ANIMAL MODELS

A New Animal Model of Gastric Lymphomagenesis *APRIL Transgenic Mice Infected by Helicobacter Species*

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APRIL is a member of the tumor necrosis factor cytokine family involved in the regulation of B-cell immunity. We present a study of the infection by *Helicobacter* species of transgenic (Tg) C57BL6 mice, ectopically expressing the human form of APRIL. Wild-type (WT) and APRIL Tg mice were infected with *Helicobacter felis* and *Helicobacter pylori* and compared with noninfected animals. Mice were euthanized 18 months after infection, and inflammatory responses and histologic alterations were analyzed. Flow cytometry results revealed that WT-infected mice had less leukocyte infiltration than APRIL Tg-infected mice. In WT-infected mice, infiltrates in gastric tissues were predominantly composed of T cells, mainly CD4⁺ for *H. pylori* and CD8⁺ for *H. felis*. In APRIL Tg-infected mice, leukocyte infiltrates were composed of B cells with few CD4⁺ T cells for both species. B cells expressed B surface markers compatible with a marginal zone origin. These results were confirmed by immunohistochemistry. B cells in particular were involved in lymphoepithelial lesions, a hallmark of gastric MALT lymphoma. Monoclonality was observed in a few infiltrates in the presence of lymphoepithelial lesions. These results confirm the importance of APRIL in the development of gastric lymphoid infiltrates induced by *Helicobacter* species *in vivo*. We believe that APRIL Tg mice infected by *Helicobacter* species may represent a novel animal model of gastric lymphomagenesis. (*Am J Pathol* 2017, 187: 1473–1484; <http://dx.doi.org/10.1016/j.ajpath.2017.03.004>)

Extranodal marginal zone—type MALT lymphomas are indolent, low-grade, mature small B-cell non-Hodgkin lymphomas that occur in a number of extranodal organs, including the gastrointestinal tract.¹ Among these, the stomach is the most frequent site for the development of MALT lymphoma. Gastric MALT lymphoma (GML) in humans can be directly related to infection by *Helicobacter pylori*, but it only appears in a small subset of infected individuals.² *H. pylori* eradication leads to complete remission in 60% to 80% of such cases.³ Long-term antigenic stimulation exerted by *H. pylori* on the gastric mucosa leads to the formation of dense lymphoid infiltrates that contain small centrocytic B-cell—expressing surface markers typical of the marginal zone B cells of normal MALT. B cells are organized in lymphoid follicles and are involved in lymphoepithelial lesions (LELs), characterized by the invasion of the

epithelium of the gastric glands by proliferating B cells and epithelial cell destruction.⁴ Nonneoplastic T lymphocytes are associated with lymphoma cells with a predominance of CD4⁺ helper T cells.⁵

To better understand mechanisms that promote gastric lymphomagenesis and because of the difficulty in obtaining primary xenografts from surgical specimens of patients with GML, animal models need to be developed. Three *Helicobacter* species—*Helicobacter felis*,^{6–9} *Helicobacter heilmannii*,^{8,10–12} and *Helicobacter pylori*,^{8,13–16}—and three rodents—C57BL6^{12,13} and BALB/c mice^{6–10,13–15} and Mongolian gerbils¹¹—are generally used.

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At 18 to 24 months post infection (PI), *H. felis*-infected BALB/c mice develop histologic lesions evocative of GML, composed of B cells with some infiltrating T cells (mainly CD4⁺) associated with LELs.^{6,8} *H. heilmannii* induced the development of GML to various degrees (25% to 66% of infected animals) and at a later time after infection (18 to 24 months PI) in BALB/c,^{8,10} C57BL6,^{6,12} or Mongolian gerbils.¹¹ For *H. pylori*, the *Helicobacter* species mostly found in human GML, the situation is more complex. C57BL/6 mice were first described as a model of high-grade GML,¹³ whereas BALB/c mice were described as a model of low-grade GML.^{8,14,15} Only a small percentage (<20%) of infected BALB/c mice developed lymphomas 24 months PI, rendering this model difficult to use. More recently, BALB/c mice that underwent thymectomy at day 3 after birth have been described as an alternative model with a much earlier onset of gastric lymphomagenesis.¹⁶ BALB/c mice infected with *H. pylori* strains that underwent thymectomy at day 3 after birth developed histologic characteristics of GML at 12 months PI in 60% of cases, whereas noninfected mice and infected nonthymectomized mice did not.¹⁶ Neonatal thymectomy seems to be an interesting model to study the pathophysiology of GML but is very difficult to implement in practice.

A previous study implicated APRIL (a proliferation-inducing ligand) in the induction and maintenance of B-cell immunity and in the survival and proliferation of B cells *in vivo*.¹⁷ APRIL promotes antigen presentation to B cells via increasing expression of co-stimulatory molecules, such as CD40.¹⁸ In Hodgkin lymphoma, APRIL activates the NF- κ B pathway and promotes the expression of anti-apoptotic proteins (BCL-2, BCL-xL, and MYC) via transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA) receptors, resulting in increased cell survival and proliferation.¹⁹ The implication of APRIL was also found in chronic lymphocytic leukemia, follicular, and Burkitt lymphomas.^{20–23} Recently, Munari et al²⁴ reported that high levels of APRIL were produced by tumor-infiltrating macrophages in a case of *H. pylori*-dependent GML and that macrophages produced APRIL after direct stimulation with both *H. pylori* and *H. pylori*-specific T cells. The number of APRIL-producing macrophages was reduced after lymphoma regression induced by *H. pylori* eradication. APRIL is a tumor necrosis factor superfamily member known to be important for B-cell development, maturation, and survival. Interestingly, several members of the tumor necrosis factor superfamily, such as APRIL, B-cell-activating factor of the tumor necrosis factor family, or TACI, a receptor for APRIL, were found to be overexpressed in *H. pylori*-infected mice that underwent thymectomy at day 3 after birth at the GML stage.²⁵

