Leptin, CD4+ Treg and the prospects for vaccination against H. pylori infection

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INTRODUCTION

H. pylori infection induces both a strong inflammatory CD4+ T cell and CD4+ Treg response

Helicobacter pylori chronically infects the mucus gel layer of the human stomach. This pathogen is highly adapted to the gastric mucosa and the natural immune response does not clear infection (Blaser, 2004). Most cases of H. pylori gastritis are subclinical, and the inflammation leads to symptomatic gastritis in only about 10% of infected persons, the implications of asymptomatic infections remain unknown (Robertson et al., 2003).

H. pylori pathogenicity is dependent on both host and bacterial factors and the mechanisms are complex and remain to be explained clearly (Blaser, 2004). Helicobacter pylori gastritis is characterized by strong infiltrates of CD4+ T cells, Neutrophils and B cells (D’Elios et al., 1997; Stromberg et al., 2003) in the gastric mucosa. When one considers the large number of persons chronically infected with H. pylori worldwide, and that a relatively low proportion of these develop symptoms, it might be seen as circumstantial evidence that this inflammation is under tight regulatory control. Studies in biopsies from infected patients and experimental animals have supported the importance of suppressor or regulatory T cells (Treg) (CD4+/CD25+/FoxP3+) in H. pylori pathogenesis. Indeed relatively high numbers of H. pylori-specific Treg are present in the stomachs of asymptomatic H. pylori-infected patients (Lundgren et al., 2003; Goll et al., 2007) and that these suppressed memory T cell function in vitro. Similarly, studies in experimentally infected mice showed that Treg suppressed proliferation of CD4+ T cells, and that this probably contributes to the persistence of the pathogen (Raghavan and Holmgren, 2005; Rad et al., 2006).

In addition, these CD4+ Treg have been shown to be specific for H. pylori antigens in humans (D’Elios et al., 1997) and mice (Raghavan et al., 2004). Further, Enarsson et al. (2006) reported antigen-specific functional Treg in the mucosa of patients with gastric tumors, it is noteworthy that greater numbers of Treg were detected in tumors as opposed to non-tumor tissue. In this circumstance, Treg may suppress natural protective anti-tumor mechanisms.

It is likely therefore that in most infected persons, H. pylori gastritis is tightly controlled by a complex balance of pro-inflammatory and suppressive T cells, and only when this balance is shifted by other factors do overtly pathogenic mechanisms move into action. The impact of these mechanisms is evident in the prevalence and clinical significance of gastric and duodenal ulcer disease, and gastric cancer. Understanding the factors influencing Treg function in the stomach will be one of the keys to progress in therapy and vaccination against H. pylori.

GASTRIC Treg AS AN OBSTACLE TO VACCINATION

The reports of successful immunization in animal models lead to early enthusiasm for a vaccine, and a variety of strategies, both oral and parenteral have been shown to be effective at reducing, but in general not completely clearing colonization (e.g., Neschcher et al., 2008b; Müller and Solnick, 2011). While there are some reports of sterilizing protection in mice (Kleanthous et al., 2001; Garhart et al., 2002; Panthel et al., 2003b) and in the guinea model (Jeremy et al., 2006), the overwhelming majority of reports report 1–3 logs reduction in colonization, but not clearance (reviewed in Zhang et al., 2003–2006).
Although this model is not entirely straightforward and we have volunteers (Aebischer et al., 2008a). Although the vaccines were one in prophylactically immunized and experimentally challenged in severe gastritis. Treg may not be a hindrance to prophylactic success this regulation, without tipping the balance toward activation. A successful therapeutic vaccination strategy would need to overcome this regulation, without tipping the balance toward activation. As to whether vaccination is even feasible, and indeed the obstacles are now a major focus. Questions have also been raised about the possibility of increasing antibiotic resistance (particularly to metronidazole and clarithromycin; Graham et al., 2007). Vaccination experiments in knockout mice showed that in the absence of functional CD4+ T cells, vaccination was not protective (Erkkola et al., 1998; Pappo et al., 1999). In addition, adoptive transfer of T cells (CD4+ T cells from vaccinated mice conferred protection in H. pylori-challenged recipients (Mohammadi et al., 1997; Lucas et al., 2001). This model offers the best possibility to determine the role for Treg sub-populations in vivo. With the importance of Treg in H. pylori gastritis in mind, and the potential obstacles for vaccination, there is regrettable almost no information from the published human vaccine trials to date on the impact of vaccination on Treg. That Treg may not interfere with prophylactic vaccination was supported by the report of Aebischer et al. (2008a), where FoxP3 positive cells were only detected in the mucosa in H. pylori-challenged volunteers after 3 months. There was however no significant effect of vaccination, at least up to 3 months when the study ended (Aebischer et al., 2008a, supplementary information).

