Mucosal-associated invariant T cells orchestrate immunopathology and augment gastritis in chronic *Helicobacter pylori* infection

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Running title: MAIT cells augment *Helicobacter pylori*-induced pathology

SUMMARY

Mucosal-associated invariant T cells produce pro-inflammatory cytokines in response to microbially-derived vitamin B2 metabolites, protecting against some human pathogens. We demonstrate that MAIT cells augment immunopathology in murine *Helicobacter pylori* infection, with implications for human infection and associated disease.

ABSTRACT

Mucosal-associated invariant T (MAIT) cells produce inflammatory cytokines (IL-17, IFNγ, TNF), and cytotoxic granzymes in response to by-products of microbial vitamin B2 (riboflavin) synthesis. Although MAIT cells are protective against some pathogens, we reasoned that they might contribute to pathology in chronic bacterial infection. We have observed MAIT cells in proximity to *Helicobacter pylori* bacilli in human gastric tissue. Here, using MR1 tetramers, we examined whether these cells contribute to chronic inflammation in gastritis in a mouse model of *H. pylori* infection. we analysed MAIT cells in chronic *Helicobacter pylori* SS1 infection. Following *H. pylori* infection of mice, MAIT cells, with an effector memory Tc1/Tc17 phenotype, accumulated in the stomach and accelerated gastritis, characterised by the augmented recruitment of neutrophils, macrophages, dendritic cells, eosinophils and non-MAIT T cells. This occurred both in MAIT TCR transgenic mice, and in wild-type C57BL/6 mice with pre-primed MAIT cells. Thus, we demonstrate a pathogenic role for MAIT cells in *Helicobacter*-associated inflammation, revealing a broader potential role for MAIT cell-driven immunopathology in chronic bacterial infection.

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INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are a subset of T cells, which are abundant in humans (1-8% in blood), but relatively rare (about 0.1% in blood) in specific-pathogen-free (SPF)-housed laboratory mice (Chen et al., 2017; Martin et al., 2009; Rahimpour et al., 2015; Reantragoon et al., 2013). MAIT cells typically express a semi-invariant αβ T cell antigen receptor (TCR), which in humans comprises TRAV1-TRAJ33 (Vα19-Jα33/20/12) TCR α-chain preferentially assembled with TRBV20- (Vβ2) or TRBV6- (Vβ13) TCR β-chain and in mice TRAV1-TRAJ33 (Vα19-Jα33) paired with TRBV19 (Vβ6) or TRBV13 (Vβ8) (Lepore et al., 2014; Reantragoon et al., 2013; Tilloy et al., 1999), and is restricted by the monomorphic Major Histocompatibility Complex (MHC) related protein-1 (MR1) (Le Bourhis et al., 2011).

MR1 presents vitamin B-based antigens (Ags), including 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), which derive from a precursor of microbial riboflavin (vitamin B2) biosynthesis and activate MAIT cells *in vitro* (Corbett et al., 2014; Eckle et al., 2015; Keller et al., 2017a, Mak et al, 2017). Indeed, a correlation between the presence of the riboflavin synthesis pathway in diverse microbes, including bacteria and yeast, with MAIT cell activation, led to the discovery of this pathway as furnishing this new class of antigen (Kjer-Nielsen et al., 2012; Corbett et al., 2014). In contrast, microbes that do not synthesise riboflavin, such as *Listeria monocytogenes* and *Enterococcus faecalis*, do not activate MAIT cells (Gold et al., 2010; Le Bourhis et al., 2010). Thus, MAIT cells, which are concentrated in tissues including lungs, intestine and liver, are ideally placed to detect metabolically active microbes producing these Ags at mucosal sites.

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In vivo stimulation of MAIT cells with intranasally-delivered synthetic 5-OP-RU, in the presence of co-stimulatory signals provided by TLR ligation, led to MAIT cell accumulation in the lungs (Chen et al., 2017). MAIT cell non-stimulatory Ags, including 6-formyl pterin (6-FP, a folic acid degradation product) and acetyl-6-FP (a derivative) are also presented by MR1 (Kjer-

Nielsen et al., 2012) and can competitively block MAIT cell activation *in vitro* (Eckle et al., 2014) and *in vivo* (Keller et al., 2017b). Human and mouse MR1-Ag-tetramers containing 5-OP-RU specifically detect MAIT cells in the respective species (Chen et al., 2017; Corbett et al., 2014; Reantragoon et al., 2013) without the need for surrogate and potentially misleading MAIT cell markers (Chen et al., 2017; Rahimpour et al., 2015).

MAIT cells rapidly secrete cytokines (including IFNγ, IL-17 and TNF) upon stimulation and play a protective role in some bacterial infections, including *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *M. bovis* BCG and *Francisella tularensis* (Georgel et al., 2011; Meierovics et al., 2013; Meierovics and Cowley, 2016; Sakala et al., 2015). They can also be activated in a TCR-independent manner by cytokines or viral infections (Loh et al., 2016; Ussher et al., 2014; van Wilgenburg et al., 2016). Correlations between MAIT cell number and disease have also been demonstrated for a range of chronic inflammatory conditions suggesting that MAIT cells may also contribute to immunopathology as well as protection against microbes. These conditions include multiple sclerosis (Held et al., 2015; Miyazaki et al., 2011; Negrotto et al., 2015; Salou et al., 2016; Willing et al., 2014), rheumatoid arthritis (Chiba et al., 2012; Sugimoto et al., 2015), inflammatory bowel diseases (Haga et al., 2016; Hiejima et al., 2015; Serriari et al., 2014; Treiner, 2015), coeliac disease (Dunne et al., 2013) and ankylosing spondylitis (Gracey et al., 2016; Hayashi et al., 2016). The basis of these correlative associations could involve MAIT cell activation but might also be attributable to treatment (Hinks et al., 2016). Indeed, few studies to date have attempted to understand the potential role of MAIT cells in immunopathology. We reasoned this might be most likely in unresolved chronic bacterial infection and tested this idea in a model of *H. pylori* gastritis noting that MAIT cell numbers are reduced in patients' blood during *H. pylori* infection versus healthy controls and human gastric MAIT cells can produce cytokines in response to *H. pylori* (Booth et al., 2015).