To study the role of APRIL during *Helicobacter* infection we used previously described APRIL transgenic (Tg) mice.²⁶ APRIL is a T-cell secreted factor, and therefore effects are systemic in this mouse strain. We hypothesized that infection with *Helicobacter* (*H. felis* versus *H. pylori*) in

mice ectopically expressing APRIL would override the natural brake observed in wild-type (WT) animals and therefore allow lymphoid infiltration. The results presented in this study indicate that APRIL Tg mice, infected by *Helicobacter* species, could serve as a novel animal model of gastric lymphomagenesis.

Materials and Methods

Mice

Helicobacter-free C57BL6 mice were obtained from Charles River Laboratories (L'Arbresle, France). APRIL Tg C57BL6 mice were provided by Dr. Michael Hahne (Université de Montpellier, Institut de Génétique Moléculaire de Montpellier, UMR5535).²⁶ All experiments were performed in specific pathogen-free animal facilities at the University of Bordeaux. The animal experiments were performed according to European Union recommendations (European Directive 2010/63/EU) on animal experimentation. The project was evaluated by a local ethical committee of the University of Bordeaux and conforms to the French Ministry of Agriculture Guidelines on Animal Care and the French Committee of Genetic Engineering, with respect to the principle of the 3Rs (replacement, reduction and refinement; approval number 50120143-A). Only female neonates were used for experiments.

Helicobacter Species Strains and Infection Experiments

H. pylori strain B47, a *H. pylori* *cag* pathogenicity island-negative human GML-associated strain, was used for its ability to colonize mouse stomachs^{27,28} and to induce GML-lesions as previously described.¹⁶ *H. felis* CS1 (ATCC 49179; ATCC, Manassas, VA) was used for its capacity to induce severe inflammation and GML lesions in mouse models.

H. pylori and *H. felis* strains were cultured under microaerobic conditions (N₂, 85%; O₂, 5%; CO₂, 10%) at 37°C, as previously described.²⁹

For infection inocula, *H. pylori* and *H. felis* strains were grown on selective agar plates and transferred to *Brucella* broth medium. Six-week-old mice were fasted to facilitate bacterial colonization and then force-fed for 3 consecutive days with a dose of 10⁸ *H. pylori* or 10⁸ *H. felis* per mouse, as previously described (20 mice for each group).³⁰

At the end of the experiment, 18 months PI, after macroscopic observation, half of the stomach of every mouse was fixed in formaldehyde, a small part was frozen at -80°C, and the remainder was used for flow cytometry analysis.

Flow Cytometry Analysis of Stomach Samples

Stomachs were cut into small fragments that were then subjected to two 20-minute incubation cycles with gentle

shaking at 37°C in 10 mL of Hanks' balanced salt solution ($\text{Ca}^{2+}\text{Mg}^{2+}$ free) (Gibco, Life Technologies, Saint Aubin, France), 1 mmol/L HEPES (Gibco, Life Technologies), 1 mmol/L dithioerythritol (Sigma-Aldrich, Saint-Quentin Fallavier, France), and 10% fetal bovine serum (Sigma-Aldrich). Debris was removed by passage through a 70- μm Falcon Cell Strainer (BD Biosciences, San Jose, CA). After filtration, the cells were centrifuged at $1870 \times g$ for 5 minutes at 4°C, the supernatant was discarded, and the cells were washed twice in 10 mL of RPMI 1640 medium (GlutaMAX; Gibco, Life Technologies) and 8% fetal bovine serum before staining. All cell suspensions were incubated with DAPI to assess cell viability (Invitrogen, Cergy Pontoise, France) in a volume of 10 mL of phosphate-buffered saline, 0.5% bovine serum albumin (Gibco, Life Technologies), and 2 mmol/L EDTA (Sigma-Aldrich) for 15 minutes at 4°C. All labeled antibodies were purchased from PharMingen/BD Biosciences. T lymphocytes were characterized using CD45 antigen-presenting cell (clone 30-F11), CD3e fluorescein isothiocyanate (clone 145-2C11), CD8a phycoerythrin cyanine 7 (clone 53-6.7), and CD4 antigen-presenting cell (clone RM4-5). B lymphocytes were characterized using CD21/CD35 antigen-presenting cell (clone 7G6), CD5 fluorescein isothiocyanate (clone 53-7.3), IgM phycoerythrin cyanine 7 (clone R6-60.2), and B220 phycoerythrin (clone RA3-6B2). Cells were washed once and analyzed on a LSRFortessa flow cytometer (BD Biosciences) using FACS Diva software version 8.0.1 (BD Biosciences).

