in gastric tissue sections. Apoptosis and proliferation were analyzed on a cell-positional basis in stomach, small intestine, and colon of CD24-null and C57BL/6 mice following γ-irradiation. Apoptosis was also assessed in HT29 cells following CD24 siRNA transfection. Of CD24-positive cells in the gastric corpus, 98% were H+/K+-ATPase-expressing parietal cells. CD24-null mice showed more prominent gastric *H. felis* colonization than C57BL/6 mice but displayed a marked reduction in corpus inflammation, reduced Ki67 labeling, and less gastric atrophy 6 wk following infection. Corpus apoptosis was elevated in CD24-null mice, but this did not increase further with *H. felis* infection as observed in C57BL/6 mice. More apoptotic cells were found following γ-irradiation in the stomach, small intestine, and colon of CD24-null mice and following CD24 knockdown in vitro. In conclusion, CD24 is expressed in gastric parietal cells, where it modulates gastric responses to *H. felis* and γ-radiation. CD24 also regulates susceptibility to apoptosis in the distal murine gastrointestinal tract.

CD24; apoptosis; stomach; small intestine; colon; *Helicobacter felis*; radiation

CD24 is a heavily glycosylated glycosyl phosphatidylinositol-linked glycoprotein, and increased expression of CD24 has been associated with poor prognosis (4, 6, 37), tumor progression (1, 14), and metastasis in several gastrointestinal (GI) cancers. The function of CD24 in the GI tract, however, remains largely unknown, although its roles in regulating immune cell apoptosis (5, 33) and differentiation (16, 17) as well as cell migration (27) have been well characterized in other tissues. Expression of the core polypeptide of CD24 has been observed in the proliferative zone of rat gastric glands and small intestinal crypts (3), and recent studies have indicated that CD24 may mark a subset of stem cells in the small intestine (36). CD24 has also been shown to label cancer stem cell subpopulations from primary gastric (39) and colorectal tumors (35). Similarly, CD24 has been demonstrated to be expressed in a subpopulation of small intestinal epithelial stem-like cells that were capable of forming organoids in vitro (11). However, in a separate study, expression of CD24 has been demonstrated in Paneth cells and intestinal stem cells of the small intestine and in cells at the colonic crypt base. The authors of this article have proposed that Paneth cells that express CD24 may provide an environment for putative Lgr5-positive stem cells to proliferate (26).

In keeping with a stem cell-regulating role for CD24 in the GI tract, cancer stem cell subpopulations of the colorectal cancer cell line HT29 and gastric cancer cell line AGS that express CD24 have been shown to initiate tumorigenesis when transplanted into nude mice (38, 39). Multilineage differentiation of CD24 expressing cells has also been observed in cells which possess goblet cell-like, endocrine cell-like, and enteroendocrine-like phenotypes; however, CD24 expression is rapidly downregulated during this differentiation process (reviewed in Ref. 34). CD24 has also been shown to be a potential target of the stem cell-regulating canonical Wnt-signaling pathway because of TCF/LEF consensus sequences present within the CD24 promoter (30).

Unlike in the distal GI tract, where the localization and importance of CD24 expression has now been reasonably well characterized, the role of CD24 in the stomach has received little attention to date. Although CD24 is known to be overexpressed in gastric adenocarcinoma (4) and gastric cancer stem cells (39), previous immunohistochemical studies have not demonstrated expression in normal human gastric epithelium (6).

We therefore hypothesized that, as in the distal GI tract, CD24 is expressed in the murine stomach and that it regulates cellular function in this tissue in ways that potentially alter susceptibility to gastric cancer development. Our investigations have used CD24-null mice, which are viable and have a normal life expectancy (17). To date, no major phenotype has been observed in the GI tract of these mice. The susceptibility of CD24-null gastrointestinal epithelia to cellular stresses, such as infection or γ-radiation has also not been extensively investigated. *Helicobacter pylori* infection of the stomach in humans is a risk factor for developing distal gastric cancer (12). Although *H. pylori* infection does not induce gastric cancer in wild-type C57BL/6 mice, the related organism, *H. felis*, causes these mice to develop evidence of gastric neoplasia 15 mo following infection. This is therefore thought to represent a good animal model of *H. pylori*-induced gastric carcinogenesis in humans (10). Because female C57BL/6 are more susceptible to the effects of *H. felis* infection than males (7), we have investigated whether CD24 deletion affects the early stages of *H. felis*-induced gastric pathology using female mice and have conducted γ-radiation experiments with male mice since no
sex difference has previously been reported with use of this stimulus (22).

