Immunization of Mice with Urease Vaccine Affords Protection against *Helicobacter pylori* Infection in the Absence of Antibodies and Is Mediated by MHC Class II–restricted Responses

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Summary

We examined the roles of cell- and antibody-mediated immunity in urease vaccine–induced protection against *Helicobacter pylori* infection. Normal and knockout mice deficient in major histocompatibility complex (MHC) class I, MHC class II, or B cell responses were mucosally immunized with urease plus *Escherichia coli* heat-labile enterotoxin (LT), or parenterally immunized with urease plus aluminum hydroxide or a glycolipid adjuvant, challenged with *H. pylori* strain X47-2AL, and *H. pylori* organisms and leukocyte infiltration in the gastric mucosa quantified. In an adjuvant/route study in normal mice, there was a direct correlation between the level of protection and the density of T cells recruited to the gastric mucosa. In knockout studies, oral immunization with urease plus LT protected MHC class I knockout mice [β_2-microglobulin (−/−)] but not MHC class II knockout mice [I-A^b (−/−)]. In B cell knockout mice [μMT (−/−)], vaccine-induced protection was equivalent to that observed in immunized wild-type (+/+) mice; no IgA^+ cells were detected in the stomach, but levels of $CD4^+$ cells equivalent to those in the wild-type strain (+/+) were seen. These studies indicate that protection of mice against *H. pylori* infection by immunization with the urease antigen is dependent on MHC class II–restricted, cell-mediated mechanisms, and antibody responses to urease are not required for protection.

Key words: *Helicobacter pylori* • knockout mice • adjuvants • gastric mucosa • T cells

Helicobacter pylori, a gram-negative, spiral bacterium that colonizes the gastric mucosa, is the principal cause of chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma in humans (1, 2). The development of vaccines to prevent *H. pylori*–associated diseases will be facilitated by an understanding of the immune mechanisms responsible for protection. Mucosal immunization with recombinant *H. pylori* urease administered with the mucosal adjuvant *Escherichia coli* heat-labile enterotoxin (LT)[1] protects mice against challenge with *H. pylori*, reducing bacterial density by ~100-fold (3). Efforts to identify specific mechanisms or cells responsible for protection using a number of *Helicobacter* antigens have failed to identify conclusively correlates of immunity (4–9). Immunized animals develop antigen-specific serum IgG and IgA, intestinal and salivary IgA, and, after challenge, a local (gastric) cellular and antibody response (3, 5, 10). Protection is also associated with the presence of $CD4^+$ cells and $CD8^+$ cells in the gastric mucosa (3, 11), and reductions in bacterial load can be achieved in the absence of active immunization by adoptive transfer of T cells from immunized donor mice, suggesting that cell-mediated immune responses play a major role in protection in this species (12).

Other than mucosal immunization with bacterial antigens combined with LT or cholera toxin (3–13), few immunization regimens have been explored as a means to study the mechanisms of protection against *H. pylori*. Subcutaneous immunization with urease plus several different parenteral adjuvants generated high levels of serum IgG and showed various degrees of protection against *Helicobacter fe-*

[1]*Abbreviations used in this paper:* β_2m, β_2-microglobulin; H & E, hematoxylin and eosin; IEL, intraepithelial lymphocytes; IN, intranasal; LT, *Escherichia coli* heat-labile toxin; urease-ACC, urease-specific antibody–containing cells.

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lis or *H. pylori* (5, 14), whereas intranasal (IN) immunization with urease without adjuvant generated moderate levels of serum IgG, salivary IgA, and fecal IgA, but was not protective (15). These findings demonstrated that although appreciable antibody responses can be generated without a mucosal adjuvant, protective immunity mediated via urease immunization can only be achieved in the presence of a mucosal or parenteral adjuvant. The lack of protection in the absence of a suitable mucosal adjuvant suggested that antibody may not be an essential mediator of protection.

Recent developments in gene knockout technology have produced a variety of experimental mouse models to study mechanisms of immunity and their roles in infectious diseases. Mice in which the I-A gene has been disrupted lack MHC class II molecules, are deficient in $CD4^+$ T cells, and have impaired cellular and antibody-mediated immunity (16, 17). Mice in which the β_2-microglobulin (β_2m) molecule is lacking are deficient in MHC class I molecules, fail to differentiate normal numbers of $CD8^+$ T cells, and have deficient CTL responses (18). Antibody-deficient mice have been produced by disruption of the immunoglobulin μ chain gene at the μMT exon (19). In these latter mice, peripheral B cells are absent, and no serum or mucosal antibody responses can be generated (20).

In this investigation, mechanisms of vaccine-induced protection against *H. pylori* were examined using mucosal and parenteral immunization regimens with recombinant urease in both wild-type and gene knockout mice. In wild-type mice, mucosal immunization with urease plus LT yielded higher levels of protection than did parenteral or combination parenteral/mucosal regimens. Protection best correlated with the density of T cells in the gastric mucosa after challenge with *H. pylori*. An essential role for MHC class II–dependent T cell responses in protection was determined using β_2m and I-A^b knockout mice. In B cell knockout mice, protection equivalent to that seen in immunized wild-type mice was demonstrated in the absence of specific antibodies against urease. These results suggest a central role of $CD4^+$ T cell–dependent cell-mediated immunity in urease vaccine–induced protection of mice against *H. pylori* infection.

Materials and Methods

Animals. All procedures were conducted with approval of the OraVax Institutional Animal Care and Use Committee. Specific pathogen–free, 8-wk-old outbred female Swiss-Webster mice, inbred homozygous (−/−) and heterozygous (+/−) I-A^b gene knockout mice, homozygous (−/−) and heterozygous (+/−) β_2m gene knockout mice, and wild-type (+/+) C57BL/6 mice free from *Helicobacter muridarum* were obtained from Taconic Farms, Inc. (Germantown, NY). Specific pathogen–free, 8-wk-old μMT (Igh −/−) gene knockout mice back-crossed to the C57BL/6 background and wild-type (+/+) C57BL/6J mice free from *Helicobacter* spp. were obtained from The Jackson Lab. (Bar Harbor, ME). Experimental groups contained 10–12 mice each.

Urease Expression and Purification. Recombinant urease was used as the model antigen in all studies and was expressed and purified from *E. coli* strain ORV214 as described previously (5). Native *H. pylori* urease was used as the coating antigen in ELISA immunoassays and was purified from *H. pylori* strain ATCC 43504 (American Type Culture Collection, Rockville, MD) as described previously (5).

Adjuvants. LT was obtained from Berna Products Corp. (Coral Gables, FL). Alum was obtained as an aluminum hydroxide gel (Rehydrogel®) from Reheis, Inc. (Berkeley Heights, NJ). The glucosylamide Bay R 1005 [*N*-(2-deoxy-2-l-leucylamino-b-d-glucopyranosyl)-*N*-octadecyldodecanamide acetate] (14, 21) was provided by Bayer AG (Wuppertal, Germany).

