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# Macrophages and neutrophils as major source of B-cells activating factors in *H. pylori* associated diseases

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# Abbreviations

| | |
|---|---|
| ABC | Activated B cells |
| AGS | Human Stomach Adenocarcinoma cells line |
| AIG | Autoimmune Gastritis |
| APC | Antigen Presenting Cells |
| APRIL | A Proliferation Inducing Ligand |
| ASPP2 | Apoptosis Stimulating Protein of p53 |
| AU | Arbitrary Unit |
| BAFF-R | B Cell Activating Factor of TNF Family Receptor |
| BCL | B Cell Lymphoma |
| BCMA | B cell maturation associates with TNF receptor |
| BCR | B cell receptor |
| BHI | Brain Heart Infusion |
| BLyS | B Lymphocyte Stimulator |
| Bp | Base Pairs |
| CagA | Cytotoxin Associated Gene A |
| CagPAI | Cytotoxin Associated Gene Phatogenicity Island |
| CD | Cluster of differentiation |
| CD | Chron disease |
| cHL | Classical Hodgkin Lymphoma |
| CIA | Collagen Induced Arthritis |
| CLL | Centrocyte Like Lymphoma |
| DAB | Diaminobenzidine |
| DC | Dendritic cell |
| DC-SIGN | Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-Integrin |
| DEPC | Diethylpyrocarbonate |
| DLBCL | Diffuse Large B Cell Lymphoma |
| EAE | Experimental Autoimmune Encephalomyelitis |
| EAIG | Experimental Autoimmune Gastritis |
| ELISA | Enzyme linked Immunoadsorbent Assay |

| | |
|---|---|
| FCS | Foetal Calf Serum |
| FL | Follicular Lymphoma |
| GCBL | Germinal Centre B cellLympohma |
| G-CSF | Granulocyte Colony Stimulating Factor |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| HL | Hodgkin Lymphoma |
| HLA | Human Leukocyte Antigen |
| HP | *H. pylori* |
| HPF | high power field |
| HP-NAP | *H. Pylori* Neutrophil Activating Protein |
| HSPG | Heparen Sulfate Proteoglycan |
| IARC | International Agency for Resench on Cancer |
| IHC | Immuno Histocompatibility Complex |
| IL | Interleukin |
| INF | Interferon |
| IPSID | Immuno Proliferative Small Intestine Disease |
| kDa | kDalton |
| KO | Knock Out |
| LPS | Lipolysaccharide |
| MALT | Mucosa Associated Lymphoid Tissue |
| MCL | Mantle Cell Lymphoma |
| M-CSF | Macrophage Colony Stimulating Factor |
| mRNA | messenger RNA |
| NHL | Non Hodgkin Lymphoma |
| NLPHL | Nodular Lymphocyte-Predominant HL |
| NSAID | Non Steroidal Anti-inflammatory Drugs |
| PBMC | Peripheral Blood Mononuclear Cells |
| PCR | Polymerase Chain Reaction |
| PMBL | Primary Mediastinal B-cells Lymphoma |
| RA | Rheumatoid Arthritis |
| RGD | Arginine-Glycine-Aspartate motif |
| ROR | Retinoic Orphan Receptor |

| | |
|---|---|
| ROS | Reactive Oxigen Species |
| SD | Standard Deviation |
| shRNA | short harpin RNA |
| STAT | Signal Trasducers and Activators of Transcripiton |
| T4SS | Type IV Secretion System |
| TACI | Transmembrane Activator and CALM Interactor |
| TCR | T Cell receptor |
| TGF | Trasforming Growth Factor |
| Th | T helper |
| TNF | Tumour Necrosis Factor |
| Treg | T regulatory |
| UreA | Urease subunit A |
| UreB | Urease subunit B |
| VacA | Vacuolating Cytotoxin A |
| WHO | World Health Organization |
| WI | Wotherspoon Index |

# Riassunto dell'attività svolta

La citochina APRIL (*A proliferation inducing ligand*) e la citochina BLyS (*B lymphocyte simulator*) sono due fattori, scoperti recentemente, facenti parte della famiglia dei TNF (*tumour necrosis factor*). Dalla loro scoperta entrambe queste citochine sono state intensivamente studiate per il loro ruolo nello sviluppo dei linfociti B. Infatti sia APRIL che BLyS sono coinvolte nella maturazione delle cellule B, ma è stato anche dimostrato che ne aumentano la sopravvivenza e la proliferazione. L'effetto di queste citochine risulta però amplificato su cellule B neoplastiche, che caratterizzano i linfomi, e sulle cellule B auto-reattive, che giocano un ruolo cruciale nelle patologie autoimmuni. In particolare, è stato dimostrato che APRIL risulta sovraespressa specificamente in diversi tipi di linfoma, mentre BLyS è molto abbondante nel siero e nei tessuti di pazienti affetti da diversi tipi di patologie autoimmuni, come ad esempio, l'artrite reumatoide, o il lupus eritematoso sistemico.

Nel nostro studio abbiamo valutato il ruolo di queste citochine in due patologie causate dal batterio *Helicobacter pylori* (*H. pylori*): i linfomi tipo MALT (I) e la gastrite autoimmune (II).

(I) L'infiammazione causata da *H. pylori* è caratterizzata da una massiva infiltrazione di cellule, che però non riescono ad contrastare efficacemente la crescita batterica. Questo porta molto facilmente alla cronicizzazione dell'infezione che porta allo sviluppo di follicoli linfatici con centri germinativi. Questo follicoli costituiscono una sorta di organo linfoide associato alla mucosa gastrica detto MALT (tessuto linfatico mucosa-associato). L'acquisizione del MALT rappresenta una condizione pre-neoplastica associata all'infezione da *H. pylori*, che può evolvere verso un linfoma a cellule B. Attualmente non è noto quali citochine o fattori solubili promuovano l'attivazione delle cellule B e la genesi del linfoma a seguito dell'infezione da *H. pylori*. Nel nostro studio, abbiamo dimostrato che nel linfoma gastrico di tipo MALT sono espressi alti livelli di APRIL. Inoltre, APRIL risulta sovraepresso anche in una situazione pre-MALT, suggerendo che questa citochina possa essere coinvolta anche nell'induzione, oltre che nel mantenimento del linfoma. Un'analisi approfondita ci ha in seguito permesso di identificare la popolazione macrofagica come fonte principale di APRIL in questo tipo di tumore. Abbiamo poi confermato con esperimenti *in vitro*

che *H. pylori* è in grado di indurre la secrezione di APRIL da parte dei macrofagi. Abbiamo infine dimostrato che anche le cellule T, specifiche per *H. pylori*, possono indurre i macrofagi a secernere APRIL.

I nostri dati rappresentano la prima evidenza di un coinvolgimento della citochina APRIL nello sviluppo del linfoma MALT in pazienti con infezione da *H. pylori* e identificano nei macrofagi le cellule chiave nel processo.

(II) Tra le malattie che conseguono all'infezione da *H. pylori* c'è la gastrite autoimmune. E' stato recentemente dimostrato il ruolo determinante della citochina BLyS in due differenti modelli di malattia autoimmune; BLyS infatti indurrebbe i linfociti T *naive* a differenziare verso il fenotipo Th17. Queste cellule T effettrici, producono IL-17 e giocano un ruolo cruciale nella genesi e nel mantenimento di numerose patologie autoimmuni. Con queste premesse, abbiamo voluto verificare se nella gastrite autoimmune da *H. pylori* fosse coinvolto l'asse BLyS/Th17, anche alla luce del fatto che era già noto che l'infezione da *H. pylori* si associa ad una risposta Th17. Per valutare questa possibilità abbiamo utilizzato come modello di studio la gastrite cronica indotta da *H. pylori*, in quanto essa può costituire il contesto da cui evolve la gastrite autoimmune. Abbiamo dimostrato che pazienti con gastrite cronica non solo presentano, come atteso, una mucosa riccamente infiltrata di linfociti Th17, ma quest'ultima è anche caratterizzata da una significativa espressione della citochina BLyS. Degno di nota il fatto che l'eradicazione del batterio si accompagna ad una riduzione di entrambe le citochine, BLyS e IL-17, nella mucosa dei pazienti, a sottolineare il ruolo determinante del batterio nel sostenere l'asse BLyS/Th17. Inoltre, abbiamo dimostrato che BLyS è in grado attivare *in vitro* monociti e macrofagi e di indurre la secrezione da parte di queste cellule di IL-1$\beta$, IL-6, TGF-$\beta$ and Il23, tutte citochine coinvolte nel differenziamento delle cellule Th17. In aggiunta abbiamo identificato i neutrofili come potenziale fonte di BLyS nella mucosa gastrica. Infatti, neutrofili stimolati *in vitro* con *H. pylori* secernono grandi quantitativi di BLyS ed inoltre sono dimostrati essere la maggior componente dell'infiltrato cellulare nell'infezione di *H. pylori*. Quindi, abbiamo ipotizzato un modello di sviluppo della gastrite autoimmune a seguito dell'infezione di *H. pylori*: il batterio stimolerebbe i neutrofili infiltranti la mucosa gastrtica a rilasciare BLyS. BLyS, potrebbe da un lato indurre la sopravvivenza delle cellule B autoreattive (con relativa produzione di auto-anticorpi), ma anche agire

sui monociti/macrofagi (anch'essi abbondanti nell'infilatrato infiammatorio) inducendo la secrezione di citochine che portano al differenziamento delle cellule Th17. Il profilo pro-infiammatorio delle cellule Th17, potrebbe aumentare l'infiammazione e peggiorare il danno tissutale tipicamente riscontrato nei pazienti con gastrite autoimmune. Tuttavia, questo studio è tuttora in corso d'opera e molte cose restano da capire per validare questo modello. Bisogna innanzitutto identificare le cellule esprimenti BLyS nella gastrite cronica; a questo scopo,diverse prove di co-immunoistochimica con marcatori specifici per i diversi tipi di cellule del sistema immunitario sono attualmente in corso. Inoltre, i risultati delle immunoistochimiche saranno corroborati con esperimenti *in vitro* mirati a: i) confermare la capacità delle cellule, identificate per immunoistochimica, di secernere BLyS a seguito di stimolazione con *H. pylori*; ii) confermare il ruolo di BLyS nel differenziamento delle cellule Th17.

Ancorché questo secondo studio sia attualmente in corso, riteniamo plausibile proporre l'idea generale che *H. pylori* possa creare un microambiente citochinico favorevole allo sviluppo sia del linfoma tipo MALT che della gastrite autoimmune. In particolare, abbiamo dimostrato che questo batterio è in grado di indurre la produzione ed il rilascio di APRIL e BLyS da parte di macrofagi e neutrofili rispettivamente, in due patologie caratterizzate da una disfunzione delle cellule B. Questi dati potrebbero suggerire che terapie mirate verso queste citochine potrebbero diventare un nuovo approccio terapeutico per la cura del MALT linfoma e della gastrite autoimmune.

# Summary

The cytokine APRIL (A proliferation inducing ligand) and the cytokine BLyS (B lymphocyte simulator) are two recently discovered factors that belong to the TNF- (tumor necrosis factor) family. Since their discovery, these cytokines have been intensively studied for their role in B cells biology. Indeed, APRIL and BLyS are involved in the maturation of B cells, but, most importantly, they increase the survival and proliferation of these cells. However, the effect of these cytokines is amplified in neoplastic B cells in lymphomas, and self-reactive B cells in autoimmune diseases. In particular, it has been demonstrated that APRIL is over-expressed in different types of lymphoma, whereas BLyS is very abundant in serum and tissues of patients with different types of autoimmune diseases, such as, rheumatoid arthritis or systemic lupus erythematous.

In our study, we evaluated the role of these cytokines in two diseases caused by the bacterium *Helicobacter pylori* (*H. pylori*): MALT lymphoma (I) and autoimmune gastritis (AIG) (II).

(I) the development of lymphoid follicles with germinal centers and an acquired MALT (mucosa-associated lymphoid tissue) are characteristic of *H. pylori*-induced gastritis. These MALT represents a pre-neoplastic condition associated with infection, which can evolve into a B-cell lymphoma. It is currently unclear which cytokines or soluble factors promote B cell activation and the genesis of lymphoma during *H. pylori* infection. In our study, we have demonstrated that gastric MALT lymphoma is characterized by high expression of APRIL. Furthermore, APRIL is over-expressed also in a pre-MALT situation, suggesting that this cytokine may be involved in the induction, as well as in the proliferation of the lymphoma. Further analysis allowed us to identify the macrophages as the main source of APRIL in this type of cancer. We confirmed this observation by *in vitro* experiments, suggesting that *H. pylori* is able to induce the secretion of APRIL by macrophages. We have also shown that the contribution to APRIL secretion is supported also by tumour infiltrating T cells specific for *H. pylori*. Thus, we have demonstrated that *H. pylori* is able to induce APRIL secretion by macrophage in a direct way, following *in vitro* infection, but even in an undirect way, mediated by *H. pylori*-specific T lymphocytes. Our data represent the first evidence of

the involvement of APRIL in the development of MALT lymphoma in *H. pylori* infected patients. Moreover, we identified macrophages as key cells in this process.

(II) Among the diseases caused by *H. pylori* there is also AIG. It has recently been reported that the cytokine BLyS plays a pivotal role in the pathogenesis of two different models of autoimmune disease (collagen induced arthritis and experimental autoimmun encephalomyelitis); in these works, it has been demonstrated that BLyS induce naive T cells to differentiate into the Th17 phenotype. These effector T cells, produce IL-17 and they play a crucial role in the pathogenesis and maintenance of several autoimmune diseases. Thus, given that it is established that *H. pylori* infection is associated with a Th17 response, we wondered to investigate whether the BLyS/Th17 axis is involved in the development of AIG following *H. pylori*-infection. To test this hypothesis, we have considered *H. pylori*-induced chronic gastritis as a model for our analysis because it is the typical inflammatory context that might evolve AIG in susceptible patients. We have demonstrated that patients with chronic gastritis not only present, as expected, a Th17-enriched cellular infiltrate in gastric mucosa, but also that in these biopses there is a significant expression of BLyS. Noteworthy, after the eradication of the bacterium there is a marked reduction of both BLyS and IL-17 cytokines in gastric mucosa, underlining the crucial role of *H. pylori* in the axis BLyS/Th17. We have also shown that BLyS is able to activate *in vitro* monocytes and macrophages, inducing the secretion of IL-1β, IL-6, TGF-β and IL-23 by these cells: all these cytokines are fundamental for Th17 cells differentiation. In addition, we identified neutrophils as a possible source of BLyS in gastric mucosa. Indeed, *H. pylori*-stimulated neutrophils secrete a great amount of BLyS *in vitro*. Accordingly, there are evidence that neutrophils represent a great percentage of cellular infiltrate during *H. pylori*-sustained inflammation. Therefore, we hypothesized a model for AIG development of following *H. pylori*-infection: the bacterium stimulates gastric mucosa-infiltrating neutrophils to release BLyS. BLyS, in turn could on one hand lead to the survival of autoreactive B cells (characterized by auto-antibodies production), but on the other hand, it might also acts on monocytes/macrophages (which are also abundant in the inflammatory infiltrate) for the induction of pro-Th17 cytokines. The Th17-pro-inflammatory profile may increase inflammation and worsen the tissue damage which is typically found in patients with AIG. However, this study is still in

progress and many things remain to be understood to validate this model. We first of all need to identify BLyS-expressing cells in chronic gastritis; to this aim different double-immunohistochemistry staining with specific markers for different immune cell types are currently underway. In addition, the results of immunohistochemical analysis will be corroborated with *in vitro* experiments aimed to: i) confirm the ability of cells, identified by immunohistochemistry, to secrete BLyS following stimulation with *H. pylori,* ii) confirm the role of BLyS in the differentiation of Th17 cells.