We have therefore assessed the immunohistochemical localization of CD24 expression in the murine stomach and have investigated whether CD24-null mice show any altered gastric phenotype in terms of apoptosis, proliferation, and differentiation. Subsequently we have assessed whether CD24-null mice showed any altered responses to the damage-inducing stimuli of *H. felis* infection and γ-irradiation in the stomach, small intestine, and colon and have determined the effects of CD24 knockdown in the HT29 cell line that was shown to express CD24 in abundance.

**MATERIALS AND METHODS**

**Animals.** C57BL/6 (Charles River, Kent, UK) and CD24-null mice on the inbred C57BL/6 genetic background (kindly provided by Professor Peter Altevogt, German Cancer Research Centre, Heidelberg, Germany) were maintained at the Biomedical Services Unit, University of Liverpool (Liverpool, UK). Animals were fed a commercially prepared pelleted diet, given water ad libitum, and maintained on a 12:12-h light-dark cycle under conventional animal house conditions. All experiments were performed during daylight hours. All animals were euthanized by cervical dislocation in accordance with UK Home Office legislation under authority of project license 40/3392 and with approval by the University of Liverpool Animal Use Committee.

*Helicobacter felis* infection. Groups of six female C57BL/6 and CD24-null mice aged 6–7 wk were orally gavaged with *H. felis* (ATCC 49179) on three separate occasions over the course of 1 wk. These mice and age-matched control animals were euthanized 6 wk later. Stomachs were removed and fixed in 4% formalin prior to being embedded in paraffin wax. Infection and inflammation scores were determined from hematoxylin and eosin (H&E)-stained 4-μm tissue sections. Twenty well-orientated antral glands per mouse were assessed for the presence or absence of *H. felis* colonization and presented as percentage of glands infected. Inflammation scores were determined based on criteria previously defined by Rogers et al. (23); briefly, a grade of 0 was given for normal gastric mucosa, 1 for patchy infiltration of mixed leukocytes in the mucosa and/or submucosa, 2 for coalescing mucosal infiltration and/or early submucosal extension, 3 for coalescing mucosal infiltration with prominent multifocal extension, and 4 for effacing transmural inflammation (23).

Irradiation. Six male mice aged 10–12 wk were used per treatment group. Animals were whole-body γ-irradiated by use of a Cs137 source delivering a dose of 2.6 GY/min. The dose and time course of γ-irradiation was different for each tissue because susceptibility of different tissues towards 80% confluent incubation at 37°C with Trypsin/EDTA (Sigma-Aldrich) in PBS; 2 × 10^5 cells were added to a T75 flask and allowed to adhere for 24 h prior to transfection with siGENOME SMARTpool CD24 or control siRNAs (Dharmacon, Perbio UK, Cramlington, UK) as per the manufacturer’s instructions. Briefly, cells were incubated with CD24 or control siRNAs for 72 h at 37°C with 5% CO_2. Cells were lysed from transfections when 80% confluent incubation at 37°C with Trypsin/EDTA (Sigma-Aldrich) in PBS; 2 × 10^5 cells were added to a T75 flask and allowed to adhere for 24 h prior to transfection with siGENOME SMARTpool CD24 or control siRNAs (Dharmacon, Perbio UK, Cramlington, UK) as per the manufacturer’s instructions. Briefly, cells were incubated with CD24 or control siRNAs for 72 h at 37°C with 5% CO_2 in a humidified chamber at 4°C. Slides were washed in 0.14, 0.5, and 0.14 M NaCl (Sigma, Gillingham, UK) and incubated with goat anti-rat FITC and goat anti-rabbit Texas red-conjugated secondary antibodies (1/800; Jackson Immuno Research Laboratories, Cambridgeshire, UK) for 1 h at room temperature. Sections were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector Laboratories, Peterborough, UK). We imaged three high-power fields per corpus, making sure that the whole gland thickness from base to gland surface was included in each image. Glands were divided into equal thirds and immunofluorescently positive cells were counted in the gland base, middle section (containing the isthmus region), and top third (constituting mainly the gastric pit) by use of a fluorescent microscope (Olympus Microscopy, Essex, UK).