Histologic and Immunohistochemistry Experiments

Tg and WT mice were euthanized at 18 months PI. Half of the stomach was fixed in formaldehyde. Sections (3- μm thick) from paraffin-embedded tissues were processed for hematoxylin and eosin (H&E) staining. H&E-stained sections were coded and examined blindly by a pathologist (P.D.) for the presence of lymphoid infiltrates and LELs. These features were graded on a 0- to 3-point scale, according to the following criteria⁸ for lymphocytic infiltration: 0, no change; 1, single or few small aggregates of lymphocytes; 2, multiple multifocal large lymphoid aggregates or follicles; and 3, extensive multifocal lymphocytic infiltration often extending through the depth of mucosa, resulting in distortion of the epithelial surface, as previously described.¹⁶ Inflammation, hyperplasia, oxyntic gland atrophy, mucinous, and pseudo-intestinal metaplasia were scored using a scale of 1 to 4, as previously described.³⁰

For immunohistochemistry (IHC), sections (3- μm thick) from paraffin-embedded tissue were rehydrated, washed, and heated to 100°C for 30 minutes and then brought to room temperature for 30 minutes for antigen retrieval using a buffer (pH 6 for CD45R and Ki-67 staining; pH 9 for CD3e and E-cadherin staining). Endogenous peroxidases were inhibited with 3% hydrogen peroxide (Sigma-Aldrich) in water for 5 minutes, and blocking with 2.5% normal goat

(for CD45R and Ki-67) or horse (for CD3e and E-cadherin) serum (ImmPRESS Reagent kit peroxidase; Vector Laboratories, Burlingame, CA) was performed for 30 minutes. B lymphocytes were identified with the anti-CD45R rat monoclonal antibody (1:100, clone RA3-6B2; Santa Cruz Biotechnology, Santa Cruz, CA), and T cells with a polyclonal goat anti-CD3e antibody (1:1000, clone M-20; Tebu-bio, Le Perray-en-Yvelines, France). A gastric cell-cell adhesion protein, E-cadherin, was detected by a polyclonal mouse antibody anti-E-cadherin (1:200, clone 36/E-cadherin; BD Biosciences). Rat anti-murine Ki-67 antibody (1:150, 16A8 clone; BioLegend, San Diego, CA) was used for detection of proliferating cells. After 1-hour incubation at room temperature (30 minutes for Ki-67), primary antibodies were detected using ImmPRESS reagent peroxidase (ImmPRESS Reagent kit peroxidase; Vector Laboratories) and revealed by an additional 10 minutes of incubation with liquid diaminobenzidine Substrate-Chromogen System (Dako, Copenhagen, Denmark) at room temperature. All slides were counterstained with hematoxylin, dehydrated, and mounted with Eukitt mounting medium (Labonord; VWR International, Fontenay-sous-Bois, France).

Slides were scanned using a digital slide scanner (Panoramic SCAN; 3DHISTECH Ltd., Budapest, Hungary) equipped with a Zeiss objective (Plan-Apochromat 40; numerical aperture, 0.95; Carl Zeiss Microscopy GmbH, Jena, Germany) and a high-resolution color camera (VCC-FC60FR19CL, 4MP; CIS Corporation, Tokyo, Japan) available at the Experimental Histopathology Platform, US 005 UMS 3427-TBM CORE. The images were read using the Panoramic Viewer software version 1.15.4 (3DHISTECH Ltd.). A semiquantitative evaluation of the percentage of proliferating cells was performed with Mercator (Explora Nova, La Rochelle, France) software version 7.12.7 as previously described.³¹

DNA Extraction and PCR Amplification of the Immunoglobulin Heavy Chain

DNA extraction and immunoglobulin heavy chain amplification were performed for all APRIL Tg mice euthanized at 18 months PI. Genomic DNA was extracted with MagNA Pure 96 (Roche Diagnostics, Basel, Switzerland) from the murine stomach specimen stored at -80°C.

To detect immunoglobulin D-JH rearrangement, PCR was performed in a reaction volume of 25 μL containing 5 μL of DNA extract (5 ng/ μL), with a mixture of 1 $\times$ PCR buffer (GoTaq; Promega, Charbonnières, France), 1 $\mu\text{mol/L}$ of each primer [DSF, 5'-AGGGATCCTTGTGAAGG-GATCTACTACTGTG-3', JH4, 5'-AAAGACCTGCA-GAGGCCATTCTTACC-3' (Eurofins, Nantes, France), described by Kawamoto et al³²], 125 $\mu\text{mol/L}$ of dNTPs (Promega), and 0.625 U of Go Taq G2 Hot Start polymerase (Promega). DNAs extracted from clonal B lymphomas from myc/CDK4R24C and myc/P53 mice were used as a positive control (a kind gift of Dr. Yves Denizot, University of

Limoges, Limoges, France).³³ PCRs were started with a 95°C DNA denaturation step for 2 minutes, followed by 40 cycles comprising three steps: a denaturation at 94°C for 30 seconds, a primer hybridization at 58°C for 30 seconds, and an elongation at 72°C for 1.5 minutes. Amplified DNA products were loaded onto a 1% agarose gel containing Midori Green Advance DNA Stain (Nippon Genetics, Düren, Germany) and were also analyzed by Agilent D5000 ScreenTape System (Agilent Technologies, Santa-Clara, CA) with a 2200 TapeStation instrument (Agilent Technologies) using TapeStation Controller Software version A.02.01 (Agilent Technologies).

A polyclonal profile was defined by the presence of several rearrangements on the CDR3 region of the *IgH* gene and represented by amplicons of different sizes of comparable intensity. A monoclonal profile was defined by 1 single PCR amplicon with a fluorescence intensity three times higher than the background peak and an oligoclonal profile by the reduction of the number of PCR amplicons.