H. pylori is a Gram negative extracellular bacterium that colonises the stomach. A large proportion of the world's population is infected with *H. pylori,* and in most people, unless treated with antibiotics, the infection persists for decades. Most infected individuals remain asymptomatic, but about 15-20% develop overt disease such as peptic ulcers and severe gastritis. A smaller proportion (1-3% of cases) develops gastric adenocarcinoma and fewer again (0.1%) mucosal-associated lymphoid tissue (MALT) lymphoma (Peek and Crabtree, 2006). The magnitude of the immune reaction to, and the pro-carcinogenic effects of *H. pylori* are determined by both pathogen-derived factors, including well-characterised virulence genes (Ohnishi et al., 2008; Wada et al., 2004), and host factors, including polymorphisms that lead to high expression of the pleiotropic pro-inflammatory cytokine IL-1β, have been linked to increased development of hypochlorhydria, gastric atrophy and distal gastric adenocarcinoma in *H. pylori* infected patients (Wroblewski et al., 2010).

Infection with *H. pylori* induces both innate and adaptive immune responses by the host, generally T helper (TH) 1-biased, and tempered by regulatory T cells (reviewed in (Larussa et al., 2015)). The production of pro-inflammatory cytokines, including IL-17 and IFNγ by MAIT cells (Dusseaux et al., 2011; Meierovics et al., 2013; Rahimpour et al., 2015), suggests their potential to play roles in the chronic inflammation (gastritis) that is characteristic of *H. pylori* infection, and possibly in the subsequent development of more severe MALT lymphoma and adenocarcinoma that occur in some patients. Since the role of MAIT cells in the immune response to *H. pylori* remains unclear, this circumstantial evidence prompted us to address this question experimentally, using Ag-specific MR1 tetramers to detect MAIT cells, thereby removing ambiguity inherent in surrogate markers of these cells.

Mouse models of *H. pylori* infection have been widely utilised to understand disease development, and show the importance of CD4+ T cells including Th17 cells (Gray et al., 2013; Shi et al., 2010). However, to date, no analysis of MAIT cells in mouse models of *H. pylori* has

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been reported, perhaps in part due to the low abundance of MAIT cells in mice and low numbers of lymphocytes in uninfected stomachs, coupled with a previous paucity of reagents (MR1 tetramers) to precisely identify murine MAIT cells. Here we use both Vα19*i*.Cα$^{-/-}$ MAIT TCR transgenic mice and wild-type C57BL/6 mice with pre-boosted MAIT cells, coupled with the capacity to specifically detect MAIT cells using MR1-tetramers, to investigate the role of MAIT cells in *H. pylori* infection, revealing for the first time, their pathogenic role in a chronic bacterial infection.

RESULTS

***Helicobacter pylori* have a riboflavin-biosynthesis pathway and are capable of activating MAIT reporter cells.**

We first tested whether *H. pylori* synthesise ligands that stimulate MAIT cells. Previously, we have shown that both human and mouse MAIT cells are stimulated by ligands, including 5-OP-RU, formed from the non-enzymatic reaction of a microbial riboflavin biosynthesis precursor with small metabolites (Corbett et al., 2014; Kjer-Nielsen et al., 2012). *H. pylori* and other *Helicobacter* species contain an intact riboflavin synthesis pathway (Kyoto Encyclopedia of Genes and Genomes database), and thus were predicted to produce MAIT cell-stimulating riboflavin biosynthesis-derived Ags. To verify MAIT cell stimulation by *H. pylori*, we tested culture supernatant from *H. pylori* SS1 cultures for its ability to stimulate a reporter MAIT cell line, Jurkat.MAIT (Kjer-Nielsen et al., 2012). Similar to *Salmonella* Typhimurium, previously shown to stimulate Jurkat.MAIT cells in this assay (Corbett et al., 2014; Kjer-Nielsen et al., 2012; Reantragoon et al., 2012), *H. pylori* culture supernatant caused up-regulation of CD69 on Jurkat.MAIT cells in the presence of MR1 expressed on C1R cells (C1R.MR1) **(Figure 1a).** This could be specifically blocked with the anti-MR1 mAb 26.5, but not an isotype control mAb **(Figure 1a)**. Importantly, Jurkat cells expressing an irrelevant TCR (Jurkat.LC13) were not

activated under the same conditions **(Figure 1b)**. This indicates that *H. pylori* is capable of producing MAIT cell-stimulating Ag. In addition, *H. pylori* supernatant caused up-regulation of MR1 on C1R.MR1 cells **(Figure 1c)**, similar to that previously seen with *Salmonella* Typhimurium supernatant or synthetic 5-OP-RU, 6-FP or acetyl 6-FP (Eckle et al., 2014; Kjer-Nielsen et al., 2012; Mak et al., 2017), a further confirmation of the presence of MR1-binding Ag.

***H. pylori* infection in mice increases MAIT cell numbers in the stomach.**

Following human *H. pylori* infection, only ~20% of individuals develop overt disease. Booth *et al.* (2015) recently reported the presence of MAIT cells in human gastric biopsies and demonstrated their capacity to respond to *H. pylori*-infected macrophages *in vitro*. Upon examining stomach tissue obtained from patients following sleeve gastrectomy bariatric procedures by immunofluorescence microscopy in 3 out of 10 cases we observed $CD3^{+}TRAV1\text{-}2^{+}$ cells within the gastric mucosa in close proximity to *H. pylori* **(Figure 2)**. Using MR1 tetramer staining MAIT cells could be detected in single cell preparations of gastric tissue where they represented ~1% of T cells **(Figure 2 and Supplementary Figure 1)**. Together, the presence of MAIT cells in gastric tissue and the ability of *H. pylori* to activate MAIT cells suggest a potential role for MAIT cells in the immune response to *H. pylori* infection. Notably, analysis of patient samples is complicated by the unknown timing and duration of infection, medication history and co-morbidities, such as obesity (Magalhaes and Lehuen, 2015) and other conditions. Hence, we directly assessed the role of MAIT cells in the well-established mouse model of *H. pylori* SS1 infection (Lee et al., 1997), given its strong similarities to human *H. pylori* gastric pathology. This allowed us to evaluate the behaviour and function of MAIT cells in mouse stomachs following controlled infection in inbred mice with a mouse-adapted *H. pylori* strain.