Immunohistochemistry. We assessed 4-μm-thick sections of stomach, small intestine, and colon using immunohistochemistry for proliferation by Ki67 (1/25; Dako), apoptosis by active caspase-3 (1/750; R and D Systems), and parietal cells by H^+-K^+-ATPase (1/1,000; Merck Chemicals, Nottingham, UK). Standard immunohistochemical methods were used. Briefly, heat-mediated antigen retrieval using ethylenediaminetetraacetic acid buffer (pH 6) was used for all antibodies except H^+-K^+-ATPase, which only required pretreatment with 0.1% Triton X-100. Appropriate biotin-conjugated secondary antibodies (Dako UK, Ely, UK) and VectaStain ABC (Vector Labs, Peterborough, UK) were applied and the signal was detected by diaminobenzidine (Sigma-Aldrich). Sections were counterstained with hematoxylin. Immunohistochemical staining was quantified on a cell-positonal basis along the crypt or gland axis as previously described (18, 22). Data are presented as mean apoptotic index (%) and as plots of percentage apoptotic cell index against cell position along the crypt or gland axis.

HT29 cell culture, siRNA transfection, and assessment of apoptosis. The transformed human colonic epithelial cell line HT29 was maintained in DMEM (Sigma-Aldrich) containing 10% FCS (Gibco Invitrogen, Paisley, UK) at 37°C with 5% CO_2. Cells were lysed from transfections when 80% confluent incubation at 37°C with Trypsin/EDTA (Sigma-Aldrich) in PBS; 2 × 10^5 cells were added to a T75 flask and allowed to adhere for 24 h prior to transfection with siGENOME SMARTpool CD24 or control siRNAs (Dharmacon, Perbio UK, Cramlington, UK) as per the manufacturer’s instructions. Briefly, cells were incubated with CD24 or control siRNAs for 72 h at 37°C with 5% CO_2 in a humidified chamber. Additionally, HT29 cells were assessed for apoptosis following transfection in 96-well plates by using a commercially available Caspase-Glo 3/7 Assay (Promega UK, Southampton, UK) according to the manufacturer’s instructions. Reduction in CD24 expression following transfection was confirmed by Western blot analysis. Cells were lysed in RIPA buffer and subjected to polyacrylamide gel electrophoresis under reducing conditions on a 12% gel. Proteins were probed on nitrocellulose membranes by using a mouse anti-human CD24 antibody (SN3 clone, dilution 1/200; Insight Biotech, Wembley, UK) or mouse anti-pan actin antibody (Neomarkers, Lab Vision Products, Runcorn, UK, dilution of 1/1,000). The secondary antibodies used were horseradish peroxidase-conjugated anti-rat or anti-mouse immunoglobulins (Dako UK, Ely, Cambridge, UK) at a dilution of 1/2,000.

Statistical analyses. A two-way ANOVA followed by Bonferroni correction for multiple comparisons was used to determine significant differences between mouse genotype and infection status, and a one-way ANOVA was used to determine differences between antral infection status between genotypes. A two-tailed two-sample Stu-
dent’s t-test assuming unequal variance was used to determine significant differences between irradiated mouse stomachs and cell line treatments. A modified median test (20) was used to determine significant differences at individual cell positions in mouse studies. Significance was defined as $P < 0.05$ by ANOVA and t-test and differences at $\geq 3$ consecutive cell positions by modified median test. Nonparametric corpus inflammation data were assessed by the Kruskal-Wallis ANOVA on ranks test with Tukey’s post hoc analysis for multiple comparisons.

RESULTS

CD24 is expressed in parietal cells of the gastric corpus. To determine whether CD24 was expressed in the stomach of C57BL/6 wild-type mice, we performed immunohistofluorescence on frozen sections. CD24 expression was detected in antral glands of C57BL/6 (Fig. 1, A and B), but this was diffuse and did not appear to occur within specific cells or a specific region of the gland. Abundant CD24 expression was also observed in the corpus parietal cells of C57BL/6 mice (Fig. 1D). To confirm that the antibody was specific for CD24, we carried out staining in parallel on CD24-null mouse antrum and corpus and were unable to detect any FITC signal, suggesting that the detection of CD24 in C57BL/6 mice was specific (Fig. 1A and F). Analysis of dual-fluorescence corpus images for CD24 and the parietal cell proton pump protein H+−K+−ATPase showed that 68 ± 7.3% of H+−K+−ATPase-positive cells were also positive for CD24 and that 98 ± 10.4% of the cells that were positive for CD24 also stained positively for H+−K+−ATPase. These proportions did not significantly change 6 wk following infection by *H. felis* (71 ± 10.0 and 89 ± 12.3%, respectively), although the absolute number of parietal cells was reduced in infected animals (Fig. 1G).