Quantitative PCR to Determine the Bacterial Load in Gastric Biopsies

A quantitative PCR using Fluorescence Resonance Energy Transfer technology targeting DNA coding for *H. pylori* 23S rRNA, previously developed in the laboratory,³⁴ was performed. Primers described by Oleastro et al³⁴ were used to amplify the 23S rRNA gene, and primers described by Laur et al³¹ were used to amplify the *GAPDH* gene. Each target was tested in duplicate on all samples. A standard curve was prepared using serial dilutions of a DNA extract from a colony-forming unit per milliliter calibrated bacterial suspension of the *H. pylori* SS1 strain. The LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), compatible with the LightCycler 480 thermocycler (Roche Diagnostics), was used according to the manufacturer's instructions and as previously described.¹⁶

A quantitative PCR using Fluorescence Resonance Energy Transfer technology targeting DNA coding for *H. felis* *flaA* was developed. Primers described by Burkitt et al³⁵ were used to amplify the *flaA* gene (60 bp). Each target was tested in duplicate on all samples. A standard curve was prepared using serial dilutions of a DNA extract from a colony-forming unit per milliliter calibrated bacterial suspension of the *H. felis* CS1 strain. The SYBR Premix Ex Taq Mix (Tli RNaseH Plus) (Takara, Saint-Germain-en-Laye, France) compatible with the PCR thermocycler CFX96 (Bio-Rad) available at the TBMCORE real-time PCR platform (University of Bordeaux) was used according to the manufacturer's instructions. The PCR started with a 95°C DNA denaturation step for 3 minutes, followed by 40 cycles comprising 2 steps: a 95°C denaturation step for 5 seconds and a 60°C primers' hybridization step for 30 seconds. After each cycle, fluorescence was measured to quantify newly synthesized DNA. At the end of the procedure, a melting curve was generated by a slow elevation

in the temperature from 65°C to 95°C and the continuous measurement of fluorescence. The generation of this melting curve permitted the verification of one specific peak at the expected melting temperature for each product, which revealed the PCR specificity. The final results were expressed as a ratio of bacteria/murine cells.

DNA extracted from the m-ICcl2 murine epithelial cell line available in the laboratory was used to express results as a bacteria/murine cells ratio. The method's detection limit was approximately 0.001 bacteria/murine cells for *H. pylori* and 0.002 bacteria/murine cells for *H. felis*.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA). The Mann-Whitney test was used as nonparametric test to compare the distributions of two unmatched groups. Differences were considered significant when $P < 0.05$.

Results

Macroscopic Appearance of Mouse Stomachs

The external appearance of the whole stomachs of noninfected and infected WT mice did not differ 18 months PI (Figure 1, A–C). The thickness of the stomach was, however, greater in infected APRIL Tg mice, particularly in those infected by *H. felis*, compared with noninfected APRIL Tg mice (Figure 1, A and C). All 10 *H. felis*–infected APRIL Tg mice and nine of the 14 *H. pylori*–infected APRIL Tg mice (64.3%) had a thicker and more rigid stomach. The gastric mucosa of noninfected mice (WT and APRIL Tg) had less visible changes [6 of 13 *H. felis*–infected APRIL Tg mice (46.2%) and 2 of 16 *H. pylori*–infected APRIL Tg mice (12.5%)]. However, some infected APRIL Tg mice presented small (in *H. pylori*–infected APRIL Tg mice) to numerous (in *H. felis*–infected APRIL Tg mice) round nodules in the corpus (Figure 1, B and C). These nodular aspects were compatible with the infiltration of the gastric mucosa by lymphoid follicles as described by others.⁹

Flow Cytometry Characterization of Leukocyte Infiltration Located in the Gastric Mucosa of WT and APRIL Tg Mice 18 Months PI

To provide quantitative information on the relative frequency of leukocyte subsets and to investigate surface markers that cannot be studied by IHC because of the lack of commercialized and/or validated antibodies for paraffin-embedded biopsy specimens from mice, flow cytometric analysis was systematically performed on noninfected and infected WT and APRIL Tg mice stomachs 18 months PI. Noninfected WT and APRIL Tg mice did not develop leukocyte infiltration ($0.7\% \pm 0.3\%$ and $0.6\% \pm 0.4\%$,

Figure 1 Macroscopic appearance of APRIL transgenic (Tg) mice and characterization of leukocyte infiltration in the gastric mucosa of wild-type (WT) and APRIL Tg mice 18 months after infection (PI) determined by flow cytometry. Macroscopic findings in the mice stomachs 18 months PI (external appearance and gastric mucosa aspect). **A:** Noninfected (NI) APRIL Tg mice that did not have abnormalities such as NI WT mice. **B:** *Helicobacter pylori* (Hp)—infected APRIL Tg mice. **C:** *Helicobacter felis* (Hf)—infected APRIL Tg mice. Several small round protrusive lesions form in the fundic area (arrows) of infected mice, especially in mice infected by *H. felis*. Characterization of the leukocyte infiltration. **D:** Viable leukocytes (CD45⁺/DAPI⁺) in the gastric mucosa of the NI ($n = 5$ in each group), *H. pylori* ($n = 15$ and $n = 13$ in the WT and Tg groups, respectively), and *H. felis* ($n = 12$ and $n = 10$ in the WT and Tg groups, respectively) WT and APRIL Tg mice were evaluated as percentages of the total number of cells that were analyzed by the cytometer. **E:** Percentage of infiltrating T lymphocytes (CD3⁺) and B lymphocytes (B220⁺ cells) in the *H. pylori*— ($n = 9$ and $n = 13$ in the WT and Tg groups, respectively) and *H. felis*— ($n = 12$ and $n = 10$ in the WT and Tg groups, respectively) infected WT and APRIL Tg mice among CD45⁺ cells. **F:** Percentages of infiltrating helper T lymphocytes (CD4⁺) and T cytotoxic lymphocytes (CD8⁺) in the *H. pylori*— ($n = 4$ and $n = 13$ in the WT and Tg groups, respectively) and *H. felis*— ($n = 12$ and $n = 10$ in the WT and Tg groups, respectively) infected WT and APRIL Tg mice among CD3⁺ cells. Data were obtained with a LSRFortessa flow cytometer using FACS Diva software. Graphic representations are box plots, with the box representing 50% of values around the median and the whiskers representing the minimum and maximum of all the data. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; [†] $P < 0.05$, ^{††} $P < 0.01$ between infected mice and respective NI controls. Scale bars = 1 cm (A–C). B Ly, B lymphocytes; T Ly, T lymphocytes.