Immunization Procedures. Mice were immunized at weekly or biweekly intervals with recombinant urease containing the appropriate dose of adjuvant (Table 1). For oral immunization, 25 μl of vaccine was delivered into the mouth (5). For IN immunization, 10 μl of vaccine was applied onto the external nares of unanesthetized mice (15). For parenteral immunization, 100 μl of solution was injected by the s.c. route along the midline of the lower back. 1 wk after the last immunization, blood samples were taken from the retro-orbital sinus to measure the antiurease systemic immune responses before challenge.

Immunization Regimens. The immunization doses and schedules for each study are outlined in Table 1. To determine the efficacy of different adjuvants and routes of urease vaccine administration, we compared mucosal immunization with parenteral immunization in an outbred mouse model. The roles of MHC-I– and MHC-II–restricted T cell responses in protection were examined in β_2m and I-A^b gene knockout mice, and the role of B cells and antibody in protection was examined in μMT knockout mice. In the adjuvant studies, urease plus LT was delivered by the IN route to give a direct comparison with IN delivery of urease without adjuvant. In the knockout studies, urease plus LT was administered by the more traditional oral route. Both IN and oral immunization with urease plus LT previously have been shown to give comparable levels of protection and recruitment of gastric T cells (3).

To determine the role of urease-specific serum IgG in protection, outbred Swiss-Webster mice were immunized by mucosal and parenteral routes as described in Table 1. 1 wk after immunization, donor mice were bled, the sera pooled, and 2 ml was injected by the i.p. route into seven recipient mice 2 d before *H. pylori* challenge. Blood from recipient mice was sampled the next day to confirm transfer of urease-specific antibodies.

H. pylori Growth Conditions and Challenge. A streptomycin-resistant mutant of *H. pylori* strain X47-2AL was used for all challenge experiments. *H. pylori* strain X47-2AL (ORV2001) was originally isolated from a domestic cat (Dr. J.G. Fox, Massachusetts Institute of Technology, Cambridge, MA) (22), and adapted to Swiss-Webster mice by sequential in vivo passages (3). To prepare the challenge inoculum, bacteria were grown on agar plates for 2 d followed by 1 d in suspension (3). 2 wk after the last immunization, mice were challenged intragastrically via a 20-gauge feeding needle with a 100-μl suspension of *H. pylori* strain X47-2AL containing 10^7 CFU/ml. At ~2 wk after challenge, mice were killed and gastric tissue processed for urease activity, *H. pylori* culture, histology, or immunohistochemistry as described below.

Serum Antibody Responses. Serum samples collected 7 d after the last immunization were evaluated for urease-specific IgG, IgG_1, or IgG_{2a} by indirect ELISA as described previously (3, 15). Urease-specific IgM or IgA was detected by antibody-capture ELISA. Plates were coated with 1 μg/ml goat anti–mouse IgM or IgA (Southern Biotechnology Associates, Birmingham, AL); test sera were added, followed by 2.5 μg/ml purified native *H. pylori*

Table 1. *Vaccine Schedule for Protection Studies*

Group	No. and route of immunizations*	Urease dose	Adjuvant dose
Adjuvant and route study in Swiss-Webster mice‡		*μg*	*μg*
Urease/no adjuvant	14 daily IN	25	—
Alum	3 s.c.	10	200
Bay	3 s.c.	10	400
LT/Alum	1 IN/2 s.c.	25/10	1/200
LT/Bay	1 IN/2 s.c.	25/10	1/400
LT	3 IN	25	1
MHC-class I and class II study in C57BL/6 mice§			
Alum	3 s.c.	10	200
LT	4 weekly oral	25	1
Passive transfer study in Swiss-Webster mice			
Urease/no adjuvant	15 daily IN	25	—
Alum	3 s.c.	10	200
LT	3 IN	25	1
μMT study in C57BL/6J mice‡			
Alum	3 s.c.	10	200
LT	3 oral	25	1

*Immunizations given biweekly unless otherwise noted; controls were untreated.
‡Included 10 mice per group.
§Included 10 (+/+) and (−/−) mice and 5 (+/−) mice per group.

urease, rabbit antiurease, and goat anti–rabbit IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates). ELISA plates were developed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) and absorbance read at OD_{405} with a V_{max} microplate reader (Molecular Devices Corp., Sunnyvale, CA). The concentrations of urease-specific serum IgG_1 and IgG_{2a} were calculated using standard curves generated by titrating pooled high-titer sera (23). The results were expressed as U/ml, and the IgG_1:IgG_{2a} ratio was calculated for each mouse. Absorbance values were converted to specific antibody measurements using standard curves generated with SOFTmax 881 software (Molecular Devices Corp.).

Gastric Tissue Analyses. The stomach was dissected along the lesser curvature and divided into strips for urease activity, *H. pylori* culture, histopathology, or immunohistochemical analyses. For histopathology, a longitudinal segment including the antrum and corpus plus a piece of attached intestine was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm. For immunohistochemistry, gastric segments were mounted on a 3-mm slice of spleen by wrapping the mucosa around the outer capsule of the spleen with the gastric mucosa facing outwards. Each piece of tissue was then mounted in O.C.T. compound (Sakura Finetek USA Inc., Torrance CA), and quick-frozen in Freon 22 cooled to its freezing point by liquid nitrogen. The spleen served as a positive control for each lymphocyte subset tested. In the knockout studies, a 5-cm-long segment of the intestine was frozen in O.C.T. by coiling the intestine around itself to form a 1–1.5-cm disk.

Urease Activity. The presence of *H. pylori* in gastric tissue was assessed by urease activity measured spectrophotometrically after 4 h of incubation using a colorimetric urease test, as described previously (3, 15).

Quantitative Culture of H. pylori from Gastric Tissue. An antral strip was placed in 1 ml Brucella broth and homogenized until the tissue was completely disrupted. 10-fold serial dilutions were then prepared and plated onto *Helicobacter*-selective agar, as described above. Bacterial counts were determined after 5 d of growth in a 7% CO_2 incubator at 37°C.

Histopathology. Histological sections from adjuvant studies and MHC-II and MHC-I knockout studies were evaluated by a pathologist blinded to the sample code (randomized histological sections of gastric mucosa) as described previously (11). *H. pylori* organisms in gastric sections were made visible with a modified Steiner silver stain (Sigma Chemical Co.). The density of *H. pylori* per longitudinal section from the intestine through the entire antrum and corpus was scored as follows: 0, no bacteria; 1, 1–5 foci containing one or more bacteria; 2, 5–10 foci; 3, 10–20 foci; and 4, >20 foci.

Gastritis and epithelial alterations were evaluated on hematoxylin and eosin (H & E) stained sections as described previously (11). For gastritis, sections were scored from zero to four based upon the intensity of the infiltration of lymphocytes, plasma cells, and neutrophils. The degree of epithelial change was scored similarly based on the loss of parietal cells, hyperplasia of the surface epithelium, and the presence of microabscesses or cystic glands.