Studies in animal models show significant populations of Treg in H. pylori-infected mice (Ragavan et al., 2004), including in the stomach (Becher et al., 2010). Other studies showed that depletion of Treg led to worsened gastritis (Kaparakis et al., 2006). Depleting Treg would be expected to improve vaccine efficacy by permitting increased pro-inflammatory responses, and this was reported by Zhang et al. (2008) who employed antigen-pulsed DCs for vaccination. There are no other published reports of this phenomenon. There remain no published data from vaccination studies employing the mouse strain developed by Rudinsky and colleagues (Kim et al., 2007) where depletion of Treg can be selectively induced by injection of diphtheria toxin. This model offers the best possibility to determine the role for Treg in vivo.

It should, however, be noted that alongside the reports of exacerbated gastritis in vaccinated mice, there are also numerous reports of effective vaccination (1–3 logs reductions in colonization) in other strains of mice, e.g., BALB/c, where gastritis minimal, or undetected by traditional methods (Gómez-Duarte et al., 1998; Lucas et al., 2001; Pantel et al., 2003a). This implies that gastritis per se is not the only requirement for a protective response and that is the first suggestion that small protective sub-populations are present. We have investigated CD4+ and CD4− Treg function in vaccinated C57BL/6 mice and compared gastric infiltrates to those in the circulation, spleen and lymph nodes. We found not only increased numbers of CD4 in the stomachs of vaccinated mice (as has been reported by others) but also decreased proportions of the Treg sub-population of CD4+ cells in the stomach. These data imply that vaccination reduced suppression of CD4+ T cell numbers and function, and predicts that these cells will have a higher proliferative capacity. This was indeed observed in in vitro studies (Becher et al., 2010).

The lack of requirement of antibody (Erkkola et al., 1998) and many inflammatory cytokines including IFNγ, for vaccine mediated reductions in colonization, was unexpected in light of the strong Th1 response induced both infection and vaccination (reviewed in Aebischer et al., 2008b). Gene expression studies were an alternative attempt to identify new pathways and mechanisms involved in protection (Mueller et al., 2003a; Rahn et al., 2004; Walduck et al., 2004).

One of the novel signature genes identified in these studies was adipokines—a family of genes associated with fat cells (Mueller et al., 2003a; Walduck et al., 2004). Adipokines are defined as cytokines secreted by adipose tissue, many of which have endocrine activity. This group includes leptin, chemein, resistin which have pro-inflammatory activity, and adiponectin which has
anti-inflammatory activity (La Cava and Matarese, 2004; Goralski et al., 2007; Wilk et al., 2011).

A ROLE FOR LEPTIN IN REGULATING INFLAMMATION

Leptin (Ob – the obese gene) is an adipocytokine that links nutrition with immunity. Adipokines are produced by mature adipocytes and other cell types (e.g., T cells, Treg, epithelial cells; Siegmund et al., 2002). Leptin (also known as Ob, the obese gene) is the best-studied adipokine to date, and acts as a hormone that regulates the perception of hunger, and therefore food and energy intake (Fantuzzi, 2005). Leptin has since also been implied in cytoprotection (Konturek et al., 2001), bone mass control (Elefteriou et al., 2004), and immune functions (Lord et al., 1998; Fantuzzi et al., 2005), including the effect of nutritional status on immune dysfunction (Papathanassoglou et al., 2006).

The leptin receptor is a single transmembrane protein that is similar in structure to the gpl30 family of receptors. Binding of the 16-kDa leptin protein triggers the receptor to form homodimers and induces signaling principally via the JAK/STAT pathway (La Cava and Matarese, 2004; Figure 1).

Ob-Res binds leptin but has no intracellular domain and although there is a report that it can initiate signaling, is thought to act chiefly as a method of storing leptin and as such prevents signaling and transport of leptin (Tu et al., 2010). Leptin receptor-signaling deficient mice are obese, and diabetic and display a marked increase in the number and suppressive function of Treg cells (Taleb et al., 2007). Leptin can also directly regulate Treg function by acting as a strong inhibitor of proliferation (De Rosa et al., 2007).

Leptin also acts as a chemo-attractant for neutrophils and macrophages (Montecuccio et al., 2006; Gruen et al., 2007), these cells are also present in high proportions in vaccinated stomachs (Becher et al., 2010). Both macrophages and neutrophils are thought to migrate in response to local leptin gradients in tissues.
Interestingly, in vitro stimulation via the leptin receptor induced migration, but not activation of neutrophils and rendered them unresponsive to "classical chemotaxins" such as IL-8 (Montecucco et al., 2006). Conversely, leptin stimulation induced migration and activation (caused Ca^{2+} influx), but did not interfere with the ability of a macrophage cell line to respond to MCP-1 (Gruen et al., 2007).