respectively) (Figure 1D). Infected WT ($2.5\% \pm 1.4\%$ for *H. pylori*—infected mice and $8.8\% \pm 6.3\%$ for *H. felis*—infected mice) and APRIL Tg mice ($19.1\% \pm 13.0\%$ for *H. pylori*—infected mice and $28.6\% \pm 5.8\%$ for *H. felis*—infected mice) developed significant leukocyte infiltration compared with their respective noninfected group. This infiltration was significantly higher in *H. felis*—infected mice compared with *H. pylori*—infected mice for WT and APRIL Tg mice (Figure 1D).

For *H. pylori*— and *H. felis*—infected WT mice, most lymphocytes were T cells (CD3⁺) ($71.5\% \pm 9.8\%$ and $68.7\% \pm 16.8$, respectively), unlike APRIL Tg mice for which a predominant B infiltration (B220⁺) was observed ($64.6\% \pm 19.9\%$ for *H. pylori*—infected mice and $78.7\% \pm 4.8$ for *H. felis*—infected mice) (Figure 1E). Percentage of B and T lymphocytes did not differ significantly between *H. pylori*— and *H. felis*—infected mice in each group. In infected APRIL Tg mice, B lymphocytes were mostly CD5⁺, CD21⁺, and IgM⁺, a typical phenotype of marginal zone B cells ($69.8\% \pm 16.0\%$ for *H. pylori*—infected mice and $71.0\% \pm 3.6$ for *H. felis*—infected mice). In *H. pylori*—infected WT and APRIL Tg mice, CD4⁺ T cells predominated over CD8⁺ T cells (CD4/CD8 ratio:

2.2 ± 1.2 and 2.0 ± 0.8 , respectively). Surprisingly, *H. felis*—infected WT mice had a clear CD8⁺ T-cell infiltration (CD4/CD8 ratio: 0.6 ± 0.2) in opposition to their APRIL Tg-*H. felis*—infected mice counterparts for which a significant switch toward CD4⁺ T cells was found (CD4/CD8 ratio: 2.8 ± 1.3) (Figure 1F).

In conclusion, APRIL promoted B-cell infiltration in *H. pylori*— and *H. felis*—infected mice and the recruitment of helper T lymphocytes (CD4⁺) in *H. felis*—infected mice.

Histologic and Inflammatory Aspects during *H. pylori* and *H. felis* Infection in WT and APRIL Tg C57BL6 Mice 18 Months PI

Noninfected WT and APRIL Tg mice did not develop signs of gastric inflammation or any other pathological indications 18 months PI (Figure 2, Supplemental Figures S1, and S2, A and B). At 18 months PI, a moderate leukocyte infiltration and a few scattered small lymphoid infiltrates were observed in stomachs of *H. pylori*— and *H. felis*—infected WT mice. No significant epithelial lesions and LELs were observed in this group. These histologic aspects were compatible with a gastritis stage. Leukocyte infiltration present at the base of

Figure 2 Scores of inflammation and lymphoid infiltration in noninfected (NI) and infected wild-type (WT) and APRIL transgenic (Tg) mice 18 months post infection. **A:** For inflammation, scores range from 0 to 4. **B:** For the lymphoid infiltration, scores range from 0 to 3 (*Materials and Methods*). Data are expressed as medians $\pm$ SD for each group. $n = 9$ NI WT and APRIL Tg mice; $n = 11$ *Helicobacter pylori* (Hp)–infected WT mice; $n = 14$ *Helicobacter pylori*–infected APRIL Tg mice; $n = 8$ *Helicobacter felis* (Hf)–infected WT mice; $n = 10$ *Helicobacter felis*–infected APRIL Tg mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ between infected mice and respective NI controls.

the mucosa was composed of polymorphonuclear cells with some B and T lymphocytes. T lymphocytes were observed within the gastric mucosa, alongside glands (*Supplemental Figures S2, C and D, and S3*), an aspect compatible with a lymphocytic gastritis.

At 18 months PI, *H. pylori*– and *H. felis*–infected APRIL Tg mice had more inflammation and lymphoid infiltrates than their respective infected WT mice counterparts (*Figure 3*). *H. felis*–infected APRIL Tg mice had more inflammation and lymphoid infiltrates than *H. pylori*–infected APRIL Tg mice. Extensive multifocal lymphocytic infiltrations deforming the gastric mucosa were observed in *H. pylori*– and *H. felis*–infected mice (*Figure 3*). They were composed of mostly B lymphocytes

surrounded or including areas with T cells (*Figure 4, A and B*). The presence of LELs was investigated by using H&E and E-cadherin IHC staining (*Figure 4, A and B*). Overall, LELs were found in 5 of 12 *H. pylori*–infected APRIL Tg mice (41.7%) and 7 of 10 *H. felis*–infected APRIL Tg mice (70%), whereas no LELs were detected in WT mice.