Immunohistochemistry of Gastric Tissue. Cryosections were blocked with avidin–biotin-blocking reagents (Vector Laboratories Inc., Burlingame, CA), and endogenous peroxidase activity blocked with 0.03% H_2O_2 and 0.01% sodium azide in PBS. The sections were incubated with rat mAbs against mouse CD4 (clone RM4-

5), CD8α (clone 53-6.7), CD103 (α^E-integrin) (clone M290), β_7-integrin (clone M293), CD45R (B220) (clone RA3 6B2), IgA (clone R5-140), IgM (clone R6-60.2), CD11b (Mac-1) (clone M1/70), or Ly-6G (clone RB6-8C5) (reactive with neutrophils) (PharMingen, San Diego, CA). Sections were then incubated with biotinylated rabbit anti–rat IgG (Vector Laboratories Inc.), followed by horseradish peroxidase conjugated to an avidin–biotin complex (ABC-*Elite* Kit; Vector Laboratories Inc.), diaminobenzidine, and methyl green. Control sections were incubated without primary mAb. Urease-specific antibody–containing cells (urease-ACC) were detected by an avidin–biotin glucose oxidase procedure, as described previously (3). The number of positive cells was counted in 0.5-mm segment lengths of gastric mucosa and expressed per mm^2 field as described previously (11).

Flow Cytometric Analyses of Lymphocyte Populations. Peripheral blood, spleen, and intestinal intraepithelial lymphocytes (IEL) were examined for depletion of T cells in knockout mice using rat mAb against mouse CD4 (clone RM4-5), CD8α (clone 53-6.7), CD103 (α^E-integrin) (clone M290), CD90 (Thy-1.2) (clone 30-H12), CD45 (clone 30-F11), CD45R (B220) (clone RA3 6B2), IgM (clone R6-60.2), I-A^b ($A_\beta{}^b$) (clone 25-9-17), or H-$2D^b$ (clone KH95) (PharMingen) as described previously (11, 24). The stained lymphocytes were analyzed (10,000 cells) using an Epics XL (Coulter Corp., Miami, FL).

Statistics. Statistical analyses were performed with either JMP™ or Graphpad Prism™ software using Wilcoxon/Kruskal-Wallis (rank sums) test for continuous or ordinal variables, or linear regression.

Results

Role of Adjuvant and Route of Immunization in Protection of Outbred Mice

Protection against H. pylori Infection. Levels of protection were determined by urease activity or quantitative culture of gastric tissue in a study using five different adjuvant regimens administered with urease (plus controls) to determine whether correlates of protection could be identified (Fig. 1). Unimmunized mice and mice receiving daily IN urease immunization without adjuvant were highly infected with geometric mean CFUs of $\sim 5.0 \times 10^4$–1.5×10^5 per gastric segment at 14 d after challenge. The greatest level of protection was observed in mice given urease plus LT by the IN route; the geometric mean *H. pylori* density (3.6×10^2) was 100–1,000-fold lower than in unimmunized mice or mice receiving the same regimen of urease without adjuvant ($P < 0.01$ versus controls; Wilcoxon/Kruskal-Wallis rank sums test). Parenteral administration of the same dose and regimen of urease plus either alum or Bay adjuvants resulted in partial protection characterized by greatly reduced gastric urease activity ($P < 0.01$) but <10-fold decrease in *H. pylori* density by culture (significance achieved versus IN per day controls, $P < 0.02$; but not versus unimmunized controls). A combined regimen of mucosal priming with urease plus LT followed by parenteral boosting with urease plus alum or Bay gave intermediate levels of protection between urease plus LT and parenteral-only regimens (10–100-fold decrease in *H. pylori* versus controls, $P < 0.01$).

Effects on Gastric Mucosa. Histopathologic evaluations of gastritis and epithelial change were performed on H & E sections of gastric mucosa from mice that received urease plus LT, urease plus alum, or no immunization, and were then challenged with *H. pylori*. As reported elsewhere (14), immunization and protection was associated with increased recruitment of immune cells to the gastric mucosa in comparison with controls (each group, $P < 0.01$). The increased cellular infiltrate was accompanied by only a slight increase in epithelial change in either the antrum or corpus (mean epithelial score in the corpus increased from 0 in controls to 1 in urease plus LT immunized mice, $P < 0.01$).

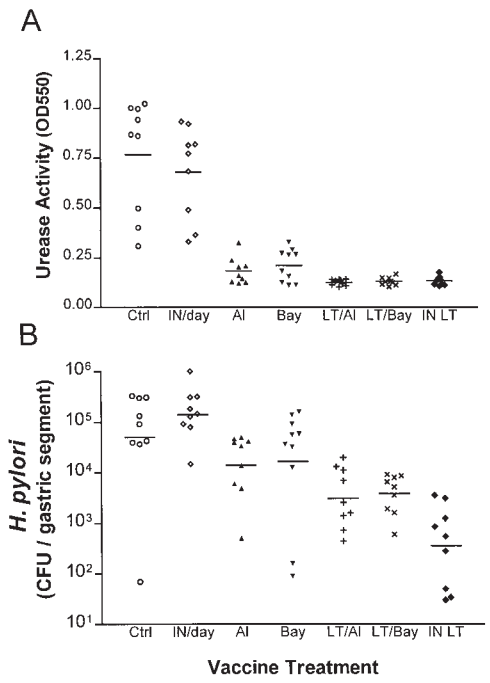

Figure 1. Protection against *H. pylori* infection in Swiss-Webster mice immunized with recombinant urease using nonprotective, partially protective, and highly protective adjuvant and route combinations. (*A*) Gastric urease activity. (*B*) *H. pylori* CFUs from gastric mucosa. All groups were challenged with *H. pylori*. (*Ctrl*) Control mice were untreated. (*IN/day*) IN urease daily over 14 d without adjuvant. (*Al*) Three biweekly s.c. immunizations with urease plus alum. (*Bay*) Three biweekly s.c. immunizations with urease plus Bay R 1005. (*LT/Al*) One IN immunization with urease plus LT followed by two s.c. immunizations with urease plus alum. (*LT/Bay*) One IN immunization with urease plus LT followed by two s.c. immunizations with urease plus Bay. (*IN LT*) Three biweekly IN immunizations with urease plus LT. Data points are from individual mice. In *A*, the bar represents the arithmetic mean of each group. In *B*, the bar represents the geometric mean CFU. Al and Bay groups showed significant protection after challenge as inferred from urease activity ($P < 0.01$), but at most a 10-fold decrease in bacterial density as determined by quantitative culture (*Al*, $P = 0.05$, and *Bay*, not significant, versus unimmunized controls; *Al* and *Bay*, $P < 0.02$, versus *IN/day* group). LT/Al and LT/Bay showed greater protection than Al or Bay alone ($P < 0.04$). IN LT was more protective than LT/Al ($P < 0.03$) or LT/Bay ($P < 0.01$). Wilcoxon/Kruskal-Wallis (rank sums) test.