Although this second study is currently ongoing, we have demonstrated that *H. pylori* might create a cytokine microenvironment which can drive the development of MALT lymphoma or AIG. In particular, we have shown that *H. pylori* is able to induce the production and release of BLyS and APRIL by macrophages and neutrophils, respectively, in two diseases characterized by dysfunction of B cells. These data may suggest that targeting these cytokines may be a new additional therapeutic approach for the treatment of AIG and MALT lymphoma.

# Introduction

## 1 *Helicobacter pylori*

*Helicobacter pylori* is a spiral-shaped, flagellated microaerophilic Gram-negative bacterium (fig. 1), that is specialized in the infection of human stomach. Due to the strong acid environment (pH 1-2), the human stomach is inaccessible for almost all bacteria; indeed, since the discovery of *H. pylori*, the human stomach was considered a sterile niche. *H. pylori* is well adapted to survive inside the human stomach and its infection persists lifelong after the first contact: for this reason *H. pylori* is considered one of the most successful human bacterial parasite, that infects more than half of human population[12][13]. Genetic analysis of different strains of *H. pylori* and patients from different ethnical and geographical areas, revealed that there is similar segregation between human's and *H. pylori*'s antigens. This suggests that the bacterium co-evolved with human race since pre-history [14].

*Helicobacter pylori* was firstly described by Giulio Bizzozzero in the end of 19$^{th}$, but it was isolated for the first time in 1982 by the two Australian physicians Barry Marshall and Robin Warren who correlated the infection of human by the bacterium with the pathogenesis of different type of gastric disorders [13]. For this discovery they were awarded with the Noble Prize in medicine in 2005.

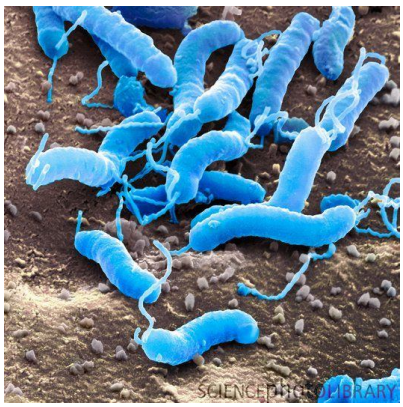


**Figure 1**: *Helicobacter pylori.* Scanning Electron Microscopy. Picture from juergen berger/Science photo library.

Despite the fact that *H. pylori* infection remains silent in the majority of infected subjects, a certain percentage of patients develops chronic, atrophic and eventually autoimmune gastritis, peptic ulcer, MALT (mucosa associated lymphoid tissue)

lymphoma and gastric adenocarcinoma. Given this strong association between *H. pylori* infection and the increased risk of developing gastric malignancies the International Agency for Research on Cancer (IARC) included this pathogen in class 1 category of carcinogens (IARC 1994).

## 1.1 Epidemiology and way of transmission

*H. pylori* causes the most frequent chronic infection in humans. Despite the difference in incidence, *H. pylori* is present worldwide. There are many factors that contribute to favour the infection by this pathogen, first of all the socio-economic condition; accordingly, in developing countries the incidence of infected subjects is more than 90%, compared with industrialized countries where less than 50% of people are infected (Fig.2) [10]. On the other hand, also the virulence of the bacterium as well as the host characteristics (i.e. genetics, acid secretion, age, and lifestyle) affect the diffusion of *H. pylori* [15].


**Figure 2**: Global incidence of *H. pylori.* Image from [10].

Currently, the way of bacterial transmissions is not fully understood. Given that the principal habitat of *H. pylori* is the human stomach, the main way of transmission is probably inter-human: gastro-oral, oro-oral or faecal-oral. *H. pylori* was indeed isolated from dental plaque, saliva, vomit and faeces of infected patients [16]. Anyway, as recently reported, there is also the possibility that *H. pylori* gain access to humans, by contaminated water or food [17].

Without antibiotics treatment, the infection of *H. pylori* persists for the entire life of an individual. In most cases, the infection remains silent, without any significant

symptoms, or with moderate inflammation detectable only by histological analysis. However, about 15-20% of patients develop different type of gastric disorders that will be described in detail below. The development of gastric diseases depends on the host immune response, but also on the bacterial strain or environmental factors.

## 1.2 Colonization of gastric epithelium

*H. pylori* infection generally occurs during childhood and it can persist lifelong without significant symptoms. However, for several, and partially unknown, reasons the bacterium triggers a strong inflammation that becomes the driving force for all the diseases related to *H. pylori*.

Despite the ability of *H. pylori* to tolerate the strong acid environment of the stomach, it does not remain in the lumen for a long period, but it reaches the mucus layer that protects gastric epithelial cells from of the acidity of the stomach lumen.

The helicoidally shape and flagella made *H. pylori* able to cross the mucus layer, swimming inside the viscous film, composed by glycoprotein and phospholipids. Once crossed the mucus layer, *H. pylori* strongly adheres to the apical membrane of gastric epithelial cells. This adhesion is mediated by several adhesins and glycolipids. The adhesins BabA binds to blood group antigens [18], while SabA binds to sialylated protein [19]; moreover, different structural proteins of the Type IV Secretion System (T4SS,see below), participate to bacterial adhesion by binding to integrin-$\beta_1$ receptor on the host cells membrane [20]. Furthermore, several other bacterial factors, for example lectins and glycolipids promote the adhesion of the bacterium to host cells. The binding to host cells is intimate and almost irreversible and causes cytoskeletal reorganization [21]. Some of the rearrangements are induced by bacterial toxins injected into the cells through the T4SS.

All the genes involved in the assembly of the T4SS are located in a 40 kb DNA portion, called *cytotoxin-associated genes Pathogenicity Island* (CagPAI), a horizontally acquired locus that is responsible for the virulence of several strains of *H. pylori [22]*. Indeed, $CagPAI^-$ strains are less virulent and usually cause a very weak or no inflammation; on the contrary, $CagPAI^+$ strains are much more aggressive and are associated with severe inflammation and mucosal damage[22].

## 1.3 *H. pylori* virulence factors

*H. pylori* produces several factors that allow it to survive in the gastric mucosa, to intimately bind to epithelial cells, to resist to phagocytosis and to cause a severe mucosal damage. These factors include: i) urease, essential for colonization because, through the generation of ammonia, it buffers *H. pylori* as it passes through the highly acidic gastric lumen; ii) flagella, which propel bacteria through the gastric mucus; iii) the cag PAI, which encodes a type IV secretion apparatus; iv) VacA, a secreted vacuolating cytotoxin that damages the epithelium and impairs lymphocyte function; v) *H. pylori*-NAP a strong pro-inflammatory and immune modulant protein which drives the Th1 inflammation during infection.

### 1.3.1 Urease

*H. pylori* is able to survive in the stomach, which is characterize by an acid pH, thanks to urease that buffers the pH near the bacterial surface. This enzyme is very important for *H. pylori*: accordingly, almost the 10% of the total bacterial protein amount is represented by urease, a $Ni^{2+}$- containing enzyme, which hydrolyses urea into $NH_3$ and $CO_2$ [23]. Several genes are dedicated to the biosynthesis of the functional urease; in particular, *H. pylori* possesses a seven-genes cluster than encodes for urease and other accessory proteins needed for the hydrolytic activity of this enzyme. Urease is a dodecamer, composed by six subunits of UreA (26,5 kDa each) and six UreB (60,3 kDa) [24]. This enzyme is located in the cytosol of the bacterium: urea enters the cytosol of the bacterium through a proton-gated channel and ammonia generated by the hydrolysis of urea, buffers cytosol and periplasm, but, most importantly, it creates a neutral layer around the bacterial surface (fig. 3). Urease is also released by dead bacteria or by autolysis and it can bind the membrane of living *H. pylori*, where it generates ammonia to increase the buffering of pH [25].

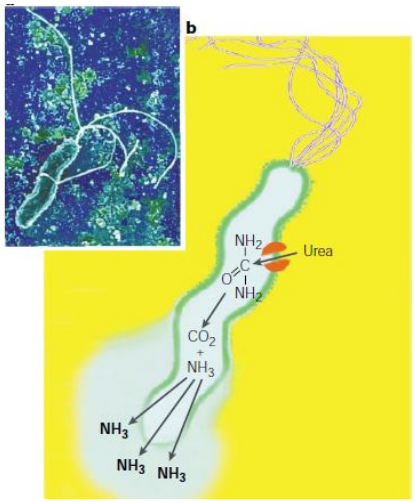


**Figure 3**: Mechanism of pH buffering exerted by *H. pylori* in the stomach. Ammonia-generated by urease buffers the strong acidic pH of gastric lumen. Urea enters in the cytoplasm through a pH-dependent channel that opens at low pH. Image adapted from [9].

This enzyme is crucial for bacterial colonization: accordingly, urease defective mutants are not able to colonize the stomach. Moreover, urease is considered one of the most important virulence factors produced by *H. pylori* since it actively participates to mucosal damage caused by the bacterium. It has been demonstrated that urease recruits and activates neutrophils and monocytes/macrophages in the inflamed tissue where they release pro-inflammatory cytokines and Oxigen Reactive Species (ROS) that, together with ammonia produced by *H. pylori* consistently damage gastric mucosa [26].

Interestingly, the diagnosis for the presence of *H. pylori* is based on the enzymatic activity of urease. Patient ingests $^{13}C$ or $^{14}C$ labelled urea: if *H. pylori* is present in the stomach, labelled urea is hydrolized in ammonia and $CO_2$ : the latter, containing the isotope, can be detected in patients' breath [27].

### 1.3.2 Flagella and Adhesins

*H. pylori* normally possess a unipolar bundle of two to six sheathed flagella that enable the bacteria to move in their ecological niche represented by the mucous layer of the gastric epithelium. Each flagellum is about 3 μm long and exhibits a typical bulb-like structure at its distal end that represents a dilation of the flagellar sheath.

The *H. pylori* flagella are composed of three structural elements like those of enteric bacteria: a basal body, which is embedded in the cell wall and contains the proteins required for rotation and chemotaxis; an external helically shaped filament that works as a propeller when rotated at its base; and a hook that serves as a joint between the basal body and the flagellar filament. The filament is a co-polimer composed by two

proteins FlaA and FlaB. They are both needed for bacterial motility, meaning that they are essential for *H. pylori* colonization [28].

*H. pylori* swims into the mucous layer to avoid the acid environment of the lumen and once it reaches gastric epithelium it strongly adheres to gastric epithelial cells. As already mentioned, the adhesion is mediated by several adhesins, in particular the blood group antigen binding adhesion BabA that binds to Lewis b antigen on epithelial cells. Another adhesin that contributes to the bacterial virulence is SabA, which binds to sialylated-glycoconjugates antigens [18][19] The presence of SabA is related to chronicization of the infection and to gastric atrophy and cancer .

### 1.3.3 The cagPAI and the Type IV secretion system

The 40 kb pathogenicity island (CagPAI) consists of approximately 30 open reading frames (fig. 4). It encodes for different virulence factors some of the encoded proteins assemble to form a molecular syringe by which the bacterium injects the cytotoxin CagA. A functional CagPAI is required for the injection into the epithelial host cells of CagA: the latter represents the only protein identified so far that enters the cytosol of epithelial cells through the T4SS [6].

Cag PAI was probably acquired by horizontal gene transfer from another species or genre, and it is integrated in chromosomal DNA. CagPAI provides *H. pylori* with two relevant properties: it increases the infectiousness and it makes *H. pylori* a pathogenic bacterium [6].

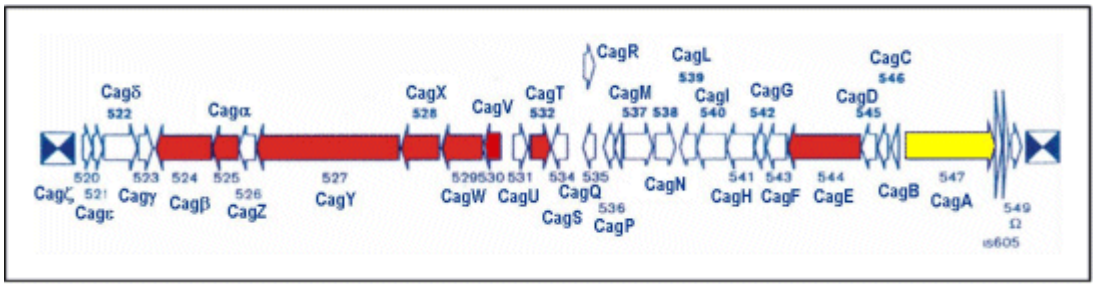


**Figure 4**: The cag pathogenicity island of *H. pylori.* Image adapted from [6].

Indeed, based on the presence or the absence of CagPAI, *H. pylori* can be divided in two groups: type I that are CagPAI$^{+}$, while type two are CagPAI$^{-}$. Type I *H. pylori*, which constitute the majority of *H. pylori* strains, are associated with the most severe outcome of infection, characterized by gastric damage, epithelial vacuolization,

necrosis that lead to ulcer and, eventually, gastric cancer. On the contrary, type II *H. pylori* cause moderate inflammation that is usually asymptomatic. It is believed that the acquisition of cagPAI conferred to *H. pylori* type I an evolutionary advantage, given that the elicited inflammatory state (and the consequent tissue damage) favours the obtainment on nutrients from host's tissue [22].

Among the 30 genes coded by cagPAI, seven are homologs to genes found in other pathogens such as *Bordetella pertussis*, *Agrobacterium tumefaciens*, *Escherichia coli*, *Legionella pneumophila*, *Rickettsia prowazekii* and *Brucella suis*. These genes code for proteins that form a multimeric complex that acts as exportation machinery. This machinery, very similar to the conjugative pilus, is called Type IV secretion system (T4SS) that acts as a molecular syringe by which *H. pylori* transfers CagA (currently the only known protein injected by theT4SS) inside the cytoplasm of host cells (fig. 5) [6]. The assembly of the secretion and the translocation of CagA are powered by three cytoplasmic NTPase, HP0525, HP0524 and HP0544; in particular, HP0524 is a coupling protein that recruits other component to the apparatus such as CagA itself [29].

T4SS binds to host cell throught the interaction between the RGD motif (Arginine-Glycine-Aspartate) on the needle of pilus, and β1 integrin on the surface of gastric epithelial cells, but recent findings suggest that it could bind host's integrins in a RGD-independent fashion . Although T4SS has been intensively studied in *H. pylori* and other bacteria, the exact working model of T4SS is still poorly understood [29].