Semiquantification of the Ki-67 IHC staining in *Helicobacter*–infected WT and APRIL Tg mice revealed that the percentage of proliferating cells among the infiltrates was significantly higher in infected APRIL mice ($11.8\% \pm 11.7\%$ for *H. pylori* and $7.6\% \pm 2.6\%$ for *H. felis*) compared with infected WT mice (2.2 ± 1.6 and 1.0 ± 0.7 , respectively) (three mice in each group, two to nine areas with lymphoid infiltrates were considered for each mouse) (*Figure 4C*). The percentage of proliferating cells among the infiltrates did not differ significantly between *H. pylori*– and *H. felis*–infected APRIL Tg mice.

Observation of epithelial lesions indicated that *H. felis* induced significant oxyntic gland atrophy associated with hyperplasia, mucinous metaplasia, pseudo-intestinal metaplasia, and dysplasia in both the WT and APRIL Tg mice (*Supplemental Figure S1*), a set of alterations compatible with established gastritis and the preneoplastic stage. *H. pylori*–infected WT mice had no significant epithelial disturbances; however, there was a clear effect of the APRIL Tg background on oxyntic gland atrophy associated with hyperplasia, mucinous metaplasia, pseudo-intestinal metaplasia, and dysplasia scores, although less spectacular than for the *H. felis*–infected APRIL Tg mice (*Supplemental Figure S1*).

In conclusion, we confirmed by histologic and IHC experiments that *Helicobacter* infection promoted B-cell proliferation and lymphoid infiltrate formation in APRIL Tg mice only. *H. felis* appeared more aggressive on the gastric mucosa because we found these lymphoid infiltrates were often associated with preneoplastic lesions. The presence of LELs in most of the infected Tg mice supports the hypothesis that APRIL may promote B-lymphoma development.

Evaluation of B-Lymphocyte Clonality

Amplification of the CDR3 region of the *IgH* gene was used to study the level of clonality of infiltrating B cells in the stomachs of all APRIL Tg mice at 18 months PI (*Table 1*). No amplification or polyclonal profile was observed in noninfected APRIL Tg mice ($n = 9$). A polyclonal profile was found in 14 of the 14 *H. pylori*–infected APRIL Tg mice (100%) and 7 of the 10 *H. felis*–infected APRIL Tg mice (70%). One of the 10 *H. felis*–infected APRIL Tg mice (10%) had an oligoclonal profile, and 2 of the 10 (20%) had a monoclonal profile with a single band observed. LELs were present in the three mice with monoclonal or oligoclonal profiles. Thus, *H. felis* appeared to induce GML-like lesions in APRIL Tg mice more efficiently compared with *H. pylori*.

Figure 3 Histologic and inflammatory aspects during *Helicobacter pylori* and *Helicobacter felis* infection in APRIL transgenic (Tg) C57BL6 mice 18 months post infection. Sections (3- μ m thick) from paraffin-embedded tissues were processed for hematoxylin and eosin staining. The large curvature of the stomach (fundus and corpus) is presented on these images. **A:** Representative histopathologic features of *H. pylori*-infected APRIL Tg mice. Lymphoid infiltrates are observed. **B:** Representative histopathologic features of *H. felis*-infected APRIL Tg mice. Extensive multifocal lymphocytic infiltrations are observed. Scale bars: 1000 μ m (main images); 200 μ m (higher magnification).

Estimation of the Bacterial Load in Gastric Biopsy Specimens from Infected Mice

The comparison of bacteria to cell ratios between *H. pylori*- and *H. felis*-infected WT and APRIL Tg mice is shown in Figure 5. WT and APRIL Tg mice were infected at 6 weeks of age with the same bacterial inoculum of *H. pylori* or *H. felis* per mouse for 3 consecutive days. At 18 months PI, bacteria/epithelial cell ratios were >100 times lower in *H. pylori*-infected WT mice (with 11 of the 16 mice being negative) than in APRIL Tg mice [mean bacteria/cell ratio = 0.08635 ($n = 14$, with *H. pylori* detected in all)] ($P < 0.001$) (Figure 5A).

To verify these results, DNA was extracted from five gastric biopsy specimens from infected mice with both *Helicobacter* species euthanized at 12 months PI to check the kinetics of the disease in this new animal model. Again bacteria/epithelial cell ratios were 10 times lower in *H. pylori*-infected WT mice (with two of the five mice being negative) than in APRIL Tg mice (mean bacteria/cell ratio = 0.00258, with *H. pylori* detected in four of the five mice) ($P < 0.01$) (Figure 5A). *H. pylori* colonization was also higher at 18 months PI versus 12 months PI in APRIL Tg mice ($P < 0.01$).

This phenomenon was also observed in *H. felis*-infected mice (Figure 5B): at 12 months PI, bacteria/epithelial cell ratios were 100 times lower in infected WT mice (with four of the five mice being negative) than in APRIL Tg mice (mean bacteria/cell ratio = 0.13644, with *H. felis* detected in four of the five mice) ($P < 0.01$). At 18 months PI, bacteria/cell ratios were 1000 times lower in infected WT mice (with seven of the 13 mice being negative) than in APRIL Tg mice [mean bacteria/cell ratio = 0.40870, with *H. felis* detected in all mice ($n = 10$)] ($P < 0.001$).