Recruitment of T and B Cells to Gastric Tissue after Challenge. Previous studies demonstrated that significant recruitment of $CD4^+$ and $CD8^+$ cells occurs in the gastric mucosa of immunized mice after *H. pylori* challenge (3). In this study, we examined the regional localization of cells af-

ter challenge. The highest density of cells localized at the junction between the antrum and corpus (Fig. 2 *a*). For example, immunized mice had three to four times as many $CD4^+$ cells in the junction region as in the antrum or corpus. In histological sections of gastric tissue from unimmunized mice examined in preliminary studies, the junction region was also the area where most *H. pylori* were located (data not shown). Therefore, quantitative analyses of gastric lymphocytes focused on this region.

The density of gastric T cells, IgA^+ plasma cells, and urease-ACC was determined for each group of mice. $CD4^+$ cells were the most numerous of any lymphocyte subset, averaging a fivefold increase in density over unimmunized controls, and were found along the muscularis mucosa and in the lamina propria between gastric pits (Fig. 2 *b*). The density of T cells varied as a function of immunization regimen (Fig. 3, *A* and *B*) and strongly correlated with the level of protection ($CD4^+$ cells, $r^2 = 0.956$; $CD8^+$ cells, $r^2 = 0.947$) (Fig. 3, *D* and *E*). Nonprotective daily IN urease immunization without adjuvant resulted in no gastric T cells, whereas immunization with urease plus adjuvant by any route produced a significant increase in gastric T cells (all groups, $P < 0.01$). IN immunization with urease plus LT induced the greatest number of gastric T cells.

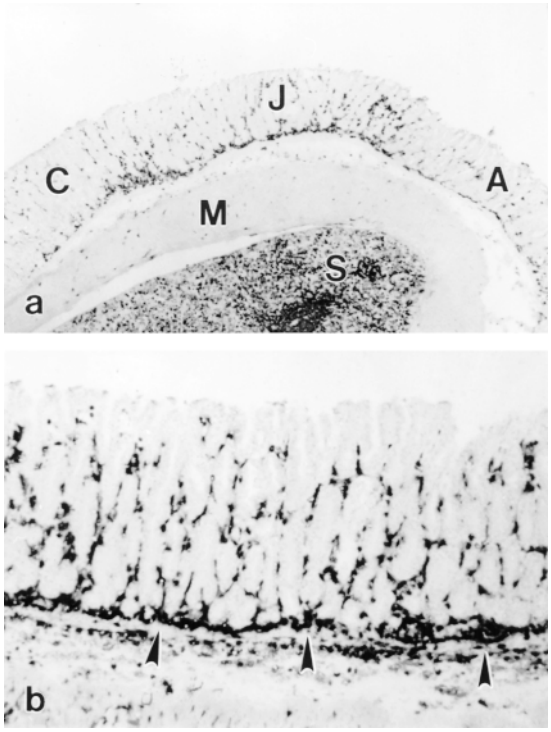

Figure 2. Localization of $CD4^+$ T cells in the gastric mucosa of urease-immunized, *H. pylori*–challenged mice. (*a*) Low-power field of gastric mucosa mounted on a slice of spleen (*S*). $CD4^+$ cells localized at the junction region (*J*) of a mouse receiving s.c. urease. *A*, Antrum; *C*, corpus; and *M*, muscularis externa. (*b*) High-power field of $CD4^+$ T cells in the gastric mucosa of a mouse receiving IN urease plus LT. T cells localized in the lamina propria between gastric pits and adjacent to the muscularis mucosa (*arrowheads*). Original magnifications: *a*, ×30; *b*, ×75.

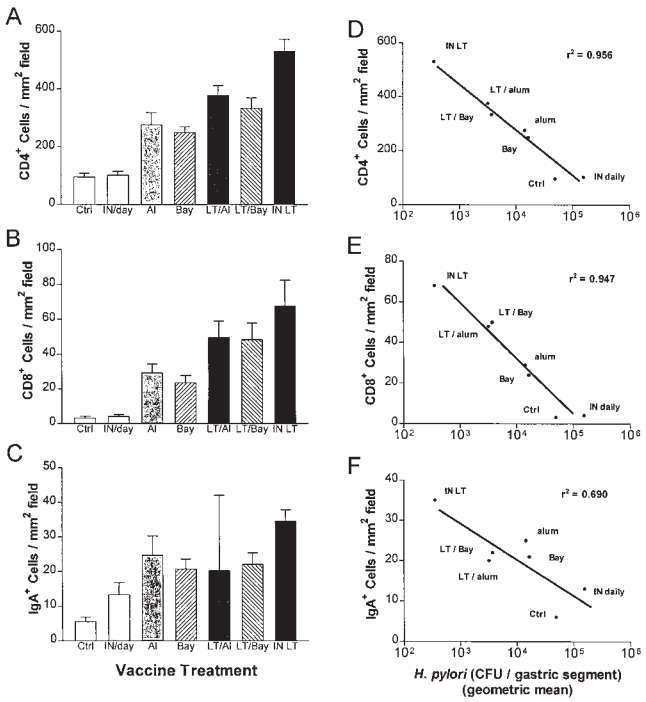

Figure 3. T and B cell subsets in the gastric mucosa of *H. pylori* challenged mice as a function of adjuvant and route of administration. (*A–C*) Quantitative evaluation of $CD4^+$, $CD8^+$, and IgA^+ cells. Mice immunized with urease plus adjuvant showed significant increases in T and B cells relative to unimmunized controls. IN/day group had no increase in T cells but did have increased IgA^+ cells ($P < 0.04$). Some combination regimens were not significantly different from single adjuvant regimens. (*D–F*) Correlation of $CD4^+$ cells, $CD8^+$ cells, and IgA^+ cells with geometric mean *H. pylori* CFUs.

IgA^+ cells were also most numerous in the urease plus LT group, increased in the nonprotective regimen of IN urease without adjuvant ($P < 0.04$), and were somewhat variable in animals receiving the parenteral and combination regimens (Fig. 3 *C*). IgA^+ cells also showed a weak positive correlation with the level of protection ($r^2 = 0.690$) (Fig. 3 *F*). Urease-ACC were detected in mice receiving urease plus LT (1–2 cells/mm^2 field in mice receiving urease plus Bay, LT/alum, LT/Bay, or LT alone), but no cells were detected in control, urease daily without adjuvant, or urease plus alum groups.