Gastric phenotype of CD24-null mice. Initial histological examination of CD24-null mouse gastric epithelium revealed no major alterations compared with C57BL/6 wild-type mice. As discussed in more detail below, gastric antral and corpus glands were unchanged in length compared with those of C57BL/6 wild-type mice (Fig. 2, D and H) and the composition of the mucosa in terms of the proportions of parietal cells was unchanged. Gastric corpus glands of C57BL/6 mice consisted of 30.4% ± 1.75 H+−K+−ATPase-positive cells compared with 29.0% ± 1.30 in CD24-null corpus glands (Fig. 4E). Assessment of basal levels of gastric mitosis (data not shown) and proliferation (Fig. 3E) were unaltered, but more apoptotic cells were observed in both the gastric antral and corpus epithelia of untreated CD24-null mice (Figs. 5E and 6, A and D). The role of CD24 expression in the stomach at this stage of the investigation therefore remained unclear. However, in view of the expression of CD24 in parietal cells and the alteration in basal gastric apoptotic index, we hypothesized that any gastric phenotype in CD24-null mice might become more apparent after induction of cellular injury.

CD24-null mice are more susceptible to *H. felis* colonization of the gastric antrum; however, they show reduced corpus inflammation. Oral gavage of *H. felis* resulted in colonization deep within antral glands of both C57BL/6 and CD24-null mice 6 wk following infection (Fig. 2, A and B). The amount...
of *H. felis* colonization, however, appeared greater in CD24-null compared with C57BL/6 mice (Fig. 2C). Very few *H. felis* organisms were observed in the gastric corpus glands of either mouse strain. Inflammation in the gastric corpus of C57BL/6 mice consisted mainly of mononuclear cells and neutrophils and was significantly more severe than that observed in control C57BL/6 mice 6 wk following *H. felis* infection. CD24-null corpus histology, however, did not show a similar inflammatory infiltrate following *H. felis* infection, and inflammation scores were not significantly different from uninfected CD24-null mice (Fig. 2, E–G). In keeping with the *H. felis* model, gastric inflammation was corpus predominant with very mild to no inflammation observed in the antrum of either strain of mice (data not shown).

Gastric corpus glands of CD24-null mice do not demonstrate increased proliferation or hyperplasia following *H. felis* infection. *H. felis* infection of wild-type mice is associated with gastric gland hyperplasia as a result of increased epithelial cell proliferation. C57BL/6 mice showed a significant 1.8-fold increase in corpus gland length 6 wk following infection by *H. felis*, whereas CD24-null corpus glands did not display any such increase in cell number per hemi-gland (Fig. 2H). A significant increase in antral gland length was observed in both C57BL/6 and CD24-null mice 6 wk following infection; however, no significant difference in gland length was observed between the strains in untreated or *H. felis*-infected groups (Fig. 2D). Ki67 immunohistochemistry (Fig. 3, A–D) was used to quantify proliferation within gastric corpus glands to determine whether the lack of hyperplastic response in CD24-null mice was associated with proliferation and not other phenomena such as excessive cell death. As expected, C57BL/6 mice showed a significant 1.7-fold increase in Ki67 labeling index whereas no significant change in Ki67 labeling was seen between control and infected CD24-null corpus glands 6 wk following *H. felis* infection (Fig. 3E). Cell-positional quantification of Ki67 labeling also confirmed reduced positively labeled cells in CD24-null (Fig. 3G) compared with C57BL/6 (Fig. 3F) mice. A greater proportion of cells in the gastric antrum compared with corpus of C57BL/6 mice were Ki67-positive. CD24-null gastric antrum showed a small but significant increase in Ki67 labeling following *H. felis* infection whereas C57BL/6 antral glands showed no significant increase (Fig. 3E).