MAIT cells are rare in wild-type (wt) C57BL/6 mice compared with humans. For instance, they represent <0.5% of T lymphocytes in the stomach of naïve mice, (data not shown). Hence,

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following *H. pylori* infection of wt C57BL/6 mice, we observed that only ~20% (2/10) of animals exhibited an increase in proportion of MAIT cells, detected by MR1-tetramers, co-incident with gastric pathology (data not shown). We reasoned that increasing mouse MAIT cell numbers to resemble the relative numbers found in adult humans might facilitate investigation of their role in immune protection or pathology. For this purpose, we turned initially to MAIT TCR transgenic mice (Vα19*i*Cα$^{-/-}$MR1^{+}). These transgenic mice express large numbers of T cells with the canonical TCRα chain (Vα19*i*) characteristic of mouse MAIT cells. They lack the endogenous TCRα chains (Cα$^{-/-}$), thereby eliminating expression of endogenous TCRα (Kawachi et al., 2006). MAIT cells from Vα19*i*Cα$^{-/-}$MR1^{+} mice are abundant, produce IFNγ and TNF and proliferate *in vitro* in response to specific MAIT cell Ag 5-OP-RU (Reantragoon et al., 2013).

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Accordingly, we infected Vα19*i*Cα$^{-/-}$ transgenic mice on MR1^{+} and MR1$^{-/-}$ backgrounds with *H. pylori* and then isolated single cell suspensions from the stomachs for flow cytometric analysis of MAIT cells at several time points. MAIT cells as a proportion of αβ-T cells significantly increased from ~30% to ~60% at 12 weeks post-infection in the Vα19*i*Cα$^{-/-}$MR1^{+} mice as compared to the control Vα19*i*Cα$^{-/-}$MR1$^{-/-}$ mice **(Figure 3a, b)**. Thus, MAIT cells are capable of responding not only to *H. pylori* Ags *in vitro*, but also to infection *in vivo*.

An increase in gastric MAIT cells upon *H. pylori* infection of Vα19*i*Cα$^{-/-}$MR1^{+} mice is associated with severe pathology.

We next determined whether MAIT cells accumulating in the stomach in chronic *H. pylori* infection contribute to pathology. Vα19*i*Cα$^{-/-}$MR1^{+} and Vα19*i*Cα$^{-/-}$MR1$^{-/-}$ were infected with *H. pylori* and stomachs processed for histological assessment by H&E staining at various time points after infection. Sections were scored blindly for cellular infiltration (neutrophilic and lymphocytic) and atrophy, a classical characteristic of chronic gastritis where there is a loss of chief and parietal cells, and a critical step in the development of gastric cancer (Park and Kim, 2015; Rogers, 2012).

An increased cellular infiltrate was seen in the stomachs of Vα19iCα^{+}MR1^{+} mice relative to Vα19iCα^{+}MR1$^{-/-}$ mice at 12 weeks **(Figure 3a, c)**, consistent with an increase in MAIT cells identified by tetramer staining **(Figure 3b)**, and as early as 2 weeks in some mice (not shown). At 12 weeks post-infection, most Vα19iCα^{+}MR1^{+} mice developed severe atrophy **(Figure 3c, d)**, suggesting that MAIT cells not only accumulate after infection, but also contribute to pathology.

Prior expansion of MAIT cells in wild-type mice results in greater MAIT cell accumulation in stomachs following in *H. pylori* infection.

MAIT cell accumulation and enhanced pathology was seen in the Vα19i (MAIT TCRα) transgenic mice. However, since the αβ-T cell repertoire in these mice is unnaturally constrained to favour cells expressing only the transgenic Vα19Jα33 TCR (Kawachi et al., 2006) and some of these T cells do not appear to be MR1-restricted (Sakala et al., 2015), we wished to determine the MAIT cell response to *H. pylori* infection in wt mice. Inbred SPF-housed mice have far fewer MAIT cells in the blood than humans, and it has been hypothesised that childhood infections in humans boost MAIT cell numbers (Koay et al., 2016). Less than 100 MAIT cells are recoverable in single cell suspensions from the stomach of naïve wt C57BL/6 mice (<0.5% stomach T cells), compared to ~1-3% in the human gastric mucosa (Booth et al., 2015 and our unpublished observations). We reasoned that boosting MAIT cell numbers in wt C57BL/6 mice by prior bacterial infection at another mucosal site might expand MAIT cells, and hence reveal their role in gastric infection as observed in the Vα19iCα^{+}MR1^{+} mice.

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For this purpose, we used a recently developed model of MAIT cell enrichment in wt C57BL/6 mice (Chen et al., 2017) that involves either intranasal infection with *Salmonella* Typhimurium or co-inoculation of MAIT Ag and TLR agonists.. Following boosting, MAIT cells rapidly expand in an MR1-dependent manner, comprising up to 50% of T cells in the lungs at day 7 post-infection. Moreover, MAIT cells were redistributed to other organs and tissues, and their frequencies

remained elevated up to 10 weeks post-infection (Chen et al., 2017). Thus, we consider that this model more closely resembles MAIT cell distribution in adult humans where a history of infection and bacterial colonisation may lead to a greater number of MAIT cells than observed in SPF-housed mice.

In order to determine the effect of boosting MAIT cell numbers on subsequent *H. pylori* infection, wt C57BL/6 mice were infected with *S.* Typhimurium BRD509 and after 7 weeks, when the *Salmonella* bacteria are cleared, mice were challenged by gastric infection with *H. pylori* SS1 **(Figure 4a)**. As expected, prior pulmonary infection with *Salmonella* not only expanded the proportion of MAIT cells in the lungs, and blood of infected mice, but also in the stomachs (**Figure 4b**). Notably, in the stomach, the proportion of MAIT cells increased to approximately 6% of T cells **(Figure 4b)**. This is similar to human MAIT cell percentages (Booth et al., 2015 and our unpublished observations). When these mice were subsequently challenged with *H. pylori*, there was an increase in MAIT cell numbers as early as 2 weeks post-infection with a significant increase over time **(Figure 4c)**.

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Stomach MAIT cells induced following *H. pylori* infection exhibit a Tc17/Tc1 profile.