Urease-specific IgG_1 and IgG_{2a} Levels as Surrogate Markers for Th2:Th1 Responses. Urease-specific antibody levels were analyzed to determine if IgG_1:IgG_{2a} ratios correlated with protection (3, 12, 14). Daily IN administration of urease without adjuvant and parenteral immunization with alum or Bay produced higher IgG_1 than IgG_{2a} levels, whereas immunization with urease plus LT produced equivalent levels of IgG_1 and IgG_{2a} (Table 2). The ratio of urease-specific IgG_1 to IgG_{2a} showed a positive correlation with protection ($r^2 = 0.703$), with a ratio of 1 being associated with the greatest protection (urease plus LT). This correlation suggested that a balanced Th2:Th1 response might be important for the high level of protection observed after immunization with urease plus LT.

Table 2. *Urease-specific Serum IgG_1 and IgG_{2a} Levels by Immunization Regimen*

Group	IgG_1 (median)	IgG_{2a} (median)	IgG_1:IgG_{2a} ratio* (median)
	U/ml	*U/ml*	
IN/day	2.61×10^6	5.71×10^4	41
Alum	7.03×10^7	1.31×10^6	43
Bay	5.35×10^7	3.20×10^6	38
LT/Alum	3.88×10^7	2.66×10^7	1.9
LT/Bay	4.82×10^7	6.73×10^6	10
LT	2.01×10^7	9.61×10^6	1

*Median IgG_1:IgG_{2a} ratio from individual mice; not the ratio of median IgG_1 to median IgG_{2a}.

Protection in I-A^b (−/−) and β_2m (−/−) Knockout Mice

Effect of Immunization on H. pylori in Gastric Tissue after Challenge. Wild-type, β_2m (−/−), and I-A^b (−/−) C57BL/6 mice received urease administered either orally with LT, parenterally with alum, or were not immunized before challenge. The doses and regimens are outlined in Table 1. Analysis of gastric tissue revealed that wild-type (+/+) and β_2m (−/−) mice receiving urease plus LT had little detectable gastric urease activity and a >100-fold decrease in geometric mean CFUs as compared with unimmunized controls ($P < 0.01$) (Fig. 4). Subcutaneous immunization with urease plus alum resulted in a significant decrease in gastric urease activity (Fig. 4 *A*) and a fivefold decrease in the bacterial density of wild-type (+/+) mice ($P = 0.02$) (Fig. 4 *B*), but had no protective effect in β_2m (−/−) or heterozygous (+/−) strains (Fig. 4). Oral immunization of both wild-type and β_2m (−/−) mice with urease plus LT clearly gave superior protection in comparison to parenteral immunization with alum ($P < 0.02$).

Neither mucosal nor parenteral immunization regimens were effective in protecting I-A^b (−/−) knockout mice as determined by gastric urease activity and quantitative culture (Fig. 4). The geometric mean bacterial densities of immunized mice were no different from those of unimmunized controls. Furthermore, *H. pylori* infection was not enhanced in the absence of the host MHC II–restricted responses of control I-A^b (−/−) mice in comparison to wild-type mice (Fig. 4).

Antibody Responses to Urease Immunization. An analysis of urease-specific serum IgG, IgM, and IgA indicated systemic immune responses to urease in mice from all immunized groups except the I-A^b (−/−) knockout mice, which did not have detectable antibody responses (data not shown). β_2m (−/−) knockout mice had reduced serum IgG titers after immunization when compared with wild-type mice, as well as equivalent or lower levels of serum IgM and IgA.

Effects on Gastric Mucosa. As in outbred mice, immunization and challenge in wild-type and β_2m (−/−) mice but not I-A^b (−/−) mice was associated with increased lymphocyte recruitment to the gastric mucosa, as determined by gastritis scores obtained from H & E sections (data not shown). Wild-type (+/+) mice immunized with urease plus LT had minimal loss of parietal cells (mean corpus epithelial score of 1.1, range 0–2) relative to controls (mean of 0.2, range 0–2) ($P < 0.04$), whereas a more substantial loss of parietal cells was seen in β_2m (−/−) mice receiving the same regimen (mean score of 2.4, range 1–3) ($P < 0.01$ versus controls). Wild-type and β_2m (−/−) mice immunized with urease plus alum, and the unprotected I-A^b (−/−) mice immunized by either regimen, had no epithelial change relative to controls.

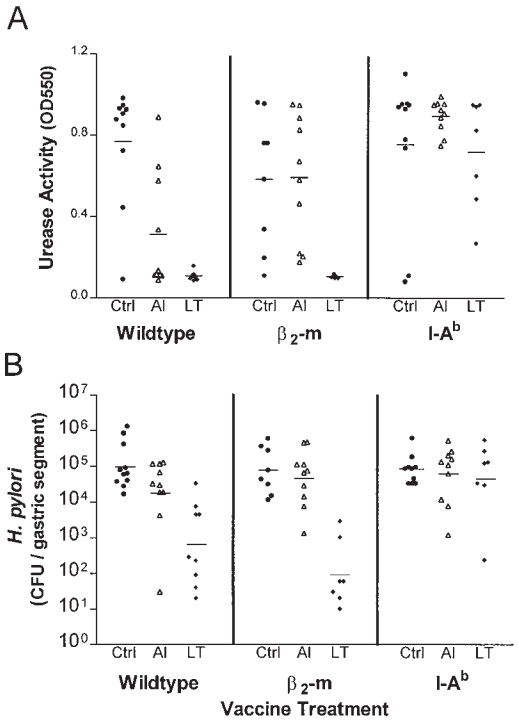

Figure 4. Protection against *H. pylori* infection in wild-type (+/+), β_2m (−/−), and I-A^b (−/−) knockout mice in response to challenge. (*A*) Gastric urease activity. (*B*) *H. pylori* CFUs from gastric mucosa. All groups were challenged with *H. pylori*. (*Ctrl*) Control mice received LT but no urease. (*Al*) Three biweekly s.c. immunizations with urease plus alum. (*LT*) Four weekly oral immunizations with urease plus LT. Data points are from individual mice. In *A*, the bar represents the arithmetic mean of each group. In *B*, the bar represents the geometric mean CFU. β_2m (−/−) knockout mice receiving urease plus alum were not protected. Not shown, wild-type heterozygous (+/−) mice were protected after urease plus LT but not after urease plus alum. Lack of protection in these mice may reflect differences among (+/+), (+/−), and (−/−) strains.

T and B Cells in Gastric Tissue. The densities of $CD4^+$, $CD8^+$, and IgA^+ cells in the gastric mucosa were evaluated as indicators of T and B cell activity in response to immunization and challenge. In addition, the densities of $CD8^+$ and $CD103^+$ (α^E-integrin$^+$) cells were used as indicators of gastric IEL. Unimmunized controls had few T or B cells in the gastric mucosa 2 wk after *H. pylori* challenge. Signifi-

cant increases in $CD4^+$, $CD8^+$, $CD103^+$, and IgA^+ cells were seen in β_2m (−/−) and wild-type (+/+) mice immunized with urease plus LT ($P < 0.05$) (Fig. 5).