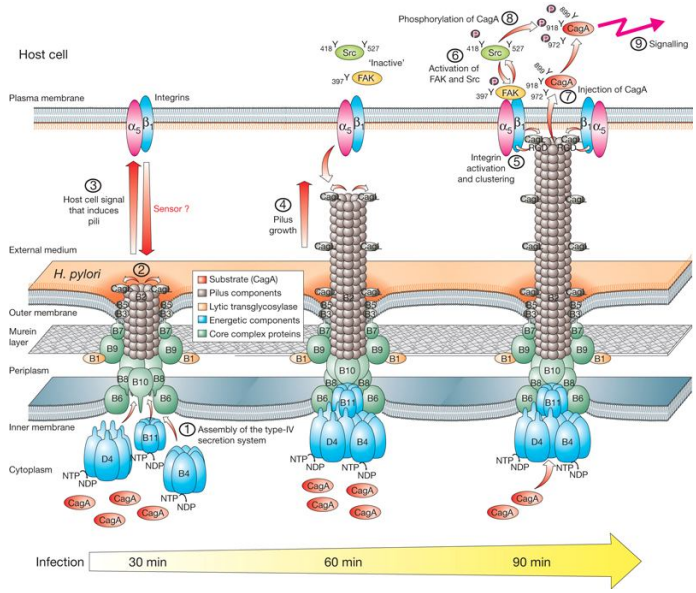


**Figure 5**: Type 4 secretion system of *H.pylori.* The structural components are assembled in the inner membrane and the pilus growths from the cytosol of the bacterium. Image adapted from[4].

### 1.3.4 Cytotoxic associated gene A (CagA)

CagA is produced only by *H. pylori* type I. CagA is a 128 kDa protein that the bacterium injects directly inside the cytoplasm of host cell using the T4SS. After the delivery into host cells, CagA localizes to the plasma membrane where it interacts with some proteins of the apical junctions, such as Jam or ZO-1, β-catenin and E-cadherin [30]. In the host cell cytoplasm CagA is phosphorylated by c-Src/Lyn and Abl kinases; this phosphorylation triggers the receptor tyrosin kinase signalling cascades, which promote loss of cell polarity [31]. Several phenotypic changes are mediated by CagA during infection of type I *H. pylori in vitro*: indeed it has been demonstrated that CagA, by binding to different cytoplasmatic proteins, is able to trigger actin cytoskeleton rearrangement. These rearrangement results in host cell elongation, giving rise to the so-called (fig. 6) [3].

CagA has been shown to be not only sufficient to disrupt the epithelial junctions, but also to profoundly alter the differentiation and behaviour of polarized epithelium.

All the properties and effects induced by CagA inside the cytoplasm of host cells might explain the development of severe gastric pathology in *H. pylori* type I infected patient, but most importantly they may explain the association between CagA and the induction of gastric cancer [32].

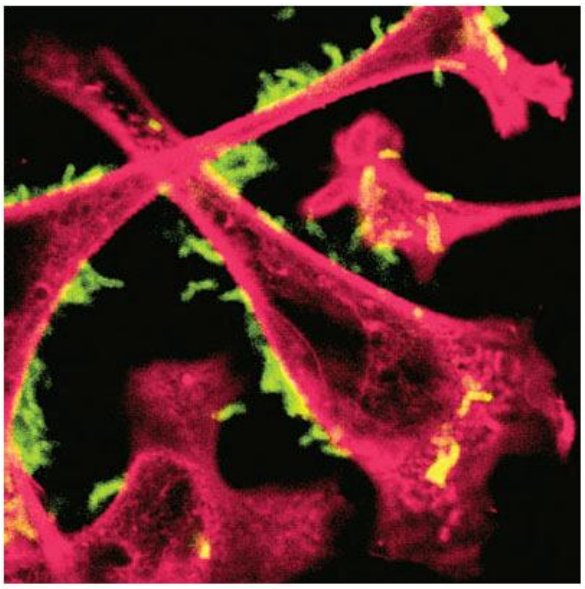

**Figure 6**: The IF image shows *H. pylori*-infected gastric cells with the typical hummingbird phenotype. The green particles identify the bacteria, while the cells are stained in red [3].

Indeed, it has been demonstrated that subjects infected with $CagA^+$ *H. pylori* have a considerably increased risk of developing gastric cancer. $CagA^-$ *H. pylori* are less strongly linked to malignancy [22].

CagA activates the transcription of different proto-oncogenes, such as c-fos and c-jun, two events that represent the first step for gastric carcinogenesis. Moreover, it has been recently demonstrated that CagA subverts the tumour suppressor function of apoptosis-stimulating protein of p53, ASPP2: the latter is a pro-apoptotic protein that activates p53. The interaction between CagA and ASPP2 results in p53 degradation and consequently, resistance to apoptosis. Thus CagA plays an active role in *H. pylori*-mediated gastric carcinogenesis [30].

### 1.3.5 The VacuolatingCytotoxin A (VacA)

The first description of the Vacuolating Cytotoxin (VacA) dates back to 1988 when it was documented the existence of a protein, in the culture supernatant of *H. pylori*, capable of inducing the formation of vacuoles in the cytoplasm of cells in culture. The perinuclear vacuoles firstly appear in perinuclear zone, but then they increase in number and size occupying the entire cell. Vacuolization reflects a cell suffering state that may eventually evolve to necrosis [33] [34].

There are 25 different allelic forms of the VacA gene, which encode a polypeptide of 140 kDa. From the N-terminus of the protein, four domains can be distinguished: i) a signal sequence of 33 amino acids that allows the translocation from the cytosol to the periplasm of the bacterium; this domain is removed by proteases; ii) a region of 37 kDa (p37) formed mainly by α-helices and β sheets with a non-structured loop which undergoes to proteolytic cleavage after protein secretion; iii) A third domain of 58 kDa (p58) enriched in β sheets. Finally, there is a carboxyl-terminal region of 35 kDa, which directs the export of the protein through the outer membrane. Part of the toxin is released into the medium, while 40-60% of mature VacA remains associated with the outer membrane via the carboxy-terminus [35]. The mature toxin is found in culture supernatant as 95 kDa protein that has a strong tendency to form oligomers. In these oligomers the subunits p37 and p58 are clearly distinguishable. Interestingly, after its release in the culture medium, VacA can be further cleaved in the two p37 and p58 kDa domains that remain bound to each other by non- covalent forces [36].

The oligomeric form of the toxin is poorly active, but if it is subjected to a transient pH variation, it is strongly activated. Indeed, the exposure of VacA to a pH between 1.5 and 5.5 determines its disassembly in the individual monomers that represent the

active form of the toxin. Upon activation, VacA become able to insert in phospholipidic membranes, where it assemble an ion-permeable pore [37].

Once VacA is inserted into the plasma membrane of host cells, it is endocytosed and transported to endosomes/lysosomes. The presence of VacA in these organelles increases the membrane permeability to anions, which lead to the stimulation of the proton pump V-ATPase that is responsible for the acidification of these organelles. Thus, if weak bases are present inside the endosome, for example the ammonium generated by *H. pylori* urease, they become protonated and accumulate inside the endosome. These osmotically active acidotropic ions favour the entry of $H_2O$ from the cytosol to the endosomal lumen, leading to the swelling of endosomes which appear as vacuoles (fig. 7) [38][39].

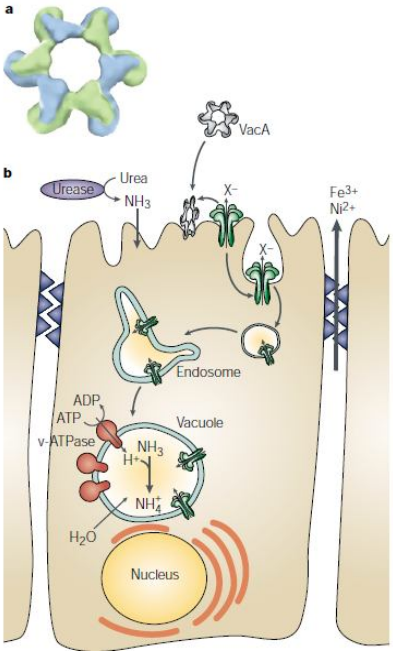


**Figure 7**: VacA induced cytotoxicity.The binding of the toxin on the apical side of gastric cells leads to interanalization. Image adapted from [9].

Interestingly VacA is endowed with both pro- and anti-inflammatory properties. Indeed, VacA activates mast cell and it induces the secretion of pro-inflammatory cytokines such as TNF-$\alpha$, IL-1$\alpha$, IL-1$\beta$, IL-6, IL-10 and IL-13 [40]. On the other hand, it has been demonstrated that VacA inhibits the stimulation of T-cells clones specific for epitopes generated in the antigen-processing compartment, by blocking the proteolytic processes and, as a consequence, antigen presentation by Antigen Presenting Cells (APC). Accordingly, VacA-specific $CD4^+$ T-cells are found at low frequency in gastric mucosa of infected patients [41].

The inhibition of local antigen processing by VacA could be a strategy of survival for *H. pylori*, contributing to chronic infection of the stomach.

### 1.3.6 *H. pylori* Neutrophil Activating Protein (HP-NAP)

This protein is highly conserved among many isolates of *H. pylori*. The structure of HP-NAP is a ball-shaped dodecamer, formed by the assembling of 17 kDa monomers [42].

It is likely that HP-NAP can be released by autolysis and, once in the gastric lumen, it can crosses the epithelium reaching the underlying tissue where it activates mast cells and starts the recruitment of monocytes, neutrophils and lymphocytes, thus triggering and amplifying the inflammation. Indeed, it has been demonstrated that *H. pylori*-NAP plays a crucial role in recruiting and activating neutrophils, in particular it is able to cross endothelium and to directly contact circulating leukocytes, triggering their extravasation. It has been reported that HP-NAP triggers the release of ROS from neutrophils and monocytes in a NADPH oxidase- dependent manner [43].

HP-NAP stimulates monocytes and dendritic cells (DC) to secrete high amount of IL-12p35, IL-12p40 and IL-23p19. According to the fact that these cytokines are crucial in promoting the differentiation of T cells towards the Th1 phenotype, HP-NAP activity on monocytes results in the polarization of adaptive response to *H. pylori* towards the Th1 profile, *in vivo* [44]

### 1.3.7 Lipopolysaccharide (LPS)

*H. pylori* LPS possesses a peculiar structure that made it less active than that of other pathogens, both in terms of biological functions and endotoxic activity. In particular, most of the *H. pylori* strains display on their LPS Lewis blood group, which are very similar to those expressed by gastric epithelial cells [45].

It has been demonstrated in mouse model that the *H. pylori* LPS contributes to the induction of the Th1 profile [45].

More in details, based on the LPS structure two different populations of infecting *H. pylori* strains can be distinguished: one population blocks the Th1 differentiation, by binding to lectine DC-SIGN (Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-Integrin) on DC; the other population, that is not able to bind DC-SIGN, induces the differentiation of T-helper cells into the Th1 phenotype. These two co-

existing variants could promote a peculiar balance between Th1 and Th2 that may facilitate the persistence of *H. pylori* colonization.

During the chronic infection by *H. pylori*, the molecular mimicry between bacterial LPS and human Lewis blood antigens can trigger the production of autoantibodies that cross-react with the gastric proton pump in genetic susceptible patients. These self-reactive antibodies may contribute to the mucosal destruction and exacerbate the gastric inflammation [46].

## 1.4 *H. pylori*-associated inflammation

During infection, *H. pylori* gains access to the stomach where it can survive thanks to urease. The bacterium swims in the mucus layer using flagella and it reaches the luminal surface of gastric epithelial cells that it binds through the adhesins. Using the T4SS *H. pylori* injects the cytotoxin CagA, and secretes several virulence factors, such as VacA or HP-NAP. Different bacterial factors together with CagA disrupt cell-to-cell junctions altering the permeability of gastric epithelium. On the other hand, *H. pylori*-NAP can cross the epithelial lining and recruits neutrophils and monocytes that extravasate into the tissue and, upon activation, they release pro-inflammatory cytokines and ROS causing a severe mucosal damage [47][44]. Moreover, due to altered conformation of gastric epithelium, *H. pylori* itself can invade gastric mucosa, where it can be phagocytized by monocytes and neutrophils that are continuously recruited in inflamed gastric mucosa [9].

There are many factors that influence the severity of inflammation during *H. pylori* infection; these factors relie both on host genetic profile and on the specific bacterial strain. For example bacteria with enhanced motility, able to secrete urease, bacteria that possess CagPAI and express adhesins, cause generally a severe gastric inflammation.

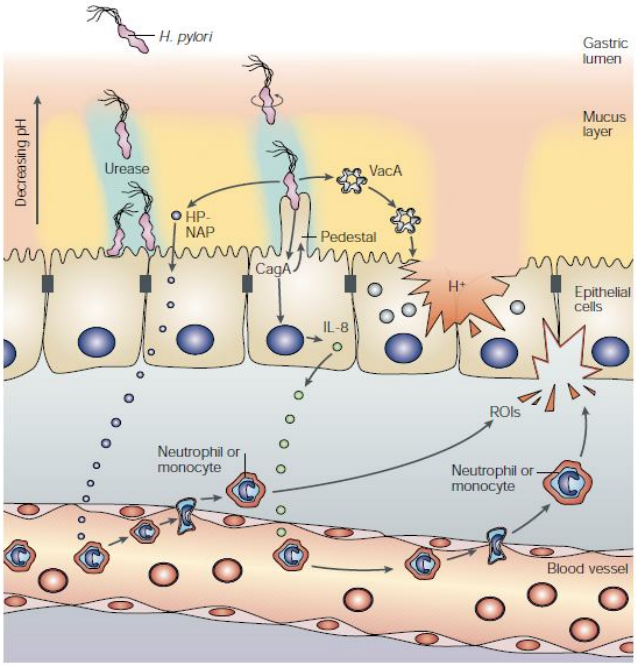


**Figure 8**: mechanism of *H. pylori* induced inflammation. *H.pylori* and its virulence factors recruit monocytes and nutrophils that causes a severe inflammation of gastric mucosa. Image adapted from [9].

On the other hand, the genetic predisposition of a subject is crucial for the outcome of infection: for example, polymorphisms of the interleukin (IL)-1β, IL-10 and Tumour Necrosis Factor (TNF) genes, are associated with the chronicization of infection and the consequent mucosal damage in addition to an increased risk of developing gastric cancer [48].

Studies on the gastric mucosal levels in *H. pylori* infection reveal that there are increased levels of several pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, INF-γ and TNF-α [49].

Recently, it has been demonstrated that also IL-17 is increased in *H. pylori*-infected patients, suggesting that also the Th17 response is crucial for *H. pylori*-induced inflammation [50]. Moreover, together with Th17 response, *H. pylori* infection induces a Th1 profile that is responsible for a severe mucosal damage, due to the chronic and persistent inflammation [51]. Gastric epithelial cells produce IL-18 in response to *H. pylori*, and this cytokine increase the production of IL-8, which enhances the recruitment and the extravasation of innate immune cells (i.e. monocytes and neutrophils) that exacerbate the inflammatory response. Moreover, IL-18 increases the production of INF-γ by activated T-helper cells and promoted the Th1 response. INF-γ alters the TGF-β signalling pathway: in particular, the anti-inflammatory cytokine TGF-β is highly expressed in gastric mucosa in presence of *H. pylori,* but its signalling is inhibited by Smad7, and the trigger for Smad7 up-regualtion is INF-γ itself. The down-

regulation of the TGF-β signalling results in an increased mucosal damage, mediated by Th1 lymphocyte [52].

It has been demonstrated that *H. pylori* can survive inside the phagosome of professional phagocytes such as macrophages. In particular after phagocytosis, the Vacuolating Cytotoxin A (VacA) inhibit the phagosome-lysosome fusion. In addition, the catalase produce by the bacterium prevents the killing after phagocytosis [53].