In conclusion, for most of the WT-infected animals the bacterial inoculum was eliminated or under the detection limit. Altogether, these results highlight the influence of the inflammatory response on bacterial persistence in *Helicobacter*-infected APRIL Tg mice.

Discussion

The association of APRIL with carcinogenesis had been alluded to previously.³⁶ High levels of APRIL were observed in *H. pylori*-dependent GML,²⁴ suggesting a role of APRIL in gastric lymphomagenesis.²⁴ To study the role of APRIL in GML, we infected APRIL Tg C57BL6 mice²⁶ with *H. pylori* or *H. felis*. Typical lesions (namely, massive B lymphoid infiltrates associated with LELs), considered a characteristic of a lymphoma stage, were observed in gastric mucosa of 60% of *H. pylori*- and all *H. felis* infected-APRIL Tg mice 18 months PI, whereas the infected WT C57BL6 mice did not develop such lesions. Flow cytometry results confirm the predominance of B cells in infected APRIL Tg mice compared with infected WT or noninfected mice. A monoclonal population was detected in a few *H. felis*-infected APRIL Tg mice. These results confirm our hypothesis on the importance of the deregulation of APRIL in the development of gastric lymphoid infiltrates induced by *Helicobacter* species.

Our first observation was the resistance of WT C57BL6 mice in developing GML-like lesions even 18 months PI by *H. pylori* or *H. felis*. In contrast, previous studies have described that these mice developed high-grade GML after *H. pylori* infection.^{13,14} Others found that *H. felis* induced GML-like lesions in BALB/c mice at 22 months PI^{6,8} or even gastric adenocarcinoma in C57BL6 mice.³⁷ All these

Figure 4 Analysis by immunohistochemistry of the lymphoid infiltrates found in infected APRIL transgenic (Tg) mice 18 months post infection. **A** and **B**: Consecutive cuts of the same gastric biopsy specimen are shown. **A**: In a *Helicobacter pylori*-infected APRIL Tg mouse, a representative infiltrate composed of B lymphocytes with some infiltrating T cells. The B cells are involved in lymphoepithelial lesions (LELs). A higher magnification of the E-cadherin staining is shown. **B**: In a *Helicobacter felis*-infected APRIL Tg mouse, representative infiltrate composed of B lymphocytes with some infiltrating T cells. This infiltrate extends through the entire mucosa level, resulting in distortion of the epithelial surface. The B cells are involved in LELs. A higher magnification of the E-cadherin staining is shown. LELs are indicated by arrows. **C**: Percentage of proliferating cells evaluated by semi-quantification of the Ki-67 staining in *H. pylori*- and *H. felis*-infected APRIL Tg and WT mice. Data are expressed as medians $\pm$ SD for each group. $n = 3$ for each group, and two to nine areas with lymphoid infiltrates were considered for each mouse. $**P < 0.01$, $***P < 0.001$. Scale bars: 250 μ m (main images); 50 μ m (higher magnification).

findings should be considered with caution because the criteria used to define GML or even gastric adenocarcinoma are sometimes unclear (eg, histologic analyses only were performed, without precise characterization of infiltrating lymphoid cells, in the study by Wang et al¹³). To circumvent these issues, we took care to demonstrate not only large lymphoid infiltrates consisting of B cells but also LELs and immunological surface markers for B cells of marginal zone origin, such as those found in human GML.

The *H. pylori* strain used in our study differs from those used by other groups, namely, *H. pylori* strains 119/95, G50, or SS1,^{13,14} used to infect C57BL6 mice. It is noteworthy that in these studies the appearance of GML-like

lesions was dependent on the strain: only *H. pylori* strains 119p and G50 induced lymphoma-like lesions, whereas *H. pylori* strain SS1 did not.

In this study, we report the first use of mice with C57BL6 genetic background with *H. pylori* strain B47. It was originally isolated from a patient with GML and is known for its ability to efficiently colonize BALB/c mice stomachs and to induce GML-like lesions in neonatal thymectomized mice.¹⁶ The fact that *H. pylori* strain B47 is *cag* pathogenicity island negative suggests that this important virulence marker is not required for MALT pathogenesis, as previously suggested^{16,27,38}; hence, GML appearance could be related to a stimuli by unknown bacterial antigens in a host with an

Table 1 Results of PCR Amplification of the CDR3 Region of the *IgH* Gene for APRIL Transgenic Mice at 18 Months after Infection

Variable	CDR3 <i>IgH</i> PCR profiles			
	No amplification	Polyclonal	Oligoclonal	Monoclonal
Noninfected ($n = 9$)	2	7	0	0
<i>Helicobacter pylori</i> ($n = 14$)	0	14	0	0
<i>Helicobacter felis</i> ($n = 10$)	0	7	1	2
All infected mice ($n = 24$)	0	21	1	2

Figure 5 Quantification of the bacterial load in gastric biopsy specimens from infected mice. Results were obtained by quantitative PCR as described in [Materials and Methods](#). **A:** *Helicobacter pylori*/murine cell ratio in wild-type (WT) and APRIL transgenic (Tg) mice infected by *H. pylori* at 12 months post infection (PI) ($n = 5$) versus 18 months PI ($n = 16$ and $n = 14$, respectively). **B:** *Helicobacter felis*/murine cell ratio in WT and APRIL Tg mice infected by *H. felis* 12 months PI ($n = 5$) versus 18 months PI ($n = 13$ and $n = 10$, respectively). Data are expressed as medians $\pm$ SD for each group. $n = 5$ *H. pylori*-infected WT and APRIL mice at 12 months PI (**A**); $n = 16$ *H. pylori*-infected WT mice at 18 months PI (**A**); $n = 14$ *H. pylori*-infected APRIL mice at 18 months PI (**A**); $n = 5$ *H. felis*-infected WT and APRIL mice at 12 months PI (**B**); $n = 13$ *H. felis*-infected WT mice at 18 months PI (**B**); $n = 10$ *H. felis*-infected APRIL mice at 18 months PI (**B**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

altered inflammatory response, such as that found in APRIL Tg C57BL6 mice.