We next examined the phenotype of the MAIT cells accumulating after *H. pylori* infection in mice that were pre-boosted with *S.* Typhimurium. MAIT cells isolated from both the lungs and the stomach retained an "effector-memory" phenotype, as previously described (Chen et al., 2017; Dusseaux et al., 2011), being $CD44^{+}$ and $CD62L^{low}$ **(Figure 5a)**. MAIT cells are known to express a transcriptional signature that includes the regulators PLZF, RORγt and T-bet. We found that stomach MAIT cells showed a mixture of $PLZF^{+}$ and $PLZF^{-}$, as well as $ROR\gamma t^{+}$ and $ROR\gamma t^{-}$ cells. T-bet was expressed on the majority of MAIT cells, 8 weeks post *H. pylori* infection **(Figure 5b and Supplementary Figure 2)**. There was a preferential accumulation of $CD8^{+}$ MAIT cells in the stomach **(Figure 5c)**, and the majority of MAIT cells expressed CD69 and CD103, consistent with

a tissue-resident phenotype (Booth et al., 2015) **(Figure 5d)**. This was also true for non-MAIT αβ-T cells accumulating in the stomach after 8-weeks of infection with *H. pylori*. Multiplex PCR and sequencing of single cells showed a TCR repertoire consistent with MAIT cells (**Supplementary Table 1**). Thus, MAIT cells accumulating in the stomach after *H. pylori* infection have a transcription factor profile consistent with Tc17/Tc1 type cells characteristic of pro-inflammatory capability.

MAIT cells are important for early recruitment of other T cells.

In order to understand the implications of MAIT cell accumulation during gastric infection, we examined the effect of MAIT cell induction on the non-MAIT αβ-T cell response. In pre-primed mice the increase in MAIT cells was also accompanied by an increase in non-MAIT αβ-T cells. This effect was not observed in mice lacking MR1 (C57BL/6.MR1$^{-/-}$), indicating that it was MR1-dependent and therefore likely to be a MAIT cell-driven **(Figure 6a)**. We have previously shown, both *in vitro* and *in vivo,* that MAIT cell responses are dependent on microbial riboflavin metabolites (Chen et al., 2017; Corbett et al., 2014; Kjer-Nielsen et al., 2012). Mice infected with a mutant strain of *S.* Typhimurium BRD509 in which key genes involved in riboflavin synthesis, *ribD* and *ribH,* are deleted (Salm.Δ*ribDH)* (Chen et al., 2017) did not accumulate MAIT cells in their stomachs following subsequent *H. pylori* infection **(Figure 6b)**. However, the response could be rescued when BRD509Δ*ribDH* was supplemented with synthetic 5-OP-RU ligand (Mak et al., 2017) **(Figure 6b)**. The non-MAIT αβ-T cell accumulation was also partially dependent on the presence of 5-OP-RU, supplied either by adding riboflavin-producing *S.* Typhimurium BRD509 bacteria or by adding exogenous synthetic ligand **(Figure 6c)**. Similar to our previous observations in the lung model (Chen et al., 2017), this result could be reproduced by intranasal introduction of specific MAIT cell antigen 5-OP-RU and a co-stimulus, such as the TLR2/6 agonist *S*-[2,3-bis(palmitoyloxy)propyl] cysteine (Pam2Cys) (Jackson et al., 2004; Lau et al.,

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2006). In contrast, non-MAIT αβ-T cell accumulation was much less apparent when Pam2Cys was combined intranasally with the MR1 ligand 6-FP, which does not stimulate MAIT cells (Kjer-Nielsen et al., 2012) **(Figure 6d, e)**. Thus, Ag-specific priming of MAIT cells leads to accumulation of non-MAIT T cells in the stomach upon *H. pylori* infection.

Recruitment of other immune cells via MAIT cells leads to accelerated pathology.

T cells and other cell types, including neutrophils and macrophages, have been implicated in pathology (gastritis) following *H. pylori* infection in humans and mouse models (Kaparakis et al., 2008; Moyat and Velin, 2014). In mice pre-primed with either *S.* Typhimurium BRD509 or Pam2Cys plus 5-OP-RU, and then challenged with *H. pylori,* we observed, in addition to MAIT cell (**Figure 4c**) and non-MAIT αβ-T cell (**Figure 6a**) accumulation, an increase in other immune cells including neutrophils, macrophages, eosinophils and dendritic cells (**Figure 7a-d**). This increase was significantly higher than in MR1$^{-/-}$ mice, indicating a role for MAIT cells in the recruitment of other cell types. The MAIT cell accumulation and recruitment of other cell types was associated with gastritis, which was evident on H&E-stained stomach sections **(Supplementary Figure 3)**, and in significantly increased pathogenic scores, compared to MR1$^{-/-}$ mice, for lymphocytic infiltrate and atrophy. Strikingly, all MAIT pre-boosted mice infected with *H. pylori* developed atrophic gastritis by 8 weeks post-infection **(Figure 7e)**; comparatively, only 2/5 mice developed atrophic gastritis (score 1/4) in the absence of MR1. Gastritis was also observed following *H. pylori* infection in wt mice primed with riboflavin-deficient *S.* Typhimurium (Salm.ΔribDH) or Pam2Cys, but this was significantly increased when the specific MAIT antigen 5-OP-RU was also added **(Supplementary Figure 4)**, further demonstrating that MR1-Ag dependent MAIT activation is a driver of gastric pathology.

DISCUSSION

We and others (Booth et al., 2015) have observed the presence of MAIT cells in human gastric mucosae, prompting us to examine the role of MAIT cells in chronic *H. pylori* infection, with the use of an established mouse infection model supported by detection of MAIT cells using Ag-specific MR1-5-OP-RU-tetramers. Given that MAIT cells were virtually undetectable in stomachs of SPF-housed wt C57BL/6 mice, we used two methods to boost MAIT cell numbers to 'human-like' proportions prior to challenge with *H. pylori*, on the basis that prior expansion of these cells is integral to their impact. First, we used Vα19iCα^{-}MR1^{-} TCR transgenic mice, where a rapid, MR1-dependent, expansion of MAIT cells was observed following infection that led to early and severe atrophic gastritis. This observation strongly suggested the association of MAIT cells and pathology.