## 1.5 *H. pylori* associated diseases

*H. pylori* infection is the most common chronic bacterial infection worldwide. As mentioned above, *H. pylori* infects more than a half of human population. Virtually all these *H. pylori*-positive patients develop chronic active gastritis [12]. In addition, a relevant proportion of infected subjects develops other, more severe, *H. pylori*-associated diseases during the course of infection. Indeed, if infection persists, *H. pylori* associated inflammation may cause chronic atrophic gastritis, peptic ulcer, autoimmune gastritis (AIG), gastric adenocarcinoma and gastric MALT (mucosa associated lymphoid tissue) lymphoma [54]. Each of these diseases are discussed in following paragraphs, except for AIG and MALT lymphoma that will be described more in details below.

### 1.5.1 Duodenal and gastric ulcers

There are many evidence for the correlation between infection by *H. pylori* and the development of duodenal ulcers. Firstly, it was observed that in the absence of treatment with anti-inflammatory drugs (NSAIDs), *H. pylori* is the cause of 70% of cases of duodenal ulcer [55]. Secondly, patients with duodenal ulcer infected with *H. pylori* infection have a lower risk of relapse when treated with antibiotics specific for *H. pylori* [56]. In addition, the mechanism of infection by the bacterium is predisposing to ulcer development: indeed, in an infected person, the inflammation that develops in the atrum of the stomach and the resulting inflammation stimulate G cells to release gastrin, a hormone that increases secretion of histamine. Histamine, in turn, binds to its type 2 receptor on the parietal cells, which subsequently release acid. Thus, the constant stimulation of parietal cells causes an increased acid production into the duodenum, which leads to an irritation of the mucous membrane. If the infection

persists or the acid load does not decrease, this situation may evolve into a duodenal ulcer [54].

Many of the evidence that support the role of *H. pylori* in duodenal ulcer development are also applicable to gastric ulcer. Indeed, the healing of gastritis follows the eradication of the bacterium and patients undergoing antibiotic treatment showed significantly lower recurrence rates compared with untreated individuals.

### 1.5.2 Chronic atrophic gastritis

Although the infection by *H. pylori* leads to an increased acid secretion that may cause ulcers, it has been demonstrated that, if the infection is not healed and it become chronic, acid secretion is impaired. Usually in patients with *H. pylori* chronic infection, the bacterium is disseminated in the whole stomach, causing inflammation in the entire corpus, a condition that is named “pan-gastritis”. Chronic inflammation leads to atrophy of the mucosa of the corpus, where parietal cells are located [54]. In this condition, the gastrin secreted after meals act on a reduced number of parietal cells, resulting in a reduction of acid secretion. Over time, there can by a completely lost of parietal cells and in this case the gastric acid secretion declines further. This hypochloridria due to gastric athrophy and metaplasia, represent the typical situation that may evolve into gastric cancer in predisposed individuals [57].

### 1.5.3 Gastric Cancer

Gastric adenocarcinoma is one of the most common forms of cancer, with more than 900.000 new cased every year and, most importantly, a leading cause of cancer-related deaths in several countries. It is now established that *H. pylori* infection is the major risk factor for gastric cancer development. Indeed, plenty of studies have been focusing in this correlation, demonstrating a decrease probability of gastric cancer development after a successful antibiotic therapy against *H. pylori* [58].

One of the main candidates for the induction of gastric cancer is the toxin CagA which is injected by the bacterium inside host cells. CagA induces several modifications in host cells (see above) and it may induce the transformation of them into neoplastic cells. Even if CagA has been extensively studied *in vitro*, a direct evidence of the oncogeniticy of CagA *in vivo* is still lacking. Anyway, many other factors together with

CagA may induce malignant transformation. Indeed the chronic inflammatory state induced by *H. pylori* infection over decades provides a milieu rich in inflammatory cytokines, as well as in ROS and nitrogen species that are expected to induce cell damage and mutagenesis [59].

# 2 Lymphomas: general aspects

A lymphoma is a neoplastic transformation of lymphoid cells. Lymphomas are divided into two groups, based on the severity of symptoms, aggressiveness and diffusion of the cancer: Hodgkin Lymphomas (HL) and non-Hodgkin Lymphomas (NHL) [60].

Usually HLs are identified as B-cells lymphomas, while T cells lymphomas are less frequent. HL accounts for approximately 11% of all malignant lymphomas.

The morphologic hallmarks of this unique lymphoma were initially described more than 100 years ago; they describe the presence of Hodgkin Reed-Sternberg cells (HRS, large and polynucleated cells that derives from B lymphocytes) in classical HL (cHL). Another type of HL is the so-called nodular lymphocyte-predominant HL (NLPHL).

Typically, malignant cells are greatly out-numbered by reactive cells in a microenvironment that includes lymphocytes, macrophages, eosinophils, mast cells, plasma cells, stromal cells, fibroblasts, and other cells. Specifically in cHL, the frequency of all these cellular components, including the HRS cells, vary considerably between cHL subtypes [61].

NHL includes a large number of cancers, divided in two categories, on the basis of aggressiveness: aggressive, characterized by a rapid growth and indolent, characterized by a slow growth rate.

Recent advances in molecular genetics have significantly deepened our understanding of the biology of these diseases. The introduction of gene expression profiling has led to the discovery of novel oncogenic pathways involved in the process of malignant transformation.

There are different categories of NHL, based on lymphocytes that are transformed: B-cells lymphomas are the most common NHL (85%), while T cells lymphomas are less frequent (15%).

There are different type of NHL: i) diffuse large B-cells Lymphomas (DLBCL), which is in turn divided in germinal centre B-cells like (GCB), activated B cells like (ABC) and primary mediastinal B-cells lymphoma (PMBL), ii) follicular B-cells lymphomas (FL), mantle cell lymphoma (MCL) and iii) Burkitt's lymphoma [62].

Similar to other types of cancer, NHLs arise by a multistep accumulation of genetic aberrations that induce a selective growth advantage of the malignant clone.

Recurrent translocations, which occur during different steps of B-cell differentiation, are often an initial step in the malignant transformation. These translocations lead to deregulated expression of oncogenes that often control cell proliferation, survival, and differentiation. Interestingly, these translocations alone are often insufficient for lymphoma development. Accordingly, secondary genetic alterations are required for the full malignant transformation.

## 2.1 Mucosa Associated Lymphoid Tissue (MALT) lymphomas

In 1983, Isaacson and Wright, observed that the histological feature of a case of IPSID (Immuno Proliferative Small Intestine Disease) was very similar to that of a low-grade B-cell lymphoma. Since that time, IPSIDs were considered diseases with similar characteristics of the yet un-defined MALT lymphoma (see below) [63].

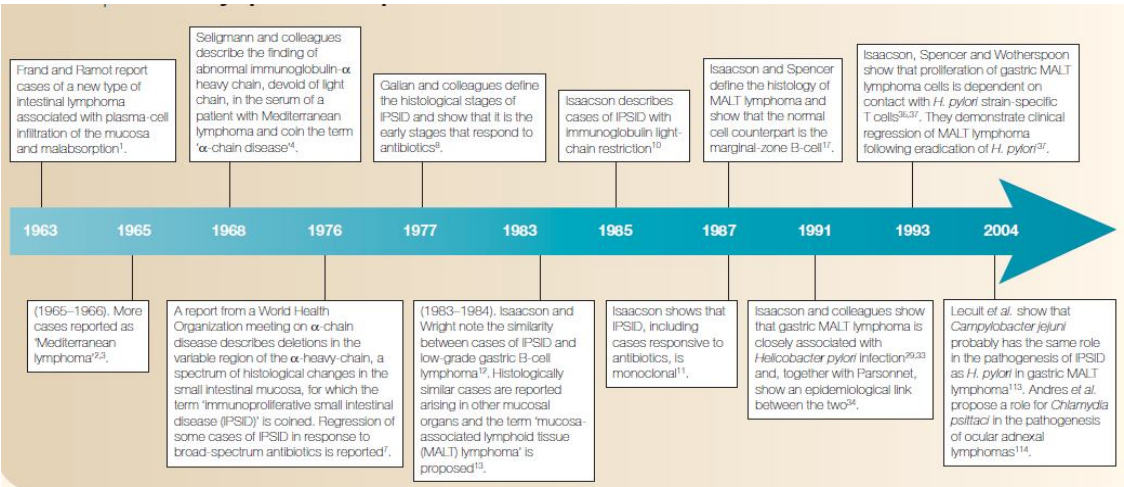


**Figure 9**: Timeline of the MALT lymphoma concept evolution. Image adapted from [7].

The histological features observed by Isaacson and Wright in IPSID were very similar to those of Peyer's patches, a very important component of the Mucosa Associated Lymphoid Tissue (MALT) in the gut. Moreover, both IPSID and low-grade B cell lymphoma show a distinctive indolent clinical course.

These observations led to the hypothesis that these cases represented a novel type of B-cell lymphoma that took place in MALT. Accordingly, the same clinical features were subsequently observed in different type of low-grade extranodal lymphomas arising in mucosal organs such as lung, salivary glands and thyroid. All these elements led to the establishment of the term "MALT lymphoma" [64].

More than 70% of MALT lymphomas arise in the stomach, while the percentage is significantly smaller for other MALT: 14% occurs in lung, 12% in lacrimal glands, 4% in thyroid and 1% in the small intestine. Given its higher frequency, most of the studies on this type of lymphoma were performed on gastric MALT lymphomas.

In 2001 the World Health Organization classified MALT lymphoma as "extranodal marginal zone B-cell lymphoma of mucosa associated lymphoid tissue". Indeed the lymphoma infiltrates around reactive B-cells follicles in the region corresponding to the Peyer's patches marginal zone, spreading into the surrounding tissues. Therefore, MALT lymphoma are characterized by the presence of lympho-epithelial lesions formed by invasion of individual mucosal glands or other epithelial structures by aggregates of lymphoid cells [64].

Transformation of MALT lymphoma to a diffuse large B-cell lymphoma can occur and it is characterized by an increased numbers of transformed blast cell. These may form sheets or clusters and finally growth to confluence and at this stage they can mask all traces of a preceding MALT lymphoma.

Among all different types of B-cells lymphomas 8% are MALT lymphoma. The disease is remarkably indolent, and especially in the case of gastric MALT lymphoma, tends to remain localized in the stomach for long periods [7].

## 2.2 Gastric MALT lymphoma and *H. pylori* infection

The fact that gastric MALT lymphoma is the most common form of MALT lymphoma is somehow paradoxical, since there is no lymphoid tissue in the mucosa of the stomach. Anyway, this paradox can be explained considering the link between the development of this disease and the infection by *H. pylori*.

The first studies based on the hypothesis that *H. pylori* can be involved in MALT lymphoma induction were carried out by Wotherspoon and colleagues in 1991 [65]. They found that almost all patients with MALT lymphoma were also infected by *H. pylori.* Subsequent epidemiological studies revealed that in regions with high incidence of gastric MALT lymphoma, there was a concomitant high frequency of *H. pylori* infection, as in the Veneto region of Italy for example. Finally, in a case-control study, an association was shown between previous *H. pylori* infection and the development of MALT lymphoma [65].

Based on these observations the same authors decided to further investigate the role played by the bacterium in gastric MALT lymphoma progression. They isolated cells (including neoplastic B-cells, T cells, macrophages and other APCs) from gastric mucosa of patients with MALT lymphoma and they observed that the stimulation with *H. pylori* antigens allowed the survival and proliferation of tumour B cells. Notably, for each case of lymphoma, the stimulation with bacterial antigens exerted proliferating effects only if the *H. pylori* strain used was that identified in the patient. On the contrary, control cells derived from nodal low grade lymphomas, did not respond to any strain of *H. pylori* [66].

After further investigations, the role of IL-2 in the clustering and proliferation of lymphoma cells was established. Indeed, clustering and proliferation are associated with enhanced IL-2 receptor expression and the release of IL-2 into the supernatants. These data indicate that the release of IL-2 following an immune response to *H. pylori* is involved in the development of the lymphoma [66][67].

Moreover, they demonstrate that T-lymphocytes play a key role in the progression of gastric MALT lymphoma, induced by *H. pylori*. In accordance, T cells are able to interact with B-cells in the context of MALT lymphoma and this interaction is mediated by the CD40 signalling [68].

Moreover, the fact that MALT lymphoma usually remains located in the stomach can be explained by the specificity of intra-tumoural T cells for *H. pylori*; indeed only intra-tumoural T cells are specific for the bacterium, while those obtained from other non-gastric tissues are not [68].

The model of MALT lymphoma induction and progression is illustrated in fig 10: *H. pylori* infection, and the subsequent inflammation is a prerequisite for MALT acquisition. The eventual transformation in MALT lymphoma and the progression of this disease is both *H. pylori*-antigen-dependent or independent and mediated by T-cells. Moreover, genetic abnormalities can be acquired as a consequence of chronic inflammation caused by *H. pylori*, which may induce DNA damage, leading to transformation of B lymphocytes in neoplastic cells.

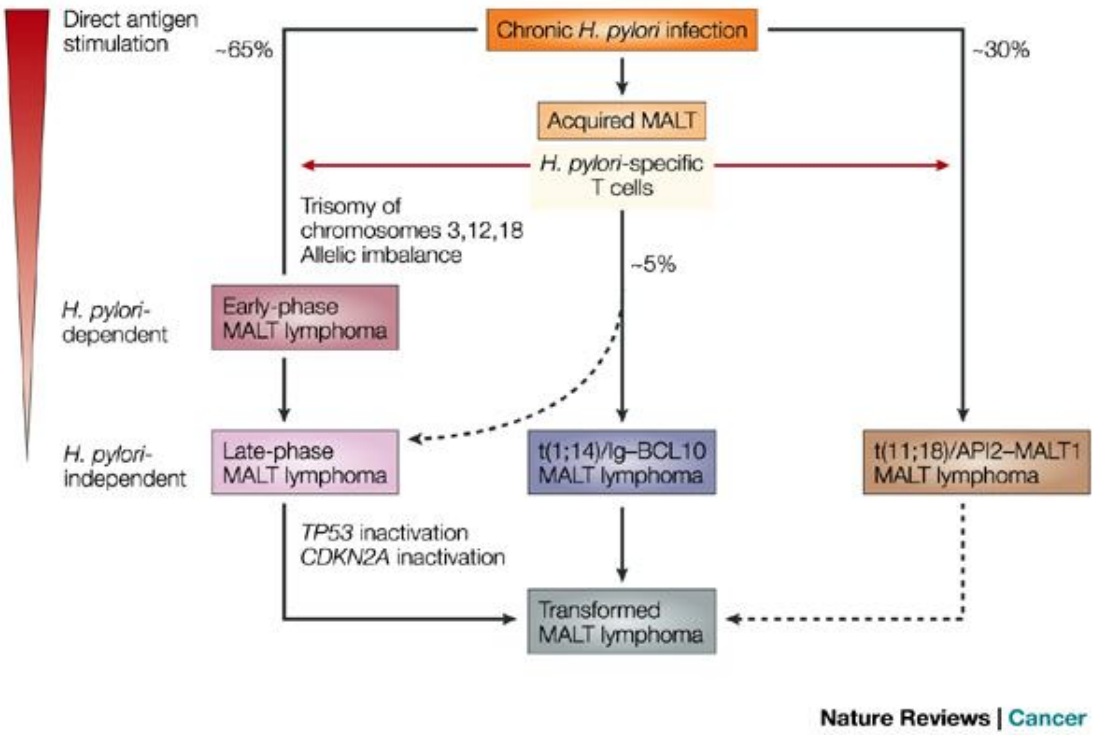


**Figure 10**: Multi-stage development of gastric MALT. Image adapted from [7].

In 1993, Isaacson's group demonstrated that patients affected by *H. pylori*-induced MALT lymphoma benefited from the treatment with antibiotics that eradicated the bacterium [69]. They performed an initial study on a group of six patients with gastric MALT lymphoma that received an appropriate antibiotic treatment for the eradication of *H. pylori.* Post-treatment biopsies, performed at regular intervals, were evaluated for the histological characteristics of lymphoma and the presence of *H. pylori*. In all six patients the bacterium was successfully eradicated and complete remission of lymphoma was achieved [70].