Smaller increases in T and B cells were seen after immunization with urease plus alum (all groups, $P < 0.05$, except $CD4^+$ cells in β_2m/alum group and IgA^+ cells in β_2m/alum group) (Fig. 5). β_2m (−/−) knockout mice had reduced $CD8^+$ and $CD103^+$ cells in the gastric mucosa after oral immunization with urease plus LT relative to wild-type mice ($P < 0.05$, except $CD8^+$ cells did not achieve significance). Most $CD8^+$ and $CD103^+$ cells in the gastric mucosa of β_2m (−/−) mice were within the epithelium, suggesting that they were IEL.

The lack of protection in I-A^b (−/−) knockout mice was accompanied by an absence of T or B cells recruited to the gastric mucosa after challenge. Although some $CD4^+$ T cells were present in peripheral blood, spleen, Peyer's patches, and intestinal lamina propria of I-A^b (−/−) mice, $CD4^+$ cells and all other T and B cell subsets in the gastric mucosa were similar to those seen in unimmunized control mice (Fig. 5).

Role of Serum IgG in Protection

The ability of parenteral immunization to afford protection against *H. pylori* raised the possibility that serum antibodies could play a role in bacterial clearance. This conclusion was supported by studies in I-A^b (−/−) mice which had no antibody response to urease and were not protected against challenge. In an effort to clarify the contribution of circulating antiurease antibodies to protection, we tested whether immunity could be transferred to naive mice by serum, as described in Materials and Methods and Table 1. Groups of donor mice were immunized with either a protective mucosal regimen (IN urease plus LT), a protective parenteral regimen (urease plus alum), a nonprotective mucosal regimen (intranasal urease without adjuvant), or were untreated. After passive transfer of sera, recipient mice had protective levels of urease-specific serum IgG similar to those of actively immunized mice before challenge (data not shown). Despite the presence of high levels of urease-specific IgG in recipient mice, including mice receiving serum from donor mice immunized according to the protective mucosal or parenteral regimens, no protection was observed in any mice after challenge.

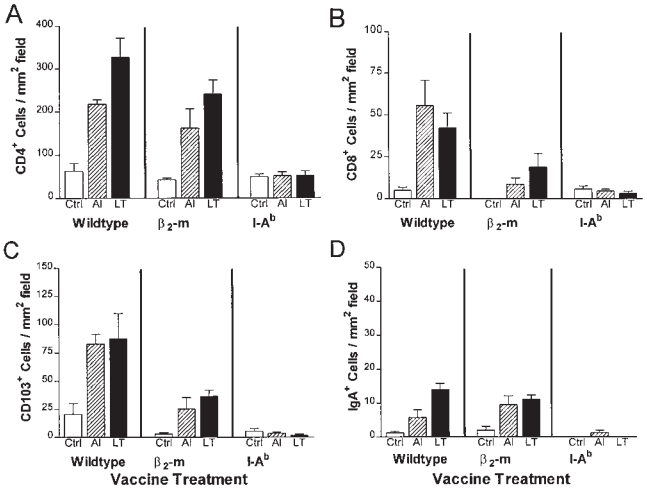

Figure 5. Quantitative phenotypic analysis of the cellular infiltrate in the gastric mucosa of wild-type (+/+), β_2m (−/−), and I-A^b (−/−) mice challenged with *H. pylori*. (*A*) $CD4^+$ cells. (*B*) $CD8^+$ cells. (*C*) $CD103^+$ (α^E-integrin$^+$) cells. Most $CD8^+$ and $CD103^+$ cells in β_2m (−/−) mice were gastric IEL. (*D*) IgA^+ cells. All wild-type and β_2m (−/−) groups had $P < 0.05$ versus controls, except the following alum groups: β_2m $CD4^+$ cells, wild-type and β_2m IgA^+ cells. I-A^b (−/−) mice showed no increase in T or B cells after immunization and challenge.

Protection against H. pylori Infection in μMT Knockout Mice

Effect of Immunization on H. pylori Infection. The inability of immune sera to passively confer protection from *H. pylori* infection to naive mice demonstrated that circulating antibodies to urease alone played a minor, if any, role in protection. To elucidate the contribution of local antibody responses in the gastric mucosa, B cell–deficient mice were used. Wild-type (+/+) and μMT (−/−) C57BL/6J mice received either oral or parenteral immunization with urease, as did the MHC-I and MHC-II knockout mice (Table 1). Oral immunization with urease plus LT decreased the gastric urease activity to baseline levels and reduced the density of *H. pylori* by more than 100-fold in both wild-type and μMT (−/−) mice after challenge (both assays, all groups, $P < 0.01$ versus controls) (Fig. 6). Parenteral immunization with urease plus alum reduced the density of *H. pylori* 10-fold in wild-type mice ($P < 0.03$) and fivefold in μMT (−/−) mice (not significant) and decreased gastric urease activity to baseline levels in more than half of the mice in both groups (Fig. 6).

Antibody Responses to Urease Immunization. No urease-specific serum IgG, IgM, or IgA responses to urease were detected in μMT (−/−) mice after oral immunization with urease plus LT or parenteral immunization with ure-

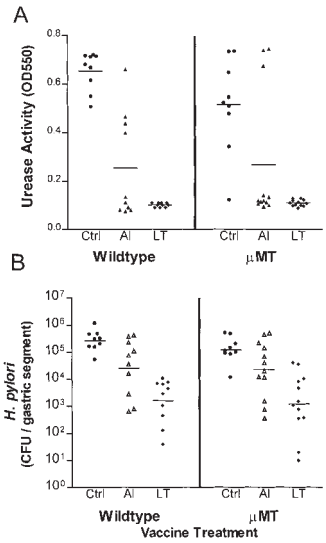

Figure 6. Protection against *H. pylori* infection in wild-type (+/+) and μMT (−/−) knockout mice. (*A*) Gastric urease activity. (*B*) *H. pylori* CFUs from gastric mucosa. All groups were challenged with *H. pylori*. (*Ctrl*) Mice were not immunized. (*Al*) Three biweekly s.c. immunizations with urease plus alum. (*LT*) Three biweekly oral immunizations with urease plus LT. Data points are from individual mice. In *A*, the bar represents the arithmetic mean of each group. In *B*, the bar represents the geometric mean CFU. LT groups, $P < 0.01$ versus unimmunized controls. In alum groups, only wild-type mice achieved a significant reduction in bacterial density ($P < 0.02$ versus unimmunized controls).

ase plus alum (data not shown). In contrast, significant increases in specific antibody levels were found in wild-type (+/+) mice immunized by all regimens. These results confirmed the defect in antibody production in μMT (−/−) mice and demonstrated that protection against *H. pylori* infection can be achieved in the absence of specific serum antibodies to urease.