**LEPTIN RECEPTOR SIGNALING IS REQUIRED FOR VACCINE-INDUCED PROTECTION**

As part of the follow-up from gene expression studies, we tested prophylactic vaccination in leptin receptor signaling deficient C57BLKS Cg-Leprdb/db mice, and found that they were not protected by prophylactic vaccination (Wehrens et al., 2008). This was in spite of developing (mild) gastritis and H. pylori-specific antibody. While the latter does not necessarily suggest a protective response, it does indicate that the db/db mouse is immunocompetent. We hypothesized that leptin signaling was required, not for mounting an H. pylori-specific response, but rather for transmitting the protective "message" from the mucosa to the gastric epithelium.

Taken together with the evidence from the literature on the influence of leptin signaling on Treg, macrophages, and neutrophils, it seems feasible that leptin signaling may also provide a link between CD4^+ function and protection in wild-type mice.

Based on the literature, and results of our own experimental data discussed below, we proposed that leptin plays a key role in signaling between protective T cells and gastric epithelium.

Hypotheses:
- That strong gastritis is not required for a protective immune response in vaccination — a small sub-population with a "protective" phenotype is sufficient.
- That leptin signaling regulates CD4^+ cell function by interfering with the function of Treg and inflammatory cells in the stomach.

The above hypotheses lead to the following predictions:

**Successful vaccination will be observed in mouse strains/models where gastritis is mild/undetectable.**

BALB/c mice develop weak, or delayed gastritis after *H. pylori* infection (Lee et al., 1997; Pantel et al., 2003). After vaccination, BALB/c mice developed (although mild) gastritis and are protected to similar level as strains that develop strong gastritis such as C57BL/6 (Gómez-Duarte et al., 1998; Koesling et al., 2001; Pantel et al., 2003; Walduck et al., 2004; Sutton et al., 2007). The reduced gastritis observed in BALB/c mice might be expected to be due to the Th2 bias and increased proportion of Treg found in this strain (Sacks and Andreason, 2004), however, Kaperszus et al. (2006) reported that depletion of Treg using anti-CD25 antibody did not affect the intensity of gastritis, but the cytokine secretion pattern. The use of anti-CD25 antibodies could also have depleted other activated T cell populations and so the approach using specific in vivo deletion of Treg discussed above will be required to clarify this question.

It is interesting to note that while CBA mice, which also develop only mild gastritis, were protected by prophylactic vaccination, therapeutic vaccination was ineffective (Sutton and Wilson, 2004).

It would appear that additional genetic factors have a role to play in gastric inflammation.

If a small sub-population is responsible for protection, then a distinct phenotype of the protective will be recognizable, and small numbers will be required for protection.

The microarray studies that display a specific gene expression signature in protected mouse stomachs are indirectly suggestive of this protective phenotype (Mueller et al., 2003b; Rahn et al., 2004; Walduck et al., 2004). Further, analysis of gene expression patterns in biopsies from vaccinated human volunteers also suggested a specific expression pattern and "T cell footprint" (Aebischer et al., 2008a).

Analysis of gene expression patterns in lymphocyte sub-populations isolated from gastric tissue of vaccinated and control mice will provide more specific information on the "protective program" induced in these cells.

Adaptive transfer studies where splenocytes or CD4^+ cells from vaccinated mice were transferred to recipients provided confirmation for the importance of these cells for protection, relatively small numbers of cells are required to achieve this effect (in the order of 5 × 10^4). These adaptively transferred cells do migrate to the gastric mucosa and are detectable in small numbers (Aebischer et al., 2008b; Becher and Walduck, unpublished observations).

That vaccination will be ineffective in the absence of leptin.

As discussed above, we reported the requirement for functional LepR signaling for protection in 2008 (Wehrens et al., 2008). The result was observed using two different prophylactic vaccination strategies. We have since also shown this in db/db mice backcrossed onto a C57BL/6 background (Becher et al., in preparation).

If our previous observations hold, then leptin will impact CD4^+ T cells and Treg proliferation and function.

The first suggestion for this comes from the analysis of gene expression patterns in the stomachs of vaccinated and non-protected db/db mice. For the most part, we observed the same genes were expressed in protected wild-type as obese mice, but interestingly, genes were more strongly up- or downregulated in obese db/db mice (Wehrens et al., 2008). This is suggestive of a lack of appropriate regulation or control of the inflammatory response in the absence of leptin signaling. Our observation is also in keeping with the reported role for LepR signaling in Treg function. De Rosa et al. (2007) proposed that Treg control their own function in an autocrine fashion, by expressing both leptin and leptin receptor.