Subsequent studies, in greater cohort of patients, revealed that 75% of patients can be successfully treated for MALT lymphoma with antibiotics. However, the remaining 25% that does not respond to this treatment shows a tumour growth that is independent from *H. pylori* stimulation [71].

## 2.3 Molecular genetics of MALT lymphoma.

The most common genetic abnormalities in gastric MALT lymphoma are translocations. The first-discovered translocation was the t(11;18)(q21;q21). This translocation is also called API2-MALT1 because results in the fusion of the API2 and

MALT1 genes. API2 inhibits the biological activity of caspases 3, 7 and 9 and is believed to be an apoptosis inhibitor [7].

The t(1;14)(p22;q32) translocation is found in 5% of MALT lymphomas and they are typically found in advanced stage lymphomas that usually growth independently from *H. pylori* and, as a consequence, they do not respond to antibiotics treatment. In two independent studies, Willis and Zhang demonstrated that the translocations brings the *BCL10* gene under the control of the Ig-heavy-chain gene enhancer and deregulates its expression. BCL10 is essential for development and function of mature B and T cells, linking antigen receptor signalling to NF-κB pathway [7].

## 2.4 Classification of MALT lymphoma staging

According to Wotherspoon and Isaacson there are 6 different stages of MALT lymphoma progression that are used nowadays by pathologists for the classification of the disease progression. This classification is based on the severity of lymphoid infiltration and lympho-epithelial lesions and it is indicated as Wotherspoon-Index (WI). It has been recently proposed that a PCR-based screening could be useful for a more precise characterization; however, the method proposed by Wotherspoon remains the gold standard for the staging of this disease (Table 1) [1].

| Score | Diagnosis | Histological features |
|---|---|---|
| 0 | Normal | Scattered plasma cells in lamina propria. No lymphoid follides |
| 1 | Chronic active gastritis | Small dusters of lymphocytes in lamina propria. No lymphoid follicles. No lymphoepithelial lesions |
| 2 | Chronic active gastritis with florid lymphoid follicle formation | Prominent lymphoid follides with surrounding mantle zone and plasma cells. No lymphoepithelial lesions |
| 3 | Suspicious lymphoid infiltrate, probably reactive | Lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely in lamina propria and occasionally into epithelium |
| 4 | Suspicious lymphoid infiltrate, probably lymphoma | Lymphoid follicles surrounded by marginal zone cells that infiltrate diffusely in lamina propria and into epithelium in small groups |
| 5 | MALT lymphoma | Presence of dense infiltrate of marginal zone cells in lamina propria with prominent lymphoepithelial lesions |

**Table 1**: Classification of MALT lymphoma progression according to Wotherspoon-index (WI). Image adapted from [1].

## 2.5 A Proliferation Inducing Ligand (APRIL)

A Proliferation Inducing Ligand (APRIL) is a recently discovered protein that is attracting the interest of scientists in the last couple of years for its involvement in several normal but, most importantly, also in pathological condition.

APRIL belongs to the tumour-necrosis factor (TNF) family, which includes 19 ligands and 29 receptors that orchestrate a wide range of biological functions such as the regulation of cell death in immune system tissue homeostasis and the regulation of its activation [72].

APRIL has a 28 aminoacid cytoplasmic domain, a transmembrane domain and a 201 residues extracellular domain. APRIL is synthesized as type II transmembrane proteins but it is cleaved by a furin convertase in the Golgi apparatus before being secreted [73].

Secreted APRIL exists as a 68 kDa non-covalent trimer, but an alternative form of APRIL exists, called TWE-PRIL, which is an hybrid mRNA transcript of APRIL and TWEAK. TWE-PRIL fusion protein is formed by the cytoplasmic and transmembrane portion of TWEAK fused to the carboxy-terminal domain of APRIL. This fusion protein represents the membrane form of APRIL [74].

There are two known receptor for APRIL, both belonging to the TNF-super family: transmembrane activator and CALM interactor (TACI) and B-cell maturation antigen (BCMA). These receptors are all expressed by B cells, but there are evidences that TACI is highly expressed in monocytes. Moreover, it has been demonstrated that APRIL can also bind to heparan sulphate proteoglycans (HSPGs) (fig. 11) [75].

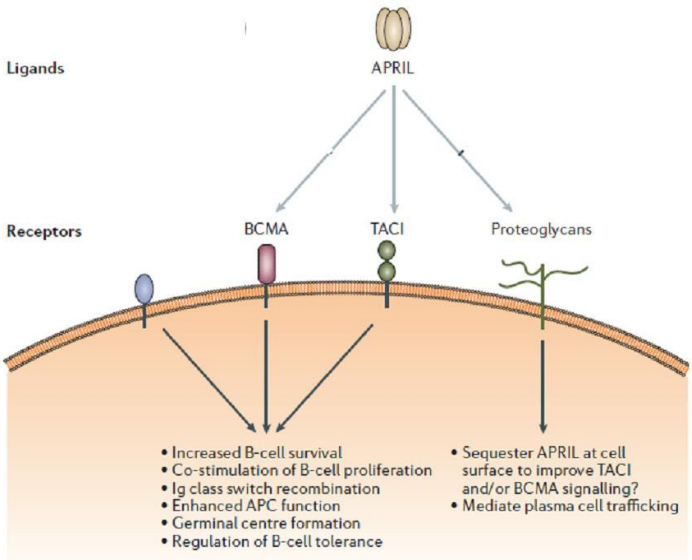


**Figure 11**: APRIL and its receptors. The binding to specific receptor triggers intracellular pathways that lead to B-cell survival and proliferation, Ig class switching, enhanced APC function, germinal center formation and regulation of B-cell tolerance. Image adapted from [11].

The binding of APRIL to TACI and/or BCMA increased B-cell survival and proliferation, triggers the Ig class switch recombination, regulates the germinal centre formation,

while the binding to HSPGs seems to help the binding to BCMA and TACI, by sequestering APRIL at cell surface [76].

## 2.6 Biological functions of APRIL

APRIL is mainly expressed by immune cells such as monocytes, DCs, macrophages and T cells and its expression is up-regulated following stimulation with INF-$\gamma$ or INF-$\alpha$ [76]. Interestingly, APRIL is expressed also in osteoclasts and tumour tissues. High levels of APRIL mRNA are detectable in colon cancers, thyroid and lymphoid tissues *in vivo*. It has been demonstrated that also gastro-intestinal tract, pancreatic, uterine and ovarian adenocarcinoma highly express APRIL [77].

APRIL, which is not expressed in normal B cells, is aberrantly expressed by malignant B cells and by various cells within the B-cell tumour micro-environment, including "nurse-like" cells derived from Centrocyte Like Lymphoma (CLL) [78].

The receptors for APRIL are expressed on B-cells at varying levels, depending on the stage of maturation and the activation state of cells.

It has been shown that APRIL promotes the generation of rapidly dividing plasmablasts from activated human memory B cells by enhancing their survival *in vitro,* via its interaction with BCMA. It has been reported that the administration of APRIL to mice results in increased spleen dimension and increased proportion of B-cells in the spleen [79]. Mice over-expressing APRIL present two-fold increase in B-cells percentage in peripheral lymph nodes and increased serum levels of IgM, without antigenic challenge. Most importantly, more than 40% of aged APRIL transgenic mice develop a B-cell associated neoplasia. This observation suggests that APRIL may support the genesis and/or the survival of B cells. [62]

## 2.7 APRIL overexpression in lymphomas: effects on B-cells survival

APRIL over-expression in lymphomas has been extensively studied in CLL. CLL is classified as non-Hodgkin's Lymphoma (NHL) and it is the most common form of leukemia in Western countries. Several studies demonstrated that APRIL is over-expressed in this disease. APRIL is highly present on neoplastic B cells; other stusies demonstrate that APRIL might be secreted by nurse-like cells also *in vivo*.

Furthermore, serum levels of APRIL are higher in CCL patient if compared to healthy donors, but most importantly, there is a significant correlation between APRIL concentration in serum and overall patient survival, with a worst prognosis for patients with the highest APRIL levels [80][81].

Several studies demonstrated that APRIL increases the survival of neoplastic B-cell *in vitro*, as well as resistance to drug-induced apoptosis.

The effect of APRIL on survival and apoptosis resistance involves the transcription factor NF-κB. Indeed, the treatment with APRIL of leukaemia cells further increases the activation of NF-κB, which is already high in neoplastic cells; moreover, this activation is dose and time-dependent.

Several other NHL over-express APRIL: its over-expression, both at mRNA and protein level, has been demonstrated for Mantle cell Lymphoma (MCL), Marginal Zone Lymphoma (MZL), Follicular Lymphoma (FL), Burkitt's Lymphoma (BL) and Diffuse Large Cell Lymphoma (DLCL). Studies performed on cell lines derived from all listed lymphomas, reveal that treatment with APRIL shift the Bcl-2 family ratio to favour B-cell survival. BCL-2 and BCL-$x_L$ (BCL: B-cell Lymphoma) are NF-κB-inducible BCL-2 family members that enhance cell survival by interfering with the release of cytochrome C from mitochondria. Cytochrome C activates caspases that start the apoptotic process. On the contrary, BAX, another BCL-2 family member enhances apoptosis by inducing the cytochrome C release. The administration of APRIL causes an up-regulation of anti-apoptotic proteins BCL-2 and BCL-$x_L$ and a down regulation of the pro-apoptotic protein BAX [61].

Lymphomas constitute a heterogeneous collection of diseases, and successful therapy depends on detailed knowledge of the pathogenic pathways involved in each lymphoma. It is clear that APRIL is involved in the survival and proliferation of several lymphomas, making this cytokine an important element of B-cell lymphomas biology.

# 3 Autoimmune diseases: general aspects

The concept of autoimmunity was described for the first time by the Nobel Prize Paul Ehrlich at the beginning of XX century and he described autoimmunity has a "*horror autotoxicus*".

In normal situations, the immune system protects the host from foreign attacks, but most importantly it is programmed for the tolerance versus self-substances; this means that the immune system normally tolerates all the antigens that are expressed by our body, avoiding an immune response against "self-antigens". This tolerance towards self-antigens can be altered and in this case the immune system recognizes self-antigens as potential threat, leading to autoimmunity [8].

The rearrangement occurring in the generation of BCR (B cell receptor) and TCR (T cell receptor) might results in more than a billion of different sites of binding to antigens. It is thought that 20-50% of BCR and TCR generated may recognize self-antigens. However, there are several mechanisms that control the survival and the expansion of these self-reactive cells (fig.12) [8].

The depletion of auto-reactive lymphocytes occurs in central lymphoid organs, bone marrow for B-cells and thymus for T cells, where auto-reactive cells undergo to apoptosis.

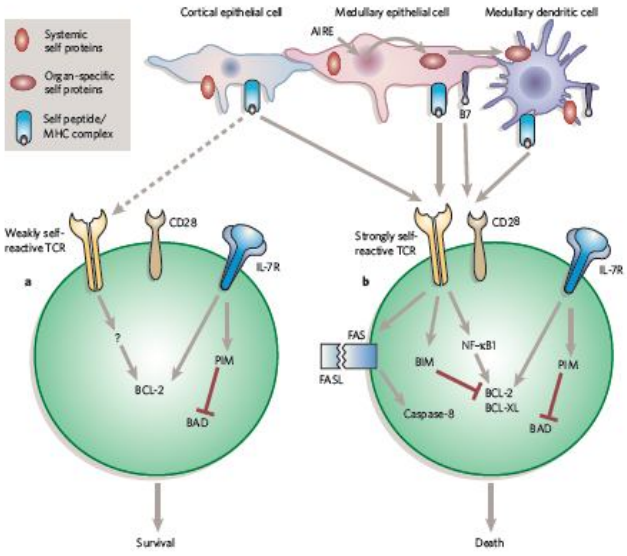


**Figure 12**: Pathways of clonal deletion for autoreactive T-lymphocytes in the thymus. Image adapted from [8].

After the evasion from thymic barrier auto-reactive cells can circulate in the body's fluid where they remain inactive until they meet their antigen. The antigen is often presented on the surface of B-cells and it can be recognized by autoreative T cells. Upon activation, the secretion of cytokines by T cells drives the activation, differentiation and proliferation of autoreactive B-cells that secrete autoantibodies.

Moreover, it is also possible that some bacterial o viral antigens may be similar to host's component and in this case autoimmunity can be achieved by a mechanism called "molecular mimicry"[46].

Summarizing, there are mainly four strategies of suppression of self-response: i) cells that express wrong receptor are induced to apoptosis, ii) the receptor is further modified toward genetic recombination (as described above) and substituted with another new receptor (receptor editing), iii) auto-reactive cells are induced to anergy towards intrinsic biochemical or genetic pathway that reduce the capability of the cells to activate the receptor. Another mechanism for self-tolerance is mediated by the activity of regulatory T-lymphocytes (T-reg). These cells express both CD25 and CD4 ($CD25^+$ $CD4^+$) and they represent the 5-10% of all $CD4^+$T cells; they are able to block proliferation and IL-2 production by $CD4^+$ or $CD8^+$ T lymphocytes, through a mechanism that needs to be clarified [82]; however, it is clear that this inhibition requires cell-cell contact and occurs through two potent immunosuppressive cytokines, IL-10 and TGF-$\beta$ secreted by T-reg cells [83].

Autoimmune diseases may develop as a consequence of an altered balance between T-reg cells and self-reactive conventional T cells. Given the dynamic nature of this equilibrium, it is conceivable that any genetic abnormalities or environmental agents that tip the balance toward self-reactive conventional T cells can cause or predispose to autoimmune disease [84].

Although Th1-type cytokines are recognized to be involved in the development of organ-specific autoimmune diseases, a recently discovered sub-population of T helper cells attracted the attention of scientist: the IL-17-producing T lymphocytes, called Th17 cells. Indeed, there is the evidence from cell transfer studies that both Th1 and Th17 cell subsets have a pathogenic role in autoimmune inflammation, perhaps acting at different stages of disease or by recruiting different effector cells.

Furthermore, the Th1 and Th17 response are close related. It has been demonstrated that IFN-γ, secreted by Th1 cells, exacerbates disease in patients with multiple sclerosis and promotes IL-1β production by human macrophages; this cytokine, in turn, enhances IL-17 production by Th17 cells. Finally, $CD4^+$T cells that express both IL-17 and IFN-γ have been found in different autoimmune disease. Thus, it appears that Th17 and Th1 cells or IL-$17^+$IFN-$\gamma^+CD4^+$T cells may have pathogenic roles in autoimmune and chronic inflammatory diseases [85].

## 3.1 Th17 cells in the pathogenesis of autoimmune diseases

Th17 lymphocytes are a recently discovered population of T helper cells that are characterized by the production of a particular set of cytokines such as IL-17A, IL-17F, IL-22 and CCL-20. They are involved in adaptive immune responses against extracellular bacteria. As already mentioned in the previous paragraph, Th17 cells are also involved in the pathogenesis of autoimmune and allergic diseases [86].