Secondly, we built upon our previous studies showing that MAIT cells are distributed to many different tissues and organs after resolution of a primary lung infection (Chen et al., 2017). We hypothesized that a previous history of infection of this kind can prime MAIT cells in naïve mice and increase their numbers to a level similar to that in humans. Indeed, we observed increased MAIT cells in the stomach after a primary lung infection, possibly due to mucosal homing markers expressed on MAIT cells and a shared cytokine/chemokine milieu between the mucosae of the lungs and stomach (Flach et al., 2012; Kohlmeier et al., 2011).

We show that when MAIT cells from wild type C57BL/6 mice are primed first with *S.* Typhimurium, or with specific antigen and a co-stimulus, they subsequently respond rapidly upon challenge with *H. pylori* (~10 fold increase at 2 weeks post-infection) and these mice develop severe inflammation, recapitulating what was observed with the MAIT TCR transgenic mice. These mice developed atrophic gastritis as early as 8 weeks post-infection, which is normally not observed in wt C57BL/6 mice infected with *H. pylori*. The response was specific to MAIT cells and dependent on MR1. The accumulation of MAIT cells in the stomach showed a biased

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enrichment of CD8^{-} MAIT cells that was not seen in the blood. This could be due to up-regulation of CD8 on the same MAIT cell population, as the cytokine milieu has been shown to influence CD8 expression on CD4^{-} T intraepithelial lymphocytes (Reis et al., 2013), or it might reflect preferential expansion of CD8^{-} MAIT cells.

Consistent with other mouse and human studies on MAIT cells (Booth et al., 2015; Chen et al., 2017; Dusseaux et al., 2011), we observed that MAIT cells in the stomach have an effector memory phenotype, characterised by CD44^{hi} CD62L^{lo} expression. A large proportion (~50-70%) expressed the tissue resident memory (TRM) markers CD69 and CD103, consistent with observations on cells isolated from *H. pylori*-infected stomachs (Booth et al., 2015), and reflecting the chronic nature of *H. pylor*i infection. MAIT cells isolated from infected stomachs also showed high expression of T-bet, consistent with a Th1-biased immune response to *H. pylori* and similar to that observed after acute bacterial lung infection (Chen et al., 2017). Interestingly, T-bet deficient mice exhibit a higher bacterial load, but lower levels of gastritis after *H. pylori* infection, indicating that a Th1-biased response may be important for both protection and immunopathology (Eaton et al., 2006). PLZF and RORγt were each expressed on ~50% of stomach MAIT cells following *H. pylori* infection of primed mice. Although MAIT cells in the stomachs of naïve mice were too few to be included in this analysis, the lower expression of these transcription factors following infection differs from that seen previously in either naïve or *S.* Typhimurium-infected lungs, where the majority of MAIT cells expressed PLZF and RORγt (Chen et al., 2017). This difference may result from re-programming of MAIT cell cytokine profiles, preferential expansion of MAIT cell subsets under different tissue-specific cytokine milieu during chronic stimulation, or other signals that differ between the two pathogens. Interestingly, natural killer T cells can down-regulate PLZF in adipose tissue due to chronic activation (Lynch et al., 2015). To our knowledge, this is the first description of such a marked shift in the expression pattern of these transcription factors by MAIT cells, and the first demonstration of enrichment of Tc1/Th1 MAIT cells.

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A number of studies using a range of mouse models have shown a Th1/Th17 type of T cell response in *H. pylori* (Gray et al., 2013). The pro-inflammatory cytokines TNF, IFNγ and IL17 are known to play an important synergistic role in regulating polymorphonuclear leukocyte migration in an inflammatory setting (Cua and Tato, 2010; Ellis and Beaman, 2004; Griffin et al., 2012). MAIT, and to a lesser extent non-MAIT αβ-T cells in our study, showed mixed RORγt+ and RORγt- populations and high T-bet expression. Although technical difficulties precluded detection of cytokine production by stomach MAIT cells, the high T-bet expression suggests MAIT cells likely express IFNγ in *H. pylori*-infected stomachs, consistent with a role for this cytokine in driving pathology. Importantly, we also showed that the presence of MAIT cells correlated with infiltration of innate immune cells, as well as increased recruitment of non-MAIT αβ-T cells. This recruitment is associated with the severe gastritis phenotype observed in the wild-type C57BL/6 with pre-boosted MAIT cells, and MAIT TCR transgenic mice infected with *H. pylori*. Indeed, previous studies have shown that T cells are major orchestrators of inflammation (Gray et al., 2013; Shi et al., 2010; Tan et al., 2008). Interestingly, IFNγ- and IL-17A-deficient mice exhibited reduced gastritis in previous *H. pylori* infection studies, consistent with a role for MAIT or other cell types in orchestrating pathology (Yamamoto et al., 2004).

In summary, we show that in *H. pylori*-induced gastritis, MAIT cells play a pathogenic role in orchestrating immunopathology. This study provides the first *in vivo* evidence that MAIT cells can drive inflammation and pathology during chronic bacterial infection. It is likely that the penetrance of gastritis in *H. pylori*-infected humans is partly influenced by a previous history of infections, and MAIT cell priming at other mucosal sites. The recognition of a common MR1-retricted Ag, derived from bacterial riboflavin synthesis, suggests that MAIT cells represent a potential therapeutic target to modulate inflammation in other chronic bacterial infections. Our findings also underscore a potential risk of vaccination, such as with *S.* Typhi (Salerno-Goncalves

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et al., 2017), which might activate MAIT cells and predispose an individual to subsequent immunopathology.