CD24-null mice do not show parietal cell atrophy in response to *H. felis* infection. Infection by *Helicobacter* sp. is associated with parietal cell atrophy, and this is reliably induced in female C57BL/6 gastric corpus 6 wk postinoculation with *H. felis* (24, 25). Because CD24 was found to be expressed in 68% of parietal cells, we investigated whether there was any alteration in parietal cell number in uninfected CD24-null compared with C57BL/6 gastric corpus and found there to be no significant difference between the two strains (Fig. 4, A and B). However, we found significantly less parietal cell atrophy in CD24-null compared with C57BL/6 mice 6 wk post-*H. felis* infection (Fig. 4, C and D). We observed a significant 53% reduction in parietal cell index in C57BL/6 following *H. felis* infection compared with only a 7% reduction in parietal cell index in CD24-null mice following the same treatment, and this was not significantly different from uninfected control CD24-null mice (Fig. 4E). Cell-positional analysis of parietal...
cell labeling showed a shift in the distribution profile toward the top of gastric glands in C57BL/6 mice following *H. felis* infection (Fig. 5F); however, no such distributional alteration was observed in infected vs. uninfected CD24-null mice (Fig. 5G).

**Basal levels of apoptosis are elevated in CD24-null gastric corpus and are not altered following *H. felis* infection.** Basal levels of apoptosis in the stomach of wild-type mice are known to be very low (22); however, an increased level of apoptosis has previously been observed following *H. felis* infection (7). We therefore determined whether CD24 deletion had any effect on levels of gastric apoptosis in uninfected CD24-null mice using active caspase-3 immunohistochemistry (Fig. 5, A and B), and by morphology using H&E-stained sections. We found a significantly elevated amount of apoptosis in CD24-null both by active caspase-3 immunohistochemistry (3.32-fold in gastric corpus and 2.47-fold in antrum) (Fig. 5E) and by H&E (corpus 2.83-fold and antrum 2.38-fold) (Fig. 6, A and D). As expected, we also observed a significant increase in active-caspase-3 labeling in the corpus of 6-wk *H. felis*-infected C57BL/6 mice; however, although levels remained elevated, no further increase in the number of active caspase-3-labeled cells was observed in CD24-null mice following *H. felis* infection (Fig. 5C-G). No significant increase in active-caspase-3 labeling was observed following *H. felis* infection in either C57BL/6 or CD24-null mice in the gastric antrum; however, there was a significant increase in active caspase-3 labeling between infected CD24-null and C57BL/6 mice (1.83-fold; Fig. 5E).

**γ-Radiation-induced apoptosis is elevated in CD24-null gastric antrum and corpus.** As basal levels of epithelial apoptosis were elevated in CD24-null stomach and no further increases were observed following *H. felis* infection, we investigated the apoptotic response of CD24-null gastric epithelial cells following a different model of gastric injury. A dose of 12-Gy γ-radiation has previously been shown to induce murine
gastric epithelial apoptosis after 6 h, with maximal levels being observed after 48 h (22). We therefore administered this dose of radiation and assessed the stomachs of C57BL/6 and CD24-null mice for morphologically apoptotic cells after both 6 h and 48 h. Amounts of apoptosis were again elevated in untreated CD24-null mice compared with C57BL/6 mice when assessed by morphology using H&E-stained sections in both antrum (Fig. 6A) and corpus (Fig. 6D).

CD24-null mice also showed a significant increase in the amount of /H9253-radiation-induced apoptosis at both the 6- and 48-h time points in the antrum (Fig. 6, B and C) and corpus (Fig. 6, E and F) compared with wild-type C57BL/6 mice. Mitosis in antral and corpus glands at this dose and time point was completely suppressed in both C57BL/6 and CD24-null mice (data not shown).