T Cells, B Cells, and Nonlymphoid Cells in Gastric Tissue. In light of the unexpected observation of protection in the absence of serum antibodies after oral immunization with urease, we examined the local gastric lymphoid and nonlymphoid cell populations that might account for immune clearance. Enumeration of T and B cell subsets revealed that the densities of $CD4^+$ in the gastric mucosa of wild-type and knockout mice that received either urease plus LT or urease plus alum were significantly higher than those in unimmunized mice (wild-type, $P < 0.01$; μMT, $P < 0.05$) (Fig. 7). An equivalent level of $CD8^+$ cells was seen in both immunized wild-type and μMT mice, but the difference relative to nonimmunized controls was not significant in the μMT mice. IgA^+ and IgM^+ cells were absent in the stomach of knockout but not wild-type mice (Fig. 7).

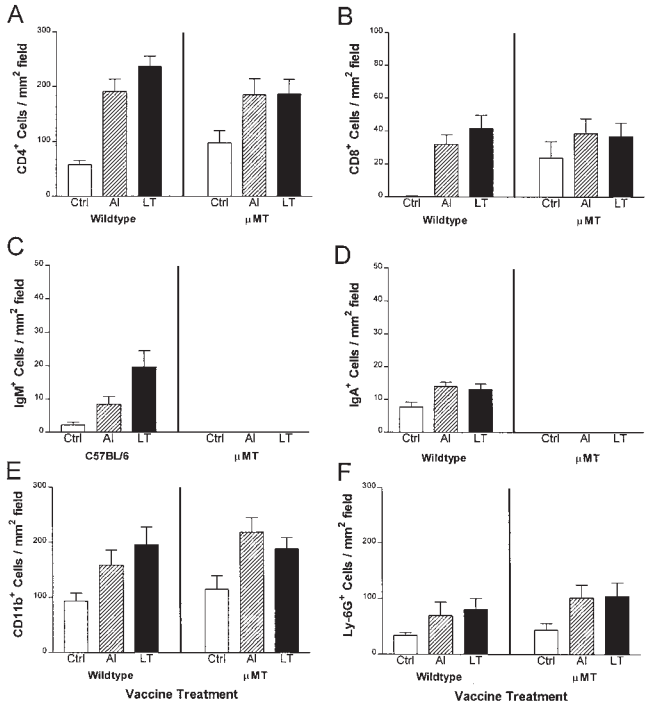

Figure 7. Quantitative evaluation of T cells, B cells, macrophages, and neutrophils in the gastric mucosa of wild-type (+/+) and μMT (−/−) mice challenged with *H. pylori*. (*A*) $CD4^+$ cells. Wild-type, both groups $P < 0.01$ versus controls. μMT, both groups $P = 0.05$ versus controls. (*B*) $CD8^+$ cells. Wild-type, both groups $P < 0.01$ versus controls. μMT mice, both groups not significant versus controls. (*C*) IgM^+ cells (mostly small lymphocytes). (*D*) IgA^+ cells (mostly plasma cells). (*E*) $CD11b^+$ cells (macrophages). Wild-type, LT group, $P < 0.03$, alum group not significant versus controls. μMT, LT group not significant, alum group, $P = 0.02$ versus controls. (*F*) Ly-$6G^+$ cells (neutrophils). Wild-type, both groups not significant versus controls. μMT mice, LT group, $P < 0.05$; alum group, $P = 0.02$ versus controls.

Immunohistochemical staining of the spleen, intestine, and Peyer's patches was performed in uninfected mice to identify sources of T and B cells in the knockout mice. No IgM^+ or IgA^+ B cells were detected in either the spleen or Peyer's patches, and no IgA^+ plasma cells were found in the lamina propria of the intestine. However, $CD4^+$ and $CD8^+$ cells were seen in the T cell areas of the spleen and Peyer's patches (composed of tiny domes and flattened T cell regions), and in the lamina propria and epithelium of the intestine.

To determine whether nonlymphoid populations were recruited to the gastric mucosa after challenge, macrophages ($CD11b^+$ cells) (25, 26) and neutrophils (Ly-$6G^+$ cells) (27, 28) were enumerated. CD11b (Mac-1) is expressed on macrophages and dendritic cells and may also be present on activated neutrophils (26). Wild-type and knockout mice showed higher levels of $CD11b^+$ and Ly-$6G^+$ cells in the junction region of the gastric mucosa after immunization and in comparison with controls (Fig. 7). $CD11b^+$ macrophages were the most numerous cell type after $CD4^+$ T cells.

Discussion

Vaccination against *H. pylori* is an important goal in the prevention and treatment of gastroduodenal diseases, including ulcers and stomach cancer. Despite the proven ability of immunization to prevent or reduce *Helicobacter* infection in murine models, the precise mechanisms of protection have remained obscured by the numerous immunological pathways stimulated. Mucosal immunization with *Helicobacter* extracts or purified recombinant proteins generates antigen-specific serum, salivary, and intestinal antibody responses as well as cellular immunity (3, 5, 12, 13, 29). Furthermore, after immunization and challenge, gastric IgA and T cell responses can be measured (3, 5, 6). Since *H. pylori* is largely an extracellular pathogen of the gastric mucosa, it was reasoned that secretory IgA might play an important role in protection through diminished bacterial–mucus or –epithelial cell interactions (5, 30). In this study, we examined mechanisms of protection using different adjuvants and routes of administration and a single *H. pylori* candidate vaccine antigen in several murine knockout models with the goal of further defining aspects of cell- and antibody-mediated immunity essential for clearance of *H. pylori*.

By comparing various prophylactic immunization schemes, including mucosal, parenteral, or combination regimens, we consistently achieved the highest level of protection by mucosal immunization with urease plus LT, which decreased the gastric bacterial load 100-fold or more after challenge compared with controls. A combined protocol of mucosal and parenteral immunization conferred a level of protection close to that of mucosal immunization. Protective efficacy was evaluated by quantitative culture of *H. pylori*, which has been shown (3) to be more sensitive than either measurement of gastric urease activity or histological enumeration of bacteria (3, 8, 31). The *H. pylori*

strain X47-2AL, originally isolated from a domestic cat (22), preferentially colonized the corpus–antrum junction region of the mouse stomach, a similar distribution to that of the human-derived *H. pylori* Sydney (SS1) strain (31). The requirement for LT with mucosal immunization was clear; no detectable protective activity could be measured in the absence of mucosal adjuvant when urease was administered by a daily IN regimen or in the form of weekly doses (15). Parenteral immunization with urease plus alum or Bay adjuvant showed less protection by comparison, demonstrating significant protection by gastric urease activity, but yielding at most a 10-fold reduction in bacterial burden after challenge.