Until the discovery of Th17 cells, T helper lymphocytes were divided in Th1 and Th2; the former are able to differentiate from *naïve* $CD4^+$ positive cells in presence of IL-12 and they secrete INF-γ and IL-2, two cytokines involved in cell-mediated immune responses; the latter differentiate in presence of IL-4 and the secrete IL-4, IL-5 and IL-13, three cytokines critical for humoral response [87].

Different studies based on the possible oligomerization partners of p40 subunits of IL-12, identified a novel subunit, IL23p19; in particular IL-23 is critical for organ specific inflammation and in particular in autoimmune inflammatory response. Moreover, the activation of T helper lymphocytes in presence of IL23. leads to an increased production of IL-17 and a parallel blocking of IL-12 and INF-γ. These cells, characterized by the ability to secrete IL-17, were identified as Th17 lymphocytes [88][89].

One of the functions for Th17 cells is the clearance of pathogens that are not counteracted by Th1 or by Th2 cells. However, Th17 cells are also potent triggers of tissue inflammation and that they are associated to several autoimmune inflammatory conditions.

### 3.1.1 Differentiation of Th17 lymphocytes

In 2006, three independent studies demonstrated that Th17 differentiate from *naïve* T cells in presence of both TGF-β and IL-6, respectively an anti-inflammatory and pro-inflammatory cytokine.

TGF-β is necessary for the differentiation towards Th17 of $CD4^+$*naïve* T cells and for the expression of IL-23 receptor (IL23R), even if high concentration of TGF-β inhibits the expression of this receptor, suggesting a biphasic regulatory effect [90][91].

Th17 express IL-6R on their surface and its engagement by IL-6 promotes the pathway of Signal Trasducers and Activators of Transcripiton (STAT) 3; however, the activation of this signalling pathway is not sufficient *per se* to induce the Retinoic acid Orphan Receptor (ROR) γt activation, one of the most important transcription factors involved in Th17 differentiation. The complete activation of RORγt occurs only with the presence of both IL-6 and TGF-β. Moreover TGF-β promotes the expression of IL-6R, maintaining the responsiveness of cells. Conversely, IL-6R is down regulated in absence of TGF-β [92].

Also the cytokine IL-21 seems to be involved, together with TGF-β, in Th17 differentiation. In particular, IL-21 participates to the cross-talk between immune cells and non-immune cells in the gut and for this reason is believed to be associated with gastric inflammation. Importantly, the expression of IL-21 is induced by IL-6 and notably, IL-21 can replace IL-6 for the activation of RORγt. Given that IL-6 is crucial for *in vivo* differentiation of Th17 cells it has been hypothesized that IL-21 is important in maintaining the Th17 pool in absence of inflammation, when the concentration of IL-6 is low [93].

RORγt, activated by TGF-β, IL-6 and IL-21, drives the expression of IL-23 that, once bound to cells, stabilizes the Th17 lineage. Indeed IL-23R is not expressed by *naïve* lymphocytes, but only in advanced stage of differentiation [92].

### 3.1.2 Effector molecules of Th17 cells

The family of IL-17 cytokines includes six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, even if those which are functionally important are IL-17A and IL-17F. Usually IL-17A is simply named IL-17, since it was the first discovered and the most

important protein of this family. IL-17F is the most homologous to IL-17A (45-50% of amminoacid identity), among the rest of IL-17 family members [94].

Both IL-17A and F have pro-inflammatory properties, and they can act on a wide range of cells. Indeed the IL-17 Receptor A (IL-17A) is expressed in hematopoietic, hepatic, pulmonary and splenic cells. IL-17 induces the expression of several pro-inflammatory cytokines and chemokines such as TNF-$\alpha$, IL-1$\beta$, IL-6, GM-CSF, G-CSF, CXCL-1, CXCL-8 and CXCL-10 [95].

Another important effector molecules produced by Th17 cells is IL-22. It has been hypothesized that the main function of IL-22 is the cross-talk between Th17 cells and the tissues. Accordingly, none of immune cells express IL-22 receptor while the latter is highly expressed on epithelial and endothelial cells. IL-22 induces the production of anti-microbial factors by endothelial cell and it is essential for the functionality of epithelial barrier [95].

The development program of Th17 and T reg cells are strictly related. Indeed the presence of TGF-$\beta$ only induces the differentiation of T reg, while the concomitant presence of IL-6 drives the differentiation of Th17. This is because TGF-$\beta$ can activate the transcriptional pathway of ROR$\gamma$t and Foxp3 (relative to Treg) while IL-6 seems to have a discriminating role in the choice of response: without pro-inflammatory stimuli, TGF-$\beta$ induces the generation of a Treg response that maintains memory cell in state of alert; during acute-phase response, IL-6 favours the differentiation of Th17 cells [96].

### 3.1.3 Th17 cells in autoimmune diseases

In contrast to their protective role during host defence, Th17 cells are thought to promote tissue destruction during inflammation, and the IL-23/IL-17 axis appears to be involved in development and maintenance of chronic inflammation. Indeed, studies performed on mice, reveal that the blockage or the absence of IL-17 or IL-23 induce resistance to different type of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD) or collagen induced arthritis. Moreover Th17 cells were isolated for the first time in patients with Chron's disease (CD), and increased expression of IL-23, IL-17 and Th17-related cytokines has been described in circulation or in inflamed tissues of patients with

diverse autoimmune diseases, such as multiple sclerosis, CD, psoriasis or rheumatoid arthritis [86]. Interestingly, the administration of anti-IL-23 antibodies to patients with autoimmune disorders results in a better outcome of the disease. Furthermore, genetic studies have shown that there is a strong correlation between polymorphism of IL-23R gene and susceptibility to psoriasis or CD [92].

## 3.2 Autoimmune gastritis

Autoimmune gastritis (AIG) is characterized by autoimmune-mediated destruction of gastric glands in the corpus of the stomach, leading to loss of acid producing parietal cells and zymogenic cells; all these features are clinically defined as corpus atrophy [97][98][99].

*H. pylori* induces autoantibodies reactive with gastric mucosal antigens in almost half of infected patients. Usually the main antigen that drives the rising of AIG is the $H^+K^+$-ATPase, a gastric proton pump located in the secretory canaliculi of parietal cells.[100].

In mouse model of experimental AIG (EAIG), *H. pylori* induces autoantibodies also against the Lewis antigens on the glycosylated β subunit of $H^+K^+$-ATPase, probably because of the similarity (molecular mimicry) between that antigen and the bacterial LPS Lewis blood group antigens [101][102].

Almost 25% of T cells that infiltrate gastric mucosa in AIG (without previous or actual evidence of *H. pylori* infection) are specific for $H^+K^+$-ATPase, as they are able to proliferate *in vitro* if stimulated with the antigen $H^+K^+$-ATPase, presented via class II MHC, and all these clones are $CD4^+$. Other evidence suggest that human AIG is driven by antigen specific polyclonal activation of autoreactive T cells, rather than by an expansion of a single autoreactive Tcell [103].

The sub-molecular epitopes of $H^+K^+$-ATPase, responsible for molecular mimicry were identified using a library of overlapping 15-mer synthetic peptides that comprises the entire sequence of the protein. Surprisingly none of the epitopes identified were part of the major antigens such as VacA, CagA or urease [104].

Based on this statement, *H. pylori* may onset or accelerate AIG in susceptible patients by two mechanisms: i) in most infected individuals the infection of *H. pylori* causes asymptomatic chronic inflammation of gastric mucosa that is kept under control by $CD4^+CD25^+$Treg. However, even apparently healthy individuals may present some

populations of auto-reactive T cells that have escaped from thymic selections. In these patients, *H. pylori*-driven chronic Th1-mediated response may be sufficient to down-regulate T reg suppression driving the activation of $H^+K^+$-ATPase-specific T cells and, as a consequence, gastric autoimmunity [105].

ii) the second plausible mechanism comprises also genetic predisposition to develop AIG. Indeed some individual express a particular HLA that facilitates the presentation of both $H^+K^+$-ATPase and *H. pylori* epitopes [46].

## 3.3 Th17 cells in the pathogenesis of *H. pylori* infection

The role of the Th17 response in different kind of infections needs to be further investigated. Indeed, on one hand, Th17 response triggers acute inflammatory reactions that enhance neutrophils recruitment and macrophages activation, as for example in *Klebisella pneumoniae* or *Mycoplasma pulmonis* infections. On the other hand, the Th17 response can contribute to the chronicization of inflammation, especially in infection sustained by *Borrelia burdorferi*, *Candida albicans*, *Schistosoma mansoni* but also *H. pylori*.[106][107][108][109].

To date, studies on the immune responses to *H. pylori* have largely focused on Th1 and Th2 cells, and, as mentioned above, it has been demonstrated that *H. pylori* induces mainly a Th1 response and that gastric inflammation and the consequent damage greatly depend on the Th1 response [44]. However, growing evidence suggest that Th17 cells are also involved in *H. pylori* infection. Studies carried on in mice by different researcher groups confirm the correlation between *H. pylori* infection and the development of a mixed Th1 and Th17 response in gastric mucosa. Notably, *H. pylori* burden and inflammation are both reduced if IL-17 is blocked *in vivo* [50]. These results suggest that the Th17/IL-17 pathway plays a pathogenic role in *H. pylori* infection, promoting mucosal inflammation and contributing to bacterial colonization [110]. Moreover, it has been demonstrated that mouse macrophages, infected with alive *H. pylori* can drive the differentiation of Th17 from naïve Th cells *in vitro*. In particular, it has been shown that the subunit B of urease is one of the bacterial factors that can promote the differentiation of Th17 [111].

Taken together, all these studies performed in mice, open the possibility that even in human *H. pylori* can induces a Th17 response.

Most importantly, it is also possible that the Th17 can exacerbate chronic inflammation, and that it can be involved in the induction and/or the progression of AIG.

## 3.4 B-Lymphocyte Stimulator (BLyS)

The B-lymphocye stimulator (BLyS) is a cytokine also known as B-cell activating Factor of the TNF family. It was firstly described at the end of '90, and it belongs to the TNF family.

BLyS acts as a potent B cell activator. It has been also shown to play an important role in the proliferation and differentiation of B cells; all the biological functions of BLyS will be discussed in details in the next paragraph.

There are three known receptors for BLyS: TACI and BCMA that are shared with APRIL and are mainly expressed by B-cells but also mesenchymal cells and activated macrophages and monocytes; the third receptor is BAFF-R (BAFF-receptor) and it is expressed by circulating B-cells but also by white blood cells, such as lymphocytes [112].

BLyS can remain bound to the membrane of the producing cells, but it can also be released in a soluble form; the latter can be as monomer, homotrimer, heterotrimer (it can be bound to the soluble form of APRIL) and even in capsid-like assembly of 20 trimers (60-mer) [113].

BLyS is produced by several cell types such as monocytes, activated T lymphocytes, memory B cells (naïve B cell are normally negative), neutrophils, DCs, macrophages but also by non-hematopoietic cells like synoviocytes, astrocytes epithelial cells [114][2].

Compared to other immune cells, neutrophils represent an important source of BLyS; neutrophils represent the most abundant fraction of blood leukocytes (50-70%) and they are the main component of cellular infitrates during the acute-phase of an inflammatory response. However, since neutrophils do not express membrane bound BLyS and that these cells do not co-localizes with naïve B lymphocytes, it is likely that neutrophils promote an influx of BLyS in lymphoid tissues in particular pathologic conditions (i.e. chronic infection or inflammation) rather than play a direct role in the proliferation of B-cells [115].

G-CSF and INF-γ induce the expression of BLyS in neutrophils. Once synthesized, BLyS is stored in intracellular organelles, where it undergoes to post-trascriptional modification by a furin-like convertase [73]; however, its secretion occurs following the application of a pro-inflammatory stimuli, such as LPS or TNF-α. Even resting cells can secrete low amount of BLyS and this suggest an important link for the regulation of B-cells homeostasis mediated by neutrophils [115].

High concentrations of G-CSF were detected in synovial tissues of patients with rheumatoid arthritis. Concerning autoimmune diseases, it could exist a link between innate immune system activation and the development of autoimmune diseases mediated by the production of G-CSF, neutrophils activation and their action on B-cells germinal centres[5].

## 3.5 Biological functions of BLyS

The main function of BLyS is to increase the survival and proliferation of B-cells. In particular, its action is on peripheral immature B-cells that differentiate in the marginal zone of the spleen. Most of these findings were obtained studying the homeostasis of B-cells in BLyS KO mice [116].

Stimulation of BAFF-R potently activates the alternative NF-κB2 pathway and weakly activates the classical NF-κB1 pathway. Both these pathways are required for B-cell survival, which can be sustained by signalling through BAFF-R alone.

NF-κB activation, which occurs downstream to BAFF-R engagement, has been linked to increased expression of anti-apoptotic proteins, to integrin-mediated localization of B-cells in marginal zone and to T-cell independent antibody class switching [2].

Together with increasing the survival of B-cells, BLyS is able to promote glycolysis, protein synthesis and cell growth, through the activation of PI3K-AKT-mTOR pathway. To confirm this observation, inactivation of mTOR (using rapamycin), renders B-cells no more responsive to BLyS in terms of survival and cell growth. Furthermore, the PI3K-AKT pathway downstream to BLyS positively regulates the transcription of MCL-1 (Myeloid cell Leukaemia sequence 1), a short living member of BCL-2 that is required for the development of haematopoietic stem cells and for the maintenance of peripheral B and T cells. Indeed MCL-1 deficient B-cells do not survive in response to

BLyS, demonstrating the crucial involvement of MCL-1 in BLyS-mediated survival [117][118].

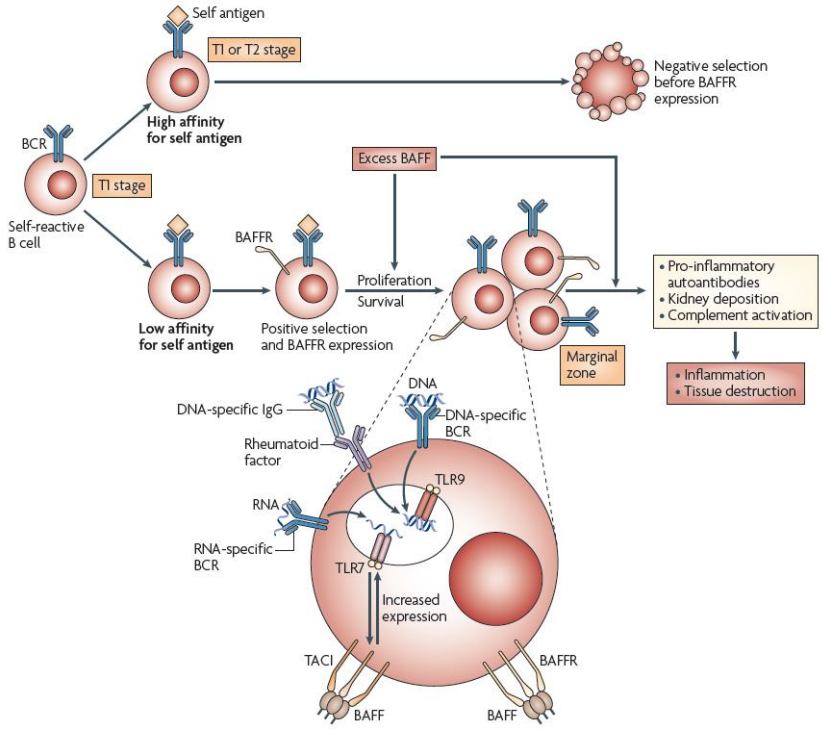


**Figure 13**: Role of excess of BlyS in B-cell tolerance and activation of self-reactive B-cells. Image adapted from [2].