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MATERIALS AND METHODS

Human gastric tissue and immunofluorescence staining. Human gastric tissue, normally discarded following sleeve gastrectomy bariatric surgery was obtained through The Avenue Hospital, Melbourne with written consent from patients, as per Ethics Approval #1442531 from The University of Melbourne and #187 from The Avenue Human Research Ethics Committees. All patients were female, between 27-57 years of age. Gastric mucosa was dissected from underlying tissue, rinsed with ice-cold HBSS, snap frozen in OCT and sectioned (8 μm) using a Leica CM3050 S cryostat. Acetone-fixed tissue was blocked with Serum-Free Protein Block (DAKO, Denmark) for 30 min and/or 10% goat serum (Sigma, Missouri, USA) at RT, prior to detection with primary antibodies targeting CD3 (CD3-12, Bio-Rad), TRAV1-2 (3C10, Biolegend, CA, USA) and *H. pylori* rabbit polyclonal (DAKO) diluted in 2.5% normal goat serum, followed by Alexa Fluor 647-Goat anti Rat, Alexa Fluor 488-Goat anti Mouse and Alexa Fluor 555-Goat anti Rabbit secondary antibodies, respectively (Life Technologies, CA, USA). Nuclei were counter-stained with Hoechst 33342 (Life Technologies) prior to mounting with ProlongGold (Life Technologies). Slides were analysed using Carl Zeiss LSM700 confocal microscope with a 20x and 100x objectives and Carl Zeiss Zen software. Images were prepared using FIJI/Image J software.

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PBMC and gastric cell preparations and flow cytometry

Tissue was transferred to 30 ml EDTA/BSS + 5% FCS + 1 mM DTT, chopped into 2-5 mm pieces, then incubated shaking at 150 rpm, 15 min, 37 °C. Cells dissociated from tissue were passed through a 70 μm strainer, washed in EDTA/BSS + 5% FCS. Patient PBMCs were prepared

by Ficoll-Plaque density gradient separation from ~20 ml fresh blood. Both gastric and blood cells were frozen in FCS + 10% DMSO and stored in liquid N_2 until use. Upon thawing, cells were washed, rested for 1-2 hours then filtered (70 μm). Cells ($3x10^5$ to $3x10^6$) were incubated in viability stain Live/Dead Aqua (ThermoFisher 1:500) for 30 min, at RT, before staining with Ab to CD3 (OKT3 AF700, eBioscience), CD45 (HI30, BV605, BioLegend), CD161 (HP-3G10, PE-Cy7, BioLegend) and human MR1-5-OP-RU-tetramer (PE), and were fixed with 1% paraformaldehyde before analysis on a BD LSR Fortessa Flow Cytometer (BD Biosciences). Data was analysed with FlowJo software (Treestar, v10).

Activation of Jurkat.MAIT cells and MR1 upregulation. MAIT cell reporter activation and MR1 expression upregulation assays were performed essentially as previously reported (Kjer-Nielsen et al., 2012). Jurkat cells overexpressing the MAIT TCR clone AF-7 (Jurkat.MAIT), were tested for activation by co-incubation with compounds and C1R antigen presenting cells over-expressing MR1 (CIR.MR1) for 16 h. Cells were subsequently stained with PE-Cy7-conjugated anti-CD3 (UCHT1, eBioscience, 1:300), and APC-conjugated anti-CD69 (BD, 1:25) before analysis by on a FACS CantoII (BD) flow cytometer. Activation of Jurkat.MAIT was measured by an increase in surface CD69 expression. In some activation experiments, blocking antibody (prepared in house from hybridoma anti-MR1 mAb 26.5, a gift from Dr. Ted Hansen, Washington University School of Medicine, St. Louis, MO (Chua et al., 2011)) or isotype control 8A5 (prepared in-house) were added (final 10 μg/ml) prior to addition of the test supernatants. For MR1 expression, cells were additionally stained with biotinylated anti-MR1 mAb 26.5, followed by PE-conjugated streptavidin. Control Jurkat.LC13 cells (Kjer-Nielsen et al., 2012) utilizing alpha chain TRAV26-2 and beta chain TRBV7-8 were activated by C1R cells expressing HLA-B8 in the presence of the Epstein-Barr viral peptide FLRGRAYGL (FLR).

Compounds and immunogens. 5-OP-RU was prepared as described previously (Mak et al., 2017). 6-FP was purchased from Schirks Laboratories (place?). TLR2/6 agonist Pam2Cys (Tan et al., 2012) was chemically synthesized and functionally verified. CpG1688 (Sequence: T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T (*phosphorothioate linkage) non-methylated cytosine-guanosine oligonucleotides was purchased from Geneworks (Australia). Poly (I:C) (HMW VacciGrade) was purchased from InvivoGen (USA).

Mice. All C57BL/6 mice, MR1$^{-/-}$ mice, Vα19iCα$^{-/-}$MR1$^{+/+}$ and Vα19iCα$^{-/-}$MR1$^{-/-}$ (all on C57BL/6 genetic background) were bred and housed in the Biological Research Facility of the Peter Doherty Institute for Infection and Immunity, University of Melbourne. MR1$^{-/-}$ mice were derived from Vα19iCα$^{-/-}$MR1$^{-/-}$ mice (Kawachi et al., 2006) obtained from Susan Gilfillan (Washington University in St. Louis School of Medicine, St. Louis, MO). MR1$^{+/-}$, mice were generated by breeding Vα19iCα$^{-/-}$MR1$^{-/-}$ mice with C57BL/6 mice, and inter-crossing of F1 mice. The genotype was determined by tail DNA PCR at the MR1 locus and by analyzing blood for the presence of intact αβ-T cells using antibodies against TCRβ. The phenotype (absence of MAIT cells) of the MR1$^{-/-}$ mouse line was verified by examining MAIT cells in the retired founder breeder mice. Female mice aged between 6-12 weeks were used in all experiments. All procedures on mice were conducted after approval by the University of Melbourne Animal Ethics Committee.

Bacterial culture and infection. *Helicobacter pylori* SS1 (Lee et al., 1997) was cultivated as described previously (McGuckin et al., 2007). Mice were infected orogastrically with *H. pylori* (10^9 cfu) suspended in 100 μL brain heart infusion broth (BHI). *Salmonella enterica* serovar Typhimurium BRD509 (Hoiseth and Stocker, 1981; Newland et al., 1992) and the *S.* Typhimurium BRD509Δ*ribDH* mutant (Chen et al., 2017) have been previously described. For *S.* Typhimurium Δ*rib*DH growth, culture medium was supplemented with 20 μg/ml riboflavin. Mice

were inoculated under isofluorane anaesthesia with *S*. Typhimurium BRD509 (10^9cfu) or Δ*rib*DH (10^7unless otherwise stated) or Ags (1.52 μM 5-OP-RU or 6-FP alone in 50 μl, or in 45 μl plus 5 μl of 20 nmol Pam2Cys) prepared in 50 μl PBS, via the intranasal route.