Basal and γ-radiation-induced levels of apoptosis are also elevated in the small intestine and colon of CD24-null mice. Having established that CD24 plays a role in the inhibition of apoptosis of gastric epithelial cells (Figs. 5 and 6), we further investigated the effects of CD24 deletion upon spontaneous and γ-radiation-induced apoptosis in the murine small intestine and colon. We showed a significant 1.7-fold increase in the basal level of apoptosis in the small intestine, and a significant 2.9-fold increase in the baseline amount of apoptosis in the colon of CD24-null mice (Fig. 7, A and E). Because small intestinal and colonic epithelial cells are both more susceptible to the effects of γ-radiation than gastric epithelial cells, we administered the lower dose of 1-Gy whole-body radiation to induce apoptosis and quantified apoptotic cells 4.5 h later. CD24-null mice again showed elevated levels of apoptosis following irradiation in both the small intestine (Fig. 7B) and colon (Fig. 7F). Cell-positional analyses showed a significantly greater proportion of apoptotic cells at cell positions 7–11 in the small intestine and 6–12 in the colon following γ-irradiation (Fig. 7, B and F). No significant difference in mean mitotic...
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index or cell-positional distribution of mitotic figures was observed in either small intestine or colon of untreated (Fig. 7, C and G) or 4.5-h 1-Gy-irradiated (Fig. 7, D and H) CD24-null compared with C57BL/6 mice.

Levels of apoptosis are increased in HT29 cells following CD24 siRNA treatment. To investigate further whether the altered susceptibility to apoptosis throughout the GI tract was specifically related to CD24 deletion, CD24 expression was manipulated in a colon cancer cell line. A panel of human gastric and colon cancer cell lines was initially assessed for expression of CD24. The HT29 cell line expressed the highest amount of CD24 protein (Fig. 8A) and was therefore chosen for CD24 knockdown. HT29 cells were transfected with either CD24 or control siRNA and apoptosis was assessed by two independent methods. Counting of morphologically apoptotic cells showed a significant increase from $2.75 \times 10^5 \pm 0.33$ cells in control siRNA-transfected flasks to $3.56 \times 10^5 \pm 0.31$ cells in CD24 siRNA-transfected flasks ($P = 0.001$, Fig. 8C). Duplicate flasks were harvested for Western blotting to confirm CD24 knockdown (Fig. 8B). Adherent viable cells were quantified as an indicator of proliferative potential of cells following CD24 knockdown. There was a significant decrease from $9.42 \pm 0.61 \times 10^5$ cells in control siRNA-transfected flasks to $6.38 \pm 0.19 \times 10^5$ cells in CD24 siRNA-transfected flasks (Fig. 8D). The observed increase in apoptosis was also confirmed by using a commercial caspase 3/7 Glo assay, which produces luminescence relative to the concentration of active caspases 3 and 7. The assay also showed a significant increase in caspase 3/7 activity in CD24 siRNA-transfected cells relative to control siRNA-transfected cells ($P < 0.001$, Fig. 8E).

DISCUSSION

CD24 is a glycoprotein involved in differentiation (16) and apoptosis (5, 33) in the immune system as well as migration in the nervous system (27). The functions of CD24 are, however, less well characterized in the GI tract, although some authors have proposed that CD24 plays a role in regulating the development and maturation of GI tissues (3, 26, 29). More recently,
evidence that CD24 is expressed predominantly within the bottom half of small intestinal crypts (36), that CD24 is expressed within subpopulations of gastric and colon cancer stem cells (35, 39), and that CD24 expression may aid in providing a suitable environment for putative Lgr5-positive stem cells to proliferate (26) has led to the proposal that CD24 acts as a potential marker or regulator of stem cells in the GI tract.