As mentioned above, BLyS is a crucial factor for B-cells survival. The excessive production of APRIL, involved in B cells survival as well as BLyS, leads to the development of B cell lymphoma, but does not cause autoimmunity and has no immediate effect on B cell homeostasis. On the contrary, B cell homeostasis together with autoimmunity strictly depends on BLyS, as will be discussed in the next session.

In physiological condition, strongly self-reactive B cells compete with normal B cells for survival factors and for entry in B cells follicles into lymphoid tissue. Given that BLyS promotes B cells survival (fig. 13), it is likely that its over-expression could rescue self-reactive B cells from apoptosis, breaking B cells tolerance. However, it has been demonstrated that strongly self-reactive cells are otherwise deleted or rendered anergic before they start expressing BAFF-R on their surface, suggesting a weak effect of BLyS on these cells [2].

On the contrary, low-affinity self-reactive B cells can survive more than strongly reactive B cells, since a basal BCR signal is required for positive selection; for this

reason the effect of BLyS over-expression increased the survival and proliferation of low-affinity self reactive B cells.

## 3.6 The role of BLyS in autoimmunty

Mice over-expressing BLyS develop a severe autoimmune disease whose symptoms are very similar to those which are typically associated to systemic lupus erythematous (SLE). However, the excess of BLyS does not result in a complete autoreactive B cells tolerance breakdown, but it seems to support only the survival of low-affinity self-reactive B cells. Therefore, the excess of BLyS alone can not explain the devastating effect of the autoimmune disease observed in BLyS transgenic mice [2].

SLE is a multifactorial disease where not only the self-reactive producing B cells are involved, but also the deregulation of other immune cells is important: innate immune cells, such as plasmacytoid DCs, which produces high levels of inflammatory mediators, but also effector T cells [119].

Increased levels of BLyS have been measured in patients with various autoimmune conditions, and, most importantly, a correlation between BLyS over-expression and the disease progression has frequently been observed. However, it remains unclear whether increased BLyS levels are the primary cause of autoimmunity or whether autoimmunity follows an enhanced production of pro-inflammatory cytokines that increase BLyS production.

## 3.7 BLyS and Th17 cells

BLyS is important not only for survival, proliferation and Ig class switching of B-cells, but several studies demonstrated that this cytokine might also activate T-lymphocytes, through BAFF-R expressed on their surface. In particular, the action of BLyS on T-cell is mainly exerted on $CD4^+$ cells, which are requested for antigen presentation and production of autoantibodies.

Recent studies have demonstrated that BLyS could be involved in the differentiation of Th17 cells, the $CD4^+$ T lymphocytes that are involved in several autoimmune disease (see above). In particular, it has been demonstrated that BLyS is crucial for the induction of Th17 response in a mouse model of rheumatoid arthritis, the Collagen

Induced Arthritis (CIA). In detail, the injection of short harpin RNA (shRNA) that blocks BLyS expression *in vivo*, suppresses the Th17 response in the joint of mice affected by CIA. Accordingly, if BLyS expression is knocked down in this model, the levels of expression of all pro-Th17 cytokines (IL-6 and IL-23 in mice) are decreased [120].

Furthermore, it has been demonstrated in a mouse model of experimental autoimmune encephalomyelitis (EAE) that the Th17 response is increased in BLyS transgenic overexpressing mouse and, accordingly, decreased in BLyS KO mice. In particular, it seems that BLyS may directly act on $CD4^+$ T lymphocytes inducing the differentiation toward the Th17 phenotype [121].

The specific role of BLyS in the induction of the Th17 response remains an unclear issue, since the literature is somehow contradictory. However, the plenty of data demonstrating that BLyS is highly expressed in autoimmune disorders and that the Th17 population of T lymphocytes is critically involved in the pathogenesis and outcome of this kind of diseases, suggest that a link between BLyS and Th17 response could be more than a simple hypothesis.

# Materials and methods

## 1 Neutrophils isolation from buffy coat

Human neutrophils were obtained from buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from healthy donors.

The blood was stratified on Ficoll-Paque (GE-Healthcare) in 3:4 ratio and centrifuged 30 minutes at 400 g without brake and accelerator. All the supernatants and the lympho-monocytes rich ring were removed. Neutrophils are concentrated in a light-red ring on top of dark-red pellet composed by red blood cells. Neutrophils-rich ring was recovered and diluited in a final volume of 40 ml with sterile phosphate buffer (PBS) without $Ca^{2+}$ and $Mg^{2+}$; dextran 4% was added to cells suspension with a ratio of 1:5, and contaminating erytrocytes were sedimented for 30 minutes. The supernatant was washed 15 minutes at 311g.

The remaining erytrocytes were lysed with a hypotonic NaCl solution: cellular pellet were resuspended in 15 ml of 0.2% (w/v) NaCl, gently shook for 1 minute and then 15 ml of 1.6% (w/v) NaCl were added to restore physiological hosmolarity; then the cells were washed with PBS and resuspended in RPMI 1640, 10% FCS, 50 μg/ml gentamycin, seeded $2x10^6$ cells in 24 wells plates and treated for different time points with the appropriate stimuli.

When required, neutrophils were primed with 1000 U/ml of G-CSF for 20 hours, before adding the appropriate stimuli.

At the end of the culture period, cell supernatants were collected for the protein level quantification; moreover, cells were lysed for the mRNA extraction using TRIzol solution according to the manifacturer's instructions. In addition intracellular proteins were extracted for ELISA assay.

## 2 Monocytes isolation from buffy coat

Human monocytes were obtained from buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from healthy donors.

The blood was diluted 1:4 with sterile PBS without $Ca^{2+}$ and $Mg^{2+}$and then dextran 5% was added diluted 1:5 and erytrocytes sedimented for 30 minutes. The supernatant

containing white blood cells was collected and centrifugated 15 minutes at 50g. The cell pellet was resuspended in 15 ml of sterile PBS, stratified on Ficoll-Paque and centrifuged 30 minutes at 400 g without brake and accelerator.

Lympho-monocytes were recovered and washed 15 minutes at 311g, and then stratified on Percoll (GE-Healthcare ) gradient (15.76 ml RPMI 1640, 10% FCS (v/v), HEPES 4 mM (Invitrogen), 50 μg/ml gentamycin, 285 mOsm; 15.54 ml 10% Percoll in 10x sterile PBS 285 mOsm), and centrifuged 30 minutes at 400g without brake and accelerator.

Monocytes were recovered and washed 15 minutes at 311 g, then the cells were risuspended in RPMI 1640, 2% FBS, 50 μg/ml gentamycin, seeded $2x10^6$ cells in 24 wells plates and separated from contaminating lymphocytes by adherence (1 hour at 37°C). Adherent monocytes were extensively washed with medium to remove residual nonadherent cells. Cells were then treated for different time points with the appropriate stimuli.

At the end of the culture period, cell supernatants were collected for the protein slevel quantification, moreover, cells were lysed for the mRNA extraction using TRIzol solution (Invitrogen) according to the manifacturer's instructions.

## 2.1 Differentiation of monocytes into macrophages

Monocytes isolated as described above were diluted at the final concentration of $5x10^5$ cells/ml and were seeded in 24 well plates 1 ml per well. After 2 hours of adhesion at 37°C 5% $CO_2$, cells were washed twice using RPMI 1640, 2% FBS, 4 mM HEPES, 50 μg/ml gentamycin, and once with RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml gentamycin; this step is required for removing non-adherent cell (i.e. dead cells, lymphocytes, erithrocytes). After washing, cells were incubated in 500 μl/well RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml gentamycin containing 100 ng/ml M-CSF (Immunological Science) for three days. At the third day, 200 μl were removed and 300 μl of RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml gentamycin containing 100 μg/ml M-CSF were added for each well. After three days all the medium was removed from the wells and cell were incubated in RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml

gentamycin for 18-20 hours. At this stage macrophages are completely differentiated and can be treated for different time points with the appropriate stimuli.

When required, macrophages were primed with 100 U/ml of INF-γfor 24 hours, before adding the appropriate stimuli.

## 2.2 Isolation of monocytes for DC differentiation

Human monocytes were obtained from Buffy coat, from the Transfusional Centre of the Universitary Hospital of Padova, derived from healthy donors.

Buffy coat was diluted 1:1 with sterile PBS without $Ca^{2+}$ and $Mg^{2+}$ and centrifuger at 220 g without brake and accelerator. Cells were resuspendend in 25 ml of PBS and stratified on 16 ml of Ficoll-Paque and centrifuged 30 minutes at 400 g without brake and accelerator. Cells were then washed four times with PBS without $Ca^{2+}$ and $Mg^{2+}$ at scalar speed for 15 minutes (240 g, 220 g, 200 g, 200 g). Cells were then stratified on Percoll gradient (15,76 ml RPMI 1640, 10% FCS (v/v), HEPES 4 mM, 50 μg/ml gentamycin, 285 mOsm; 15,54 ml 10% Percoll in 10x sterile PBS 285 mOsm), and centrifuged 30 minutes at 300 g without brake and accelerator. Cells were washed with PBS, and the resuspended in RPMI 1640, 2% FCS, HEPES 4 mM, 50 μg/ml gentamycin for cell count. Cells were diluted at final concentration of $1,5*10^6$ cells/ml, and seeded in 24 well plates (600 μl/well) and separated from contaminating lymphocytes by adherence (1 hour at 37°C). Adherent monocytes were extensively washed with medium to remove residual nonadherent cells.

Cells were incubated in RPMI 1640, 10% FCS, HEPES 4 mM, 50 μg/ml gentamycin containing 20 ng/ml IL-4 (Immunological Science) and 50 ng/ml GM-CSF (Immunological Science)for 4 days. At the fourth day, 200 μl were removed and 300 μl of RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml gentamycin containing 20 ng/ml IL-4 and 50 ng/ml GM-CSF were added for each well. After 2 day all the medium was removed from wells and cell were incubated in RPMI 1640, 20% FBS, 4mM HEPES 50 μg/ml gentamycin for 18-20 hours. At this stage DC are completely differentiatied and and could be treated for different time points with the appropriate stimuli.

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# 3 RNA extraction and gene expression analysis

## 3.1 mRNA extraction from cells

Total RNA extraction from neutrophils and monocytes was performed using TRIzol reagent, a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer instruction. Briefly, cells were lysed directly in the culture well by adding 150 μl of TRIzol and passing the lysate several times through a pipette. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 30 μl of chloroform were added, tubes were shaken vigorously for 15 seconds and incubated on ice for 15 minutes. Samples were centrifuged at 12,000 × g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase which contained RNA. Aqueous phase was transferred into a new tube and RNA was precipitated by the addition of 70 μl of isopropyl alcohol. Following 60 minutes incubation at -20°C, samples were centrifuged again at 12,000 × g for 15 minutes at 4°C and supernatant was discarded. RNA precipitate was washed two times with 500 μl of 70% ethanol RNase free. The RNA was air dried and dissolved in 10 μl RNase-free DEPC (diethylpyrocarbonate)-treated water.

An aliquot was diluted in water and the absorbance of the solution was measured spectrophotometrically at 260 and 280 nm to determine the purity and the concentration of isolated RNA. Preparations with the ratio A260/A280 higher than 1.8 were considered for further analysis.

## 3.2 mRNA extraction from biopsies

Samples were embedded in solid paraffin, and the mRNA was isolated using the *RecoverAll Total Nucleic Acid Isolation Kit* (Ambion). Briefly samples were cut from paraffin block, crumbled and placed in a microcentrifuge tube with 1 ml of xylene. Paraffin was melted by heating samples at 50°C for 3' and the tissues were pelleted by centrifugation for 3 minutes at $v_{max.}$ Xylene was discard and the pellet was washed twice with 1 ml of 100% ethanol (add ethanol, vortex, centrifugation 3 minutes at $v_{max}$). Pellet was air-dried for 45 minutes. Samples were digested using the protease

mix provided by the kit, for 15 minutes at 50°C, the 15 minutes at 80°C. Samples were resuspended in Isolation additive (provided by the kit), loaded in the appropriate Filter Cartridge (provided by the kit) and centrifuged 30 seconds at $v_{max}$. Filters were washed using appropriate washing buffers (provided by the kit) and incubated with DNase enzyme to digest genomic DNA at R.T. for 30 minutes. mRNA was finally eluted using nuclease free water. The absorbance of the solution was measured spectrophotometrically at 260 and 280 nm to determine the purity and the concentration of isolated RNA. Preparations with the ratio A260/A280 higher than 1.8 were considered for further analysis.

## 3.3 cDNA synthesis

Reverse transcription reactions were performed using from 1 to 5 μg of total RNA.

Before the RNA was denatured in:

| | |
|---|---|
| DTT | 10 mM |
| Buffer | 1X |
| dNTPs | 0,5 mM |
| Random pdNG primer | 2 μM |

RNA was denatured at 72°C for 5 minutes and then placed on ice.

Samples were retrotranscribed in cDNA using the following mix:

| | |
|---|---|
| Superscript II (Invitrogen) | 140 U |
| RNasi out (Invitrogen) | 98 U |
| DEPC water | |

The retrotranscription was performed in a final volume of 25 μl usimg following conditions:

42°C 50 minutes
95°C 5 minutes
4°C

cDNA was then precipitated with 0,1 Vol Sodium Acetate 3 M and 2,5 Vol of ethylic alcohol at -80°C for 1 hour. cDNA was centrifuged at 12000 x g for 30 minutes at 4°C, supernatant was discarded and cDNA was dissolved in 20 μl of sterile water.

## 3.4 Real-time PCR

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product

(DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green ) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. It is possible to start with minimal amounts of nucleic acid and quantify the end product accurately.

In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase.

The initial amount of template DNA is inversely proportional to a parameter measured for each reaction, the threshold cycle (Ct).

SYBR Green specifically binds double-stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. Detection of the fluorescent signal occurs during the PCR cycle at the end of either the annealing or the extension step when the greatest amount of double-stranded DNA product is present. However, SYBR Green detects any double-stranded DNA non-specifically. Therefore, the reaction must contain a combination of primers and master mix that only generates a single gene-specific amplicon without producing any non-specific secondary products.

SYBR Green detection also uniquely allows to check the specificity of the PCR using melting (also known as dissociation) curves.

At low temperature, the PCR DNA product is double stranded, and it binds SYBR Green, which fluoresces. With increasing temperature, the DNA product melts or dissociates becoming single stranded, releasing SYBR Green and decreasing the fluorescent signal. The inflection point in the melting curve then becomes a peak.

Single peaks indicate a single product while, multiple peaks usually indicate multiple products. These other products can have many sources including primer dimers, and genomic DNA contamination, but also they arise from unreported (un-annotated) splice variants of the gene of interest.

To compare gene expression between biological samples, first calculate the ΔΔCt. A ΔCt value is calculated for each sample as the difference between the Ct values for the gene of interest and the housekeeping gene in each sample. The ΔΔCt value is the difference between the ΔCt values of an experimental sample and the control sample. The fold-change in gene expression is equal to $2^{-\Delta\Delta Ct}$ if the PCR replication efficiency for all genes is 100 percent. That is, the amount of the amplicon product perfectly doubles with each cycle. The PCR amplification efficiency can be determined from the slope of the calibration curve. A slope (m) equal to -3.3 indicates 100 percent efficiency. If the PCR efficiency is less than 1 (that is, if the slope of the calibration curves is greater than –3.3), then the fold-change in gene expression is equal to $10^{\Delta\Delta Ct/m}$, where m is the average slope of the calibration curves for the gene of interest and the housekeeping gene. Finally, the fold chance is expressed ad arbitrary units (AU).