Preparation of organs and isolation of cells. Single cell suspensions of lungs, blood, mesenteric lymph nodes, and spleen were prepared as described previously (Chen et al., 2017). Gastric immune cells were isolated by perfusion from longitudinally halved stomachs, as described previously (Ng and Sutton, 2015).

Generation of MR1 tetramers. Mouse or human MR1 and β2-Microglobulin proteins were expressed in *E. coli* inclusion bodies, refolded and purified as described previously (Patel et al., 2013). MR1-5-OP-RU labelled with BV-421 and MR1-6-FP (unlabelled) tetramers were generated as described previously (Corbett et al., 2014) and prepared simultaneously.

Antibodies and flow cytometry. Antibodies against CD19 (1D3, PerCP-Cy 5.5), CD3 (UCHT1, PE or 145-2C11, PE-Cy7) CD4 (GK1.5, APC-Cy7), CD45.2 (104, FITC), CD69 (FN50, APC) CD8α (53-6.7, PE), TCRβ (H57-597, APC or FITC) were purchased from BD?. Antibodies against MHC II (M5, AlexaFluor700), PLZF (Mags.21F7, PE), RORγt (B2D, APC) and T-bet (4B10, PE-Cy7) were purchased from eBioscience. To block non-specific staining, cells were incubated with MR1-6-FP tetramer (unlabelled, 1:100) and anti-Fc receptor (2.4G2) for 15 min at RT prior to antibody staining. Cells were then incubated at RT with antibody cocktails including MR1-5-OP-RU tetramer in PBS/2% FCS. Cells were then further incubated with 7-aminoactinomycin D (5 μl/sample) for 10 min in the dark at RT and fixed with 1% paraformaldehyde prior to analysis on LSR Fortessa (BD Bioscience). Data was analysed with Flowjo X 10 software (Treestar). Cell counts were obtained using blank calibration particles (BD

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Pharmingen). Transcription factor staining was performed using a commercial transcription buffer staining set (eBioscience) according to the manufacturer's instructions.

Assessment of gastritis. Longitudinally dissected half stomachs were fixed in 10% neutral buffered formalin, embedded in paraffin; 5 µm sections were stained with H&E and scored by a blinded operator under light microscopy. A pathologist also scored sections independently and the grading system is based on a previously described system (Sutton et al., 2001) with modifications. Briefly, inflammation was assessed in two separate tissue sections for each animal using 3 parameters: (i) neutrophil infiltration and (ii) lymphocyte infiltration: scored from 0 - 4 on the basis of number of cells and whether widespread or multifocal infiltrate was observed, and (iii) atrophy: scored from 0-4 on the basis of percentage loss of chief and parietal cells from the gastric glands.

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PCR amplification and sequencing of TCR repertoire. Single cells were sorted from *S*. Typhimurium BRD509-primed, *H. pylori*-infected C57BL/6 mouse stomachs on a FACSAria flow cytometer. PCR amplification and TCR sequencing was performed as previously described (Reantragoon et al., 2013; Wang et al., 2012).

Statistical analysis. Statistical tests were performed using Prism software (version 6, GraphPad software, La Jolla, CA, USA). Comparisons between groups were performed using one-way ANOVA, Student's t test or Kruskal-Wallis test (histopathology scores)..

AUTHOR CONTRIBUTIONS

CD'S, TP, HW, LK, RE performed experiments, LL, JM, SE, BM provided unique reagents, HC generated and tested the ribDH mutant *S*. Typhimurium. AS conducted histological pathology

assessment, CD'S, AE, AC, ZC, JM conceptualised the study, designed and analysed experiments and wrote the paper, J-PS, RS, JR, DF provided intellectual input and edited the paper.

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FIGURE LEGENDS

Figure 1. *H. pylori* culture supernatant stimulates a MAIT cell line. **A)** *In vitro* activation of Jurkat.MAIT cells by filtered *S.* Typhimurium SL1344 (Salm., grey bars) or *H. pylori* SS1 (black or dotted bars) supernatant (s/n) or media controls (LB or BHI respectively, striped bars) in a co-culture assay with C1R.MR1 cells with or without MR1 blockade (mAb 26.5 or isotype control 8A5). Activation was detected by staining with anti-CD69. **B)** Activation of control Jurkat.LC13 cells cultured with C1R.HLA-B8 cells with *H. pylori* s/n (black bars) or 285nM FLRGRAYGL peptide from Epstein Barr Virus (FLR, grey bar). Data shows MFI (mean ± SEM) of gated Jurkat.MAIT or Jurkat.LC13 cells from three independent experiments. **C)** MR1 expression on

gated C1R.MR1 cells following overnight incubation with *H. pylori* s/n or BHI media. Data shows MF1 minus background (Nil stimulus) (mean ± SEM from three experiments).

Figure 2. MAIT cells are observed in human gastric mucosa in proximity to *H. pylori*. **A)** Immunofluorescence staining of human gastric tissue. Magenta: CD3, Grey: TRAV1-2, Yellow: *H. pylori*, Blue: nuclear stain. 100 x magnification. Blue arrows $CD3^{+}TRAV1\text{-}2^{+}$ cells, Yellow arrow: *H. pylori*. Multiple sections from three patients showed similar results. **B)** Detection of MAIT cells by flow cytometry in PBMCs or single cell suspensions of gastric tissue from patients undergoing sleeve gastrectomy. Gate shows MAIT cells, defined as $CD3^{+}CD45^{+}CD161^{+}MR1\text{-}tetramer^{+}$ live cells. Numbers indicate the MAIT cell percentage of $CD3^{+}CD45^{+}$ live cells.