To the best of our knowledge, CD24 localization has never previously been described in the murine stomach. We have shown that CD24 is expressed by more than half of the parietal (H⁺-K⁺-ATPase positive) cells in the murine gastric corpus (Fig. 1) and that this proportion did not change following infection by *H. felis*. We have also demonstrated that a small number of nonparietal cells express CD24. Currently available antibodies against CD24 require the use of frozen sections, which compromises the preservation of tissue architecture compared with paraffin-embedded sections; therefore the exact identity of these CD24-positive H⁺-K⁺-ATPase negative cells was not fully determined. The remaining CD24-positive, H⁺-K⁺-ATPase-negative population was distributed along the gland axis and may consist of a small number of epithelial, enteroendocrine, immune, or mesenchymal cells. Similarly to the stem cell-related functions of CD24 in the small intestine and colon (26), CD24-expressing parietal cells of the gastric corpus may provide a supportive niche for gastric stem cells to proliferate. We have observed a greater number of CD24-expressing parietal cells in the middle third along the corpus gland axis, which mainly constitutes the isthmus region (Fig. 1G) and is where gastric stem cells are thought to reside. Because the stomachs of CD24-null mice initially appeared morphologically normal, we investigated whether the response to gastric injury was altered in these animals. *H. pylori* colonizes the stomach and proximal duodenum of humans and is a susceptibility factor for distal gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (12). *H. felis* infection is a well-characterized murine model of distal gastric cancer in humans and is characterized by bacterial colonization predominantly deep within antral glands and inflammation present in the gastric corpus, with little inflammation detected in the antrum. Similar characteristics are also observed when mice are infected with a mouse adapted strain of *H. pylori* (25). Infection with *H. felis* causes parietal cell atrophy, an important preneoplastic pathological feature, 6 wk postinfection (24, 25). Because CD24 was expressed predominantly in gastric parietal cells, potentially modulating parietal cell homeostasis, this was the first model that we investigated. A greater abundance of *H. felis* colonization was observed deep within the antral glands of CD24-null mice, suggesting that stomachs from these mice are susceptible to infection (Fig. 2, A–C). However, a reduced inflammatory infiltrate was observed in the gastric corpus of CD24-null animals (Fig. 2, E–G). T cell maturation has been shown to take place in CD24-null mice (17); however, the reduction in immune response that we have observed in CD24-
null mice may be due to the reduced capacity of CD24-negative T cells to proliferate (13). Rag1−/− mice that are deficient in B and T cells and TCRβ−/− mice that lack T cells do not display gastric pathology following *H. felis* infection; however, micro MT mice that only lack B cells respond similarly to immunocompetent C57BL/6 mice, showing that the immune response to *H. felis* infection is predominantly T cell mediated (24). CD24 expression has been previously demonstrated on the surface of a variety of immune cells including various stages of maturation of B cells, T cells, and neutrophils (5, 9, 16, 17, 33).

CD24-null gastric corpus glands were no different in length compared with C57BL/6 under normal conditions; however, CD24-null glands did not significantly change in length 6 wk following infection whereas C57BL/6 glands became hyperplastic (Fig. 2H). The hyperplastic response of gastric glands 6 wk following *H. felis* infection is normally associated with an increase in the proliferation of epithelial cells (7, 25). We showed no significant increase in proliferation using Ki67 immunohistochemistry in 6-wk *H. felis*-infected CD24-null mice; however, we did observe the expected increase in infected C57BL/6 mice (Fig. 3), suggesting that CD24 is necessary for the proproliferative effect observed in gastric epithelial cells in response to infection. This is likely to be via an indirect mechanism.

We did not observe a significant difference in gastric parietal cell numbers between untreated CD24-null and C57BL/6 mice. However, although C57BL/6 mice showed marked parietal cell loss 6 wk following *H. felis* infection, CD24-null mice showed no significant change in parietal cell number compared with control mice (Fig. 4). This suggests that CD24 expression in parietal cells makes them more susceptible to the effects of *H. felis*. *H. felis* infection has not been associated with autoimmune gastritis (25) and therefore protection of parietal cell destruction in CD24-null corpus is unlikely to be due to the prevention of production of H+-K+-ATPase autoantibodies. Generation of gastric epithelial cell- and immune cell-specific murine CD24 knockout models would in future be useful to determine whether the observed effects are all mediated by
actions of the immune system in the gut, by epithelial cells directly, or by a combination of both.