Based on the results of our previous descriptive studies on local gastric responses (Becher et al., 2010), we predicted that the role for leptin would be in a local gastric signaling network. Consistent with this proposal, we have detected both gastric epithelial cells and infiltrating lymphocytes express leptin receptor in *H. pylori*-infected mice (Figure 2).

As a result of studies in models of both autoimmunity and infection, leptin is now accepted to play an important role in regulating the Th1/Th2/Treg balance (Procaccini et al., 2012), and in this manner to regulate disease. A number of recent publications point to the importance of leptin in Treg-associated disease which may well be applicable to the *H. pylori* system. Treg play an important role in the complex inflammatory response in atherosclerosis (Gotsman et al., 2007). Huertas et al. (2012)
Walduck and Becher Leptin, CD4+ Treg and vaccination

FIGURE 2 | Both leptin and leptin receptor are expressed locally in the gastric mucosa. Immunohistochemistry using specific antibodies, detected with a Cy2 labeled secondary antibody. Both leptin (A) and leptin receptor (B) are expressed in the gastric musosa of vaccinated mice at day 21 post-challenge with H. pylori SS1. Mice were vaccinated with recombinant S. typhimurium expressing H. pylori urease as previously described (Becher et al., 2010). Original magnification ×20.

reported that dysfunctional endothelial cells from idiopathic pulmonary arterial hypertension (IPAH) patients produced increased levels of leptin, and a higher proportion of their Treg expressed leptin receptor. The authors hypothesize that leptin disregulates Treg and contributes to disease (Huertas et al., 2012). In addition, studies in mouse models of atherosclerosis revealed a critical role for leptin in the alteration of the regulatory immune response. Defective Lepr signaling improved Treg function and led to dramatic decrease in lesion size (Taleb et al., 2007).

Given that leptin is ubiquitous in normal serum, in vitro studies on CD4+ T cell and Treg function will not have addressed the situation of limiting leptin concentration, which has also been raised by other authors (De Rosa et al., 2007). Available leptin concentrations in the tissue may well be lower than in standard tissue culture media. We are currently addressing this issue by examining the proliferative and suppressive ability of H. pylori-specific Treg under leptin-limiting conditions.

Because commercially available antibodies detect all receptor isoforms, there is also little information on the expression of the various isoforms of on immune cell populations. We speculate that leptins’ influence on Treg, CD4 and neutrophil function is orchestrated by expression of different receptor isoforms. Our preliminary analyses using an RT-PCR approach on sorted cell populations have shown that at least three different isoforms of Lepr are expressed by CD4+ cells alone (Figure 3). We anticipate that further studies will reveal this repertoire may also be tissue-specific.

It remains to be demonstrated whether leptin receptor expression at the level of lymphocytes (CD4+ T cells or Treg), or indeed the gastric epithelium are most important in mediating the protective effects of vaccination. It is conceivable that leptin secreted by T cells may signal via LepR on gastric epithelial cells, and therefore mediate a protective response (Figure 4). Alternatively, LepR signaling on CD4+ T cells and/or Treg may regulate inflammation and therefore the protective response. We are currently investigating these possibilities (Becher et al., in preparation). If these interactions are shown to play a significant role, it will be interesting to determine the extent to which they interact with the previously
demonstrated role for IL-17 in protection and inflammation in *H. pylori* infection (Velin et al., 2009; Flach et al., 2011).

Our current studies are addressing a series of questions which we believe will provide a “road map” for testing the hypothesis:

- Are the effects of leptin signaling mediated by direct action on epithelial cells, or immune (bone marrow-derived) cells the gastric mucosa?
- Does local leptin production can affect T cell and Treg function in the mucosa? Are the effects on these cells direct or indirect? Or is systemically produced leptin required?
- And finally, and more speculatively is there a relationship between the effects of IL-17 and leptin in the stomach? It is possible that these mediators are part of the same local “program,” or that their effects are specific and sequential.

In conclusion, we have provided evidence for the proposal that leptin has an important role in regulating Treg and CD4+ T cell function in the gastric tissue. More detailed studies testing these hypotheses are underway and will ultimately clarify this. In the interim, we believe at least that this adipokine should be considered as an important player in the control of gastric inflammation.

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**USE OF EXPERIMENTAL ANIMALS**

All studies were performed with the approval of the University of Melbourne Animal Ethical and Experimentation Committee, in accordance with NHMRC guidelines.

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Walduck and Becher Leptin, CD4\textsuperscript{+} Treg and vaccination

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