Figure 3. MAIT TCR transgenic mice show greater pathology following *H. pylori* infection. **A)** Representative plots showing MAIT and non MAIT $CD3^{+}$ cells in $V\alpha19iC\alpha^{+/-}MR1^{+}$ and $V\alpha19iC\alpha^{+/-}MR1^{-/-}$ mice at 12 weeks post-infection with *H. pylori*. **B)** MAIT cells as a percentage of $CD3^{+}$ T cells in $V\alpha19iC\alpha^{+/-}MR1^{+}$ and $V\alpha19iC\alpha^{+/-}MR1^{-/-}$ mice over a time course of 2 to 12 weeks post-infection. The experiment was performed three times with similar results: 5-8 mice per group were examined (mean ± SEM, one-way ANOVA, $^{**}p<0.001$). **C)** Pathology scores of $V\alpha19iC\alpha^{+/-}MR1^{+}$ and $V\alpha19iC\alpha^{+/-}MR1^{-/-}$ mice at 12 weeks post infection. Sections were scored for neutrophilic infiltration, lymphocytic infiltration and atrophy (Kruskal-Wallis test, $^{*}p<0.05$). **D)** Representative photomicrographs of H&E-stained sections of mouse stomachs showing infected $V\alpha19iC\alpha^{+/-}MR1^{+}$ with severe gastritis, uninfected $V\alpha19iC\alpha^{+/-}MR1^{+}$ (normal) and infected $V\alpha19iC\alpha^{+/-}MR1^{-/-}$ (normal architecture).

Figure 4. MAIT cells expand in response to *H. pylori* infection in wt C57BL/6 mice after pre-priming. **A**) Schematic depicting the model used for the experiments. **B)** Percentage distribution of

MAIT cells in different organs 7 weeks after intranasal infection with *S*. Typhimurium BRD509. **C)** Absolute numbers of MAIT cells from the stomachs of wt mice infected with *S*. Typhimurium BRD509, and then challenged (at 7 weeks) with *H. pylori* or left unchallenged over time (one-way ANOVA, *p<0.05, **p<0.001; n=8).

Figure 5. Characterisation of the phenotype of MAIT cells following infection. Analysis of effector memory (CD62L and CD44) **(A)**, and tissue residency (CD103 and CD69) marker expression **(D)** on MAIT cells and non-MAIT T cells isolated from the lungs and stomachs of *S*. Typhimurium-primed mice infected with *H. pylori,* (or left uninfected) at 8 weeks post-infection. **(B)** Transcription factor expression by MAIT cells and non-MAIT T cells isolated from the stomachs of *S*. Typhimurium-primed *H. pylori*-infected mice at 8 weeks post-infection. **C)** $CD4^+$, $CD8^+$ and DN MAIT cells expressed as a percentage of total MAIT cells in the blood and stomachs of *S*. Typhimurium-primed mice, challenged or not with *H. pylori* at 8 weeks post *H. pylori* infection. The experiments were performed three times with similar results. Representative plots are shown.

Figure 6. MAIT cells orchestrate accumulation of other T cells in the gastric mucosa. **A)** Absolute numbers of non-MAIT αβ-T cells from the stomachs of wt and $MR1^{-/-}$ mice infected with *S*. Typhimurium BRD509 (Salm.), and then challenged (at 7 weeks) with with *H. pylori* or left unchallenged over time (one-way ANVOA, *p<0.05, **p<0.001; n=8). Data is from the same experiment shown in Fig. 4c. **B-E)** Absolute numbers of MAIT cells **(B, D)** and non-MAIT αβ-T cells **(C, E)** from stomachs of wt and $MR1^{-/-}$ mice primed with *S*. Typhimurium BRD509 (Salm.) or BRD509Δ*ribDH* (Salm.Δ*ribDH*) plus or minus 5-OP-RU **(B-C)** or Pam2Cys plus either 5-OP-RU or 6-FP **(D-E)**, and then either challenged with *H. pylori* or left unchallenged (data shown is 8

weeks post-*H. pylori* infection) (one-way ANOVA, *p<0.05, **p<0.001). The experiment was performed twice with similar results. Data represent mean ± SEM (n=5-8).

Figure 7. Prior expansion of MAIT cells leads to recruitment of innate immune cells in wt mice and reveals pathological role in *H. pylori* infection. Absolute numbers of neutrophils **(A)**, macrophages **(B)**, eosinophils **(C)**, and dendritic cells **(D)** from stomachs of wt and MR1$^{-/-}$ mice infected with *S*. Typhimurium BRD509 (Salm.) or Pam2Cys plus 5-OP-RU, and then challenged with *H. pylori* (at 7 weeks) or left unchallenged over time (one-way ANOVA, *p<0.05, **p<0.001; n=8). (**E)** Pathology scores of *S*. Typhimurium-primed wt and MR1$^{-/-}$ mice at 8 weeks post *H. pylori* infection. Sections were scored for neutrophilic infiltration, lymphocytic infiltration and atrophy (Kruskal-Wallis test, *p<0.05). Representative scores from one experiment are shown. The experiment was repeated 3 times with similar results.

Supplementary Figure 1. Gating strategy for flow cytometry of PBMCs and gastric cell suspensions. Cells were gated sequenctially from left to right for lymphocytes, single cells, live cells, CD45+CD3+ cells, and finally MAIT cells.

Supplementary Figure 2. Scatter plots showing MAIT cell transcription factor expression from lung or, stomach MAIT cells from primed or primed and challenged mice (each dot represents data from 2-3 pooled mice).

Supplementary Table 1. Table of representative stomach TCR sequences identified from single sorted MAIT cells and multiplex PCR sequencing (n=30). Cells were obtained from stomachs of *S*. Typhimurium BRD509-primed and *H. pylori*-challenged wt mice at 2 weeks post *H. pylori* infection.

Supplementary Figure 3. Representative photomicrographs of H&E-stained sections of mouse stomachs from wt mice immunised with *S*. Typhimurium BRD509 (Salm.) (showing normal mucosa) and primed with *S*. Typhimurium BRD509 (Salm.) and *H. pylori*-challenged wt (severe gastritis) and MR1$^{-/-}$ mice (mild inflammation).

Supplementary Figure 4. Pathology scores of wt and MR1$^{-/-}$ mice primed with *S*. Typhimurium BRD509 (Salm.) or BRD509Δ*ribDH* (Salm.Δ*ribDH*) **(A)** or Pam2Cys and 5-OP-RU or 6-FP **(B)** then challenged with *H. pylori* or left unchallenged at 8 weeks post *H. pylori*-infection. Sections were scored for neutrophilic infiltration, lymphocytic infiltration and atrophy (Kruskal-Wallis test, *$p<0.05$).