Because a range of protective efficacy was observed with the immunization regimens tested, we examined several immunological readouts for correlation with protection. Previous analyses of the levels of urease-specific antibodies in sera and mucosal secretions after immunization with urease failed to identify a single isotype-restricted response which correlated with protection (3). However, the analysis of antigen-specific IgG_1/IgG_{2a} ratios as markers of Th activity in this investigation and a comparative study of several different parenteral adjuvants (14) determined that a more balanced (i.e., 1:1) profile was associated with greater protection, suggesting that the ability to stimulate both Th1 and Th2 responses was important for protection. These observations supported the emerging concept from vaccination studies that cell-mediated immunity is required for clearance of *H. pylori* from the murine gastric mucosa.

We focused our attention on the constituents of the cellular infiltrate that are present in the gastric mucosa after immunization and challenge. A common feature of the infiltrate was its preferential localization at the corpus–antrum junction, which coincided with the distribution of *H. pylori*. Interestingly, in humans, the junction region is also the site where intestinal metaplasia, gastric atrophy, and cancer are often found (32). When comparing the constituents of the infiltrate elicited by the various regimens, we found that certain immunization schemes stimulated recruitment of distinct cellular profiles in response to *H. pylori* challenge. For example, the nonprotective regimen of IN urease immunization without adjuvant (15) induced B cells but few T cells to the gastric mucosa. In contrast, IN immunization with urease plus LT was followed by extensive recruitment of both T and B cells to the gastric mucosa. Furthermore, the density of the local infiltrate after immunization and challenge correlated with enhanced bacterial clearance. Because the appearance of the cellular infiltrate was dependent on prior exposure to antigen, required *H. pylori* challenge, and could be modulated by adjuvants, an antigen-specific cellular response after local stimulation with *Helicobacter*-associated urease is the likely mechanism responsible for the extensive cellular trafficking to the gastric mucosa. Despite the recruitment of mononuclear cells to the gastric mucosa after protective vaccination and *H. pylori* challenge, extensive epithelial damage was not found in wild-type mice. At this time, it is not clear how these observations in the murine model will translate to humans, since it remains to be determined whether vaccination will limit both infection and pathology. In unimmunized humans, the severity of inflammation correlates with the level of infection (33–35), suggesting that controlling infection may also reduce inflammation.

Use of MHC-I and MHC-II knockout mice has been instrumental in elucidating mechanisms of immunity and vaccine-induced protection to a number of viruses, bacteria, and parasites (36–38). Using these research tools, we were able to demonstrate that protection against *H. pylori* can be achieved in MHC-I–deficient mice receiving oral urease plus LT. Mice that are lacking β_2m are deficient in $CD8\alpha\beta^+$ T cells and fail to generate cytotoxic T cell responses (39, 40). This finding strongly suggests that MHC-I–restricted $CD8\alpha\beta^+$ cell responses do not play a major role in protection against murine *H. pylori* infection. Because $CD8^+$ cells typically contribute to host defenses against viruses and intracellular bacteria (36), it is not surprising that these cells are not key players against this extracellular organism. The present study does not rule out a possible role of MHC-I in protection afforded by parenteral (alum) immunization, nor does it exclude a possible role of MHC-I–independent $CD8^+$ cell responses such as that performed by gastric $CD8\alpha\alpha^+$ IEL, which differentiate independently of MHC-I (39–41). However, considering the relatively low density of these cells in comparison to $CD4^+$ cells or macrophages it seems likely that they are not the principal cellular component involved in protection based upon urease vaccination.

In contrast to MHC-I–deficient mice, immunization and challenge of MHC-II knockout mice revealed a profound defect in the ability of immunization to afford protection. The lack of protection was associated with an absence of $CD4^+$ and $CD8^+$ T cells and IgA^+ B cells in the gastric mucosa. Although the MHC class II–deficient mice did not mount a protective immune response, the absence of detectable urease-specific antibodies in sera and IgA^+ cells in the gastric mucosa of these mice did not clarify the relative roles of antibodies and $CD4^+$ T cells as effectors of protection.

In an effort to determine the contribution of circulating antibodies to protection, we performed passive transfer studies using immune sera from mice vaccinated via mucosal or parenteral routes. Despite high antibody titers to urease in recipient mice, no detectable protection after *Helicobacter* challenge was observed, demonstrating that antigen-specific circulating antibodies play little or no role in protection in this model. However, these experiments could not definitively rule out the potentially protective role of locally synthesized antibodies. Our previous studies using mucosal immunization suggested that urease-specific IgA cells recruited to the lamina propria secreted antibody into the gastric pits (3, 5), and thus theoretically could play a role in immune clearance. It has also been proposed that local IgG antibodies in the gastric mucosa may play a role in protection (10). Conflicting data with these studies showing that mice deficient in IgA were protected against *H. felis* challenge (42) further contributed to the confusion as to the role of antibodies in protection against *Helicobacter*.

To define the role of humoral immunity in protection from *H. pylori* more precisely, we used the μMT (−/−) mouse model of B cell and antibody deficiency. This model has been instrumental in determining the role of humoral immunity in clearance of infectious diseases (43–45). Immunization followed by challenge of μMT (−/−) mice revealed, unexpectedly, that they were capable of mounting a protective response to *H. pylori* which was indistinguishable from that of wild-type mice. However, the results obtained with knockout mice do need to be interpreted with caution, as it is known that ablation of specific immune functions can result in compensation by alternative pathways (20, 43). Nonetheless, these results demonstrated that clearance of *H. pylori* from the gastric mucosa can occur by antibody-independent mechanisms and suggest that, in normal mice, neither gastric IgA (3) nor IgG antibodies (10) play an essential role in protection after immunization with urease.

In the absence of demonstrable antibody responses in μMT (−/−) mice, we enumerated various cell populations present in the stomach that were associated with protection. We found that $CD4^+$ T cells and $CD11b^+$ macrophages were the most numerous cells, followed by Ly-$6G^+$ neutrophils. The local density of macrophages may in part be due to a compensatory component of the μMT (−/−) background, since these mice have been reported to have higher levels of macrophages relative to wild-type mice (20, 43). Nonetheless, the requirement of $CD4^+$ T cells for protection, as demonstrated by their absence in the gastric mucosa of I-A^b (−/−) mice after immunization and challenge, in conjunction with their relative abundance in the stomach of immunized protected wild-type and B-cell–deficient mice, implicates Th cells as primary mediators of vaccine-induced protection in the mouse model. This conclusion is supported by adoptive transfer of protection against *H. felis* with antigen-specific $CD4^+$ T cells (12). The precise mechanisms responsible for bacterial clearance in the *H. pylori* mouse model remain undefined and under investigation.

In conclusion, this study demonstrated that, in a murine model, protection against *H. pylori* can be achieved by urease in the absence of B cells and antibodies but requires MHC class II $CD4^+$ T cell responses. These findings reveal a previously unreported cell-mediated mechanism of immune clearance of a predominantly intraluminal pathogen of the gastrointestinal tract. We cannot predict that a similar mechanism is responsible for protection after vaccination with other *Helicobacter* antigens, either during long-term challenge or after therapeutic immunization. Future studies will determine whether this is a universal mechanism used in these various settings.

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