Apoptotic cells were found in greater abundance in CD24-null corpus compared with C57BL/6; however, no further increase was observed following H. felis infection. This suggests that CD24 is protective against spontaneous apoptosis, but it is not implicated in H. felis infection-induced apoptosis (Fig. 5). To address whether CD24 also regulates apoptosis in different GI tissues, and following different modes of injury, we used γ-irradiation to determine the susceptibility of CD24-null mice to induction of apoptosis in the stomach, small intestine, and colon. Apoptosis was significantly increased in the small intestine, distal colon, and stomach of untreated CD24-null mice compared with the C57BL/6 background strain, and the cell-positional distributions of apoptosis were consistent with previous studies in wild-type mice (15, 21), with apoptotic bodies being observed most frequently at the postulated stem cell zone of each tissue (Figs. 6 and 7). Mitosis was not significantly altered by deletion of the CD24 gene in any portion of the GI tract. However, there was no significant alteration in cell number in gastric glands or intestinal crypts. The proportion of apoptotic compared with nonapoptotic cells was low. We have not assessed the longevity or shedding rate of cells, which may be different between wild-type and CD24-null mouse strains, and changes in these parameters may negate the need for increased proliferation rates. Therefore the data suggest that CD24 functions as an antia apoptotic protein under normal conditions in the GI tract. Following radiation, this phenomenon was maintained. In the small intestine and colon, 1-Gy γ-radiation induced apoptosis 4.5 h after exposure and at this dose and time point, small intestinal and distal colonic epithelial apoptosis was significantly increased in CD24-null mice compared with wild-type controls. Similarly, in the gastric antrum and corpus, significantly elevated amounts of apoptosis were observed 6 and 48 h following 12-Gy γ-radiation in CD24-null mice. CD24 therefore has a role in protection against apoptosis induced by γ-radiation, suggesting that the role of CD24 in maintaining tissue homeostasis is context dependent. The altered response to H. felis infection in CD24-null mice is likely to be immune mediated because of the observed inflammatory infiltrate in C57BL/6 wild-type mice that was absent in CD24-null mice, with no effect on corpus epithelial cell apoptosis. Damage induced by γ-radiation directly affects the epithelium, and in this context CD24 deletion in epithelial cells resulted in increased sensitivity to the induction of apoptosis.

We subsequently used a colonic epithelial cell line model (HT29) to determine whether CD24 also regulated apoptosis in a human GI cell line in vitro. This also allowed us to determine the direct effects of CD24 deletion on epithelial cells without immune involvement. A significantly increased proportion of morphologically apoptotic cells were found floating in the media following CD24 siRNA-transfection of HT29 cells compared with control siRNA transfection, suggesting an antia apoptotic function of CD24 in these cells. Since some apoptotic cells may have been in the early stages of apoptosis and not yet detached from the flask, or may have undergone cell fragmentation, these cells would not have been counted. Therefore, we

Fig. 8. A: Western blot showing CD24 expression in HT29, HCT116, Caco-2, DLD-1, and LS1747–3 human colon cancer cell lines and AGS, MKN-45, and HGT-1 gastric cancer cell lines. B: Western blot showing protein expression of CD24 following control scrambled or CD24 siRNA treatment of HT29 cells. Percentage of apoptotic compared with nonapoptotic cells (C) and viable cells (D), 72 h following control (black) or CD24 siRNA (gray) knockdown. Caspase 3/7 activation in relative luminescence units (RLUs) 72 h following control (black) or CD24 (gray) siRNA (E). Significant differences by t-test (**P < 0.001).
sought to confirm our observations using a second, independent method of measuring apoptosis based on caspase 3/7 activation. Here we found that CD24 siRNA-transfected HT29 cells had increased caspase 3/7 activation compared with control siRNA-transfected cells. Ahmed et al. (2) reported no alteration in HT29 cell apoptosis following RNA interference; this discrepancy between results may, however, be due to different siRNAs and apoptosis quantification methods used between laboratories and a different stimulus (staurosporine) to induce apoptosis. Studies performed in other epithelial cell lines have demonstrated that CD24 knockdown using either siRNA or shRNA caused increased susceptibility to apoptosis (31, 32) and an immunocomplex of anti-CD24 and Pseudomonas exotoxin has also been shown to induce apoptosis in the HT29 cell line (28). Our in vivo studies now strongly suggest that CD24 is also involved in the regulation of apoptosis in untransformed GI epithelial cells.

In summary, our data suggest that CD24 is not solely a stem cell marker in the GI tract since it is expressed in differentiated gastric parietal cells. Nevertheless, it appears to be involved in regulating the homeostasis of GI tissues. The roles of CD24 are complex and the interplay between CD24-deficient immune cells and CD24-deficient epithelial cells is likely to determine the response to injury, particularly in response to a stimulus such as *H. felis* infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.A.D. and D.C. performed experiments; C.A.D. and D.C. analyzed data; C.A.D., D.C., and D.M.P. interpreted results of experiments; C.A.D. prepared figures; C.A.D. drafted manuscript; C.A.D., D.C., and D.M.P. approved final version of manuscript; D.C. and D.M.P. edited and revised manuscript; D.M.P. conception and design of research.

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