More inflammation and gastric lesions were observed with *H. felis* compared with *H. pylori* in WT C57BL6 mice.³⁹ Indeed, *H. felis* is described as being able to induce a potent proinflammatory response in mice, greater than that induced by *H. pylori*.⁸ This is, no doubt, reflected in the incidence and severity of GML-like lesions, which varied between *H. pylori*- and *H. felis*-infected APRIL Tg mice.

Indeed, more extensive and severe lesions were found to be induced by *H. felis* compared with *H. pylori*. In particular, *H. felis* induced preneoplastic lesions, such as dysplasia, even in WT-infected animals, whereas *H. pylori* induced lesions in infected APRIL Tg mice only.

Surprisingly, we found that for both *Helicobacter* species, the level of colonization was lower in WT than in APRIL Tg animals. Because both groups of mice were infected the same day and with the same bacterial suspension, we can exclude a problem of oral gavage for the WT group. These results were unprecedented because we found in our previous study³¹ a decrease in the level of colonization with these scores in infected animals that underwent thymectomy at day 3 after birth in line with the conventional wisdom of increased gastritis and decreased colony-forming units. The differences in inflammatory responses could explain these data. Our hypothesis is that the strong Th1 response that is expected in WT C57BL6 infected mice may participate in bacterial clearance or reduction of colonization levels as already shown by others.^{40–42} An inflammatory balance (Th2 or T-regulatory cells) may be present in infected APRIL Tg animals, which allows bacterial colonization. It would be interesting to compare the inflammatory responses induced by both species in WT and Tg animals to understand the differences in inflammatory responses in this new animal model.

The second main point of our study is the confirmation of the importance of APRIL in the development of gastric lymphoid infiltrates and GML-like lesions after *H. pylori* or *H. felis* infection. Flow cytometry and histologic analyses revealed the presence of lymphoid infiltrates with a predominance of B cells with the phenotype expected for marginal zone B-cell lymphoma (CD5⁺, CD21⁺, IgM⁺).⁵ There was an emergence of lymphoid infiltrates associated with LELs, which constitutes a characteristic criterion of GML. Our in-depth histologic analysis also revealed that *H. pylori*- and *H. felis*-infected APRIL Tg mice developed mucosal atrophy, metaplasia, dysplasia, and lesions that are frequently observed at the time of GML diagnosis in humans.^{43,44} These lesions were not present in *H. pylori*-infected WT mice.

Despite oligoclonality not being a prerequisite for the diagnosis of GML,⁴⁵ monoclonal B-cell populations are detected in approximately 80% to 90% of human GML cases.^{46,47} Interestingly, oligoclonality or monoclonality of the lymphoid infiltrates has been observed in only 30% of *H. felis*-infected APRIL Tg mice and in none of the *H. pylori*-infected mice. Reduced clonality could be explained by a lack of sensitivity of the detection method used (DNA extraction of the entire stomachs and not just the lymphoid infiltrates) or a low abundance of the clonal population among the entire population of lymphocyte infiltrates. Tissue collection may also have been performed too early after infection. Nevertheless, the gastric lesions were severe and the lymphoid infiltrates were extensive in APRIL Tg infected mice. Alternatively, a rapid B-cell

proliferation could have been induced by APRIL without clonal selection.

We also identified indicators related to infiltrating T cells that are similar to those described in human GML, especially the predominance of CD4⁺ over CD8⁺ T lymphocytes.^{48,49} The predominance of the former subset is compatible with helper T-cell–dependent B-cell proliferation occurring early in low-grade GML, whereas the low number of CD8⁺ T cells is consistent with the impaired cytotoxicity of tumor-infiltrating T cells described in humans,⁴⁹ both features contributing to lymphoma development. The T-cell response switched between *H. felis*–infected WT and APRIL Tg mice, confirming the importance of CD4⁺ T cells to promote marginal zone B-cell proliferation. The effect of APRIL on T-cell subsets during infection is novel and represents a new direction in this research, requiring further development. Interestingly, these T cells were mainly found among the lymphoid infiltrates in GML-developing mice, whereas they were scattered into the gastric mucosa in infected WT animals. This may be explained by the proinflammatory response that recruits T cells, which in turn helps B cells to proliferate in GML APRIL Tg mice or contributes to epithelial lesions in WT animals.

Another major objective of our study was to compare the ability of *H. pylori* versus *H. felis* to induce GML-like lesions. *H. pylori* and *H. felis* were both able to induce lymphoid infiltrates, with a predominance of B cells having the phenotype expected for marginal zone B-cell lymphoma in APRIL Tg mice, but more LELs were observed after *H. felis* infection compared with *H. pylori* infection. Moreover, monoclonality or oligoclonality of B infiltrates was detected only in *H. felis*–infected APRIL Tg mice. *H. felis* appeared *prima facie* more efficient in induction of GML-like lesions in a shorter time frame.

In conclusion, our model is new and original and confirms the implication of APRIL in GML development. Consequently, it will be easier to use and be more productive than the previous models.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.03.004>.

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