For each sample, the following PCR mix was prepared:

| | |
|---|---|
| cDNA (500 ng/ml) | 1 ml |
| Primers (50 mM) | 0.36 ml |
| SYBR Green mix (Applied Biosystem) | 5 ml |
| DNase free $H_2O$ | 2.64 ml |

PCR reaction was performed using instrument 7900HT Fas Real-Time PCR System (Applied Bioststem) in a 96- or 384-well plates, by applying the following cycling parameters:

| | | |
|---|---|---|
| Initial denaturation | 15 minutes 95°C | |
| Denaturation | 15 seconds 95°C | |
| Annealing | 20 seconds 60°C | X 50 cycles |
| Extension | 10 seconds 72°C | |

The primers used for Real-time PCR analysis are the following:

| | |
|---|---|
| GAPDH | 5'-AGCAACAGGGTGGTGGAC-3' |
| | 5'-GTGTGGTGGGGGACTGAG-3' |
| IL-23p19 | 5'-TCCACCAGGGTCTGATTTTT-3' |
| | 5'-TTGAAGCGGAGAAGGAGACG-3' |

| | |
|---|---|
| IL-12p40 | 5'-ACAAAGGAGGCGAGGTTCTAA-3' |
| | 5'-CCCTTGGGGGTCAGAAGAG-3' |
| IL-6 | 5'-AACCTGAACCTTCCAAAGATGG-3' |
| | 5'-TCTGGCTTGTTCCTCACTACT-3' |
| TGF-β | 5'-AGTGGTTGAGCCGTGGAG-3' |
| | 5'-CCATGAGAAGCAGGAAAGG-3' |
| IL-1β | 5'-CTGTCCTGCGTGTTGAAAGA-3' |
| | 5'-TTGGGTAATTTTTGGGATCTACA-3' |
| IL-17 | 5'-TGGGAAGACCTCATTGGTGT-3' |
| | 5'-TGGGAAGACCTCATTGGTGT-3' |
| APRIL | 5'-AAGGGTATCCCTGGCAGAGT-3' |
| | 5'-GCAGGACAGAGTGCTGCTT-3' |
| BLyS | 5'-CGGGACTGAAAATCTTTGAACC-3' |
| | 5'-TGAGTGACTGTTTCTTCTGGAC-3' |
| INF-γ | 5'-GCAACAAAAAGAAACGAGAT-3' |
| | 5'-CAGGCAGGACAACCATTACT-3' |

# 4 Protein Extraction and quantification

For protein extraction 6 x $10^6$ cells were seeded on a 12-well plate and stimulated with appropriate stimuli in RPMI 1649, 1% Nutridoma-SP, 50 μg/ml gentamicin and 4mM HEPES at different time points. Cells were collected, washed in ice-cold PBS and lysed with ice-cold lysis buffer (Tris 20 mM pH 7.15, NaCl 150 mM, NP40 0.25%, supplemented with protease inhibitors PMSF 1 mM, leupeptin 1 μg/ml, and aprotinin 1 μg/ml) for 30 min on ice. Lysates were centrifuged at 12,000 x g for 20 min at 4°C, and the supernatants were collected and quantified on 96 well-plate using BCA Assay according to manufacturer's instructions. The plate was placed into a plate reader (Packard Fusion) and the optical density at 562 nm determined for each well.

# 5 Protein extraction for ELISA assay

Cells were harvested at the appropriate time points and pelleted for 5' at 4000 rpm in a table-top centrifuge. Supernatants were collected and kept at -80° for further analysis; cells were resuspended in the same volume of the supernatant (to ensure no dilution of the samples) of lysis buffer: EDTA 0.5 mM, NP40 0.05% v/v, Leupetin 1 ng/ml, Pepstatin 1 ng/ml, PMSF 0.1 mM in PBS.

For cell lysis, samples were kept for 20 min at 4°C; to enhance lysis, samples were vigorously mixed every 5'. After lysis, samples were centrifuged at $v_{max}$ for 20' to pellet DNA and membranes. Supernatant were transferred into a new eppendorf, frozen in liquid nitrogen and kept at -80°C.

# 6 Generation of gastric T cell clones and assay for T cell clone helper function

Upon approval of the local Ethical Committee (Department of Internal Medicine, University of Firenze), 5 *H. pylori*-positive patients with gastric low grade MALT lymphoma and 5 patients with uncomplicated *H. pylori*-positive chronic gastritis gave their informed consent and were enrolled in the study. None of the patients had taken antibiotics or gastric proton pump inhibitors within 2 months before the study. Biopsy specimens were obtained during endoscopy from the gastric mucosa for (a) histology and *H. pylori* detection, (b) rapid urease test, and (c) culture of infiltrating lymphocytes. Diagnosis of *H. pylori* infection was based on positive urease test, histologic detection of *H. pylori*, and positive 13C-urea breath test. Biopsy specimens of gastric mucosa were cultured for 7 days in complete medium supplemented with IL-2 (50 U/ml) to preferentially expand in vivo–activated T cells. Mucosal specimens were then disrupted, and single T cell blasts were cloned by limiting dilution (0.3 cells/well) in round-bottom microwell plates containing $10^5$ irradiated peripheral blood mononuclear cells (PBMC) as feeder cells, phytohaemagglutinin (0.5% v/v; Life Technologies, Grand Island, NY) and IL-2 (20 U/ml). Cell surface marker analysis of T cell clones was performed on a BD FACSCalibur, according to the CellQuest software, using anti-CD3, anti-CD4 and anti-CD8 mAb (BD) T cell clones were then screened in triplicate cultures for responsiveness to medium or *H. pylori* lysate (10 μg/ml) by

measurement of [3H]TdR uptake after 60 hours in the presence of autologous PBMC ($5 \times 10^4$) as antigen presenting cells. Mitogenic index (ratio of mean cpm of stimulated to unstimulated cultures) *>5* were considered as positive. T cell blasts of *H. pylori*-specific clones ($8 \times 10^5$ per ml) were co-cultured for 16 h with autologous macrophages ($4 \times 10^5$ per ml) in the presence of medium or *H. pylori* antigen (10 μg/ml).

# 7 Cytokines' quantification by ELISA assays

Culture supernatants of monocytes, neutrophils, DCs and macrophages harvested for quantification of mRNAs or intracellular protein content, were collected and stored at -80°C for subsequent quantification of cytokine content by ELISA assay: specific kits for IL-6, IL-1β, IL-23, TGF-β, APRIL and BLyS were used following manufacturer's instructions.

# 8 *H. pylori* growth

Growth of *H. pylori* was performed on blood agar plates after incubation for 2 to 3 days at 37°C under microaerophilic conditions or 5% $CO_2$. For liquid growth, media have to contain a hemin source (heat inactivated Foetal Calf Serum) and growth has to be realized at 37°C under microaerophilic conditions (preferred method) or 5% $CO_2$ with vigorous shaking (190 rpm).

When cultured under favorable conditions *in vitro*, the majority of *H. pylori* bacteria have the spiral or bacillary appearance, while aging or exposure to a variety of unfavorable conditions (including antibiotics) results in conversion to the coccoid form. There is no question that the spiral form is quite effective as infective agent. The role played by coccoid forms in pathogenesis is still unclear, even if they could represent a transition state to a viable but non-culturable state, in which the bacterium can survive in the environment.

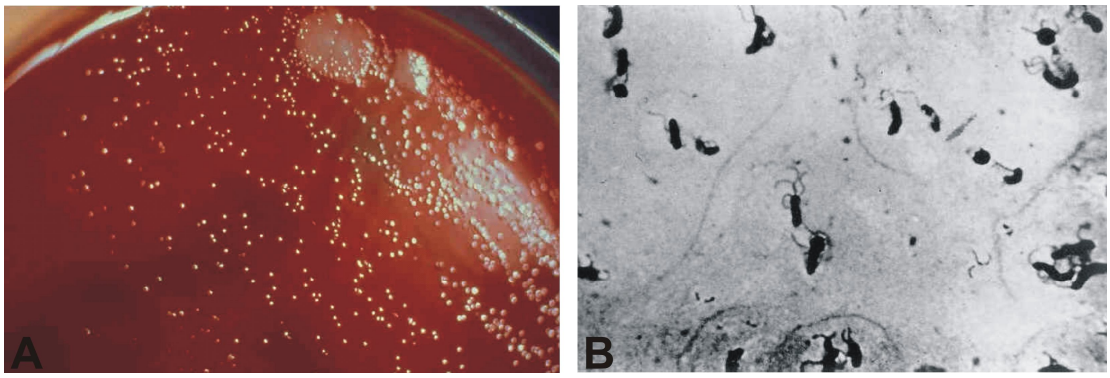


**Figure 1:** A: Two-days colture of *H. pylori* on solid blood-agar plate. B: two days culture of *H. pylori* showing typical comma or spiral shaped roads, both Gram- and motile with multiple sheathed unipolar flagella. Spherical coccoid forms may be present especially in older cultures (x1000).

B

## 8.1 Growth on plate

Plate for *H. pylori* culturing were constituted as it follows: Columbia Blood Agar Base (Oxoid) supplemented with : 5µg/ml Vancomycin (Sigma), 2.5µg/ml Trimethoprim (Sigma), 2.5 µg/ml Cefsulodin (Sigma), 2.5 µg/ml Amphotericin B (Sigma), 0.2% β-cyclodextrin (Sigma), 5% defibrinated horse blood (Oxoid).

For solid plate, 19.5 g of Columbia agar were added to 500 ml of milli-Q water. The solution was boiled to dissolve the powder and sterilized by autoclaving. The solution must be cool to 50°C in a water bath for 1-2 h. Antibiotic were aseptically added, together with β-cyclodextrin to 0.2% final concentration and 25 ml of horse blood (5% final concentration). 25 ml were added in each sterile Petri dishes (10 cm diameter). Plates were cooled overnight at room temperature and then stored inverted at 4°C.

For the preparation of *H. pylori* frozen stocks, approximately $5x10^8$ CFU/ml (0.5 $OD_{535}$ /ml) were resuspedend 1 ml of BHI (brain-hearth infusion) + 5% FBS + 20% glycerol and stored at -80°C.

## 8.2 Growth on liquid

*H. pylori* bacteria were grown in BHI 37 g/l + 5% FBS without antibiotics.

Plate-grown bacteria were collected into 1-2 ml of either BHI broth (see above) with a sterile loop. The bacterial concentration was determined by reading suspension $OD_{600}$, at 1:50 dilution, assuming that 1 $OD_{535}$ = $10^9$ CFUs/ml. 10 µl of bacterial suspension were checked under a direct microscope. If 80-90% of bacteria (by visual inspection)

were spiral form and motile with no large aggregates evident, $2x10^9$ bacterial CFUs were inoculated in 35 ml of BHI broth into a 125 ml Erlenmeyer flask. The cap of the flask should not be completely close. The flask was put into the Oxoid anaerojar with a CampyGen sachet inside, the jar was hermetically closed, and bacteria were grown o/n (16-18 h) at 37° under vigorous agitation (190 rpm).

The expected growth after 16-18 hours would be of around $6x10^8$ CFU/ml on average. The bacteria are checked by visual inspection: if >80-90% of bacilli are spirals and motile with no large aggregates visible, *H. pylori* is ready for infection experiment.

### 8.3 *H. pylori* infection experiments

After checking that the bacteria were spiral and motile (best conditions for infection), 1 ml of bacterial suspension was quantified using a spectrophotometer at $OD_{600}$. This aliquot was then centrifuge at 3000g, for 15' to pellet all bacteria. BHI supernatant was discharged, and pellet was resuspended in 1 ml of fresh BHI, to have the same concentration of the initial aliquot.

For monocytes, DCs, neutrophils and macrophages, the bacterial concentration used for infecting the cells was $5x10^5$ CFU/ml.

## 9 Tissues

Tissues were obtained from the archive of the Department of Pathology (Spedali Civili di Brescia, Brescia, Italy, San Raffaele Hospital, Milan, Italy and Padova Hospital). For APRIL evaluation, samples included normal gastric mucosa (five cases), *H. pylori*-associated gastric lymphoid infiltrates Wotherspoon-Isaacson (WI) grade 1 (five cases), 3 (three cases) 4 (two cases) and grade 5 (nine cases, resection specimens), gastric diffuse large B-cell lymphomas (five cases) and *H. pylori*-negative gastritis (six cases). In the latter group *H. pylori* was assessed by histology (modified Giemsa staining) and confirmed by clinical history, rapid urease testing, and/or ELISA (*H. pylori*-specific IgG antibodies, Gastropanel, Biohit). Another group consisted of matched endoscopic biopsies (pre- and post-treatment) from six patients with gastric MALT lymphoma, which underwent clinical and histological remission following *H. pylori*-eradication therapy. WI-5 cases lack AP12/MALT1 translocation as proved by FISH analysis (MALT1

FISH DNA Probe, Split Signal; Dako Cytomation) and the large majority of them (7 out of 9; 78%) show IgH clonal rearrangements. For the IgH clonality assay, genomic DNA was extracted from tumour tissue sections by using the QIAamp Tissue Kit (Qiagen). Polymerase chain reaction (PCR) was performed using genomic DNA (200 ng) with primers targeting the variable region FR2 or FR3 and joining regions of the IgH gene. The amplified products were visualized in 10% polyacrylamide gel.

For BLyS evaluation samples included normal gastric mucosa (thirteen cases), *H. pylori* infected (thirteen cases), AIG (thirteen cases), AIG with concomitant infection of *H. pylori*, matched endoscopic biopsies (pre- and post-treatment) from nine patients with AIG, which underwent clinical and histological remission following *H. pylori*-eradication therapy, matched endoscopic biopsies (pre- and post-treatment) from eleven patients with *H. pylori* driven chronic gastritis, which underwent clinical and histological remission following *H. pylori*-eradication therapy and *H. pylori* un-related (i.e. Non-Steroidal Anti Inflammatory Drug, NSAID-driven) chronic gastritis (fifteen cases).

## 9.1 Immunohistochemistry

Four micron formalin-fixed paraffin embedded tissue sections were stained with anti-APRIL (Ms IgG1, Clone Aprily-2, Alexis Biochemicals) and anti-*Helicobacter pylori* TMDU (Ms, Clone W4-1, provided by Prof. Eishi , Department of Human Pathology, Graduate School of Medical Science, Tokyo Medical and Dental University, Tokyo, Japan). Upon appropriate antigen retrieval (water bath at 98°C for 40 min in EDTA buffer pH 8.0), reactivity was revealed using NovoLink Polymer HRP linked (Novocastra Laboratories) followed by Diaminobenzidine (DAB). Characterization of APRIL positive cells was performed by double immunohistochemistry. After completing the first immune reaction, the second was realized using primary antibodies to the following antigens: CD11c (Clone 5D11, Novocastra Laboratories Ltd); CD68 (Clone KP-1, Dako); CD20 (Clone L26, Dako); CD163 (Clone 10D6, Thermo Scientific); CD1a (Clone 010, Dako); CD15 (Clone MMA, Thermo Scientific) and visualized using Mach 4-AP (Biocare Medical), followed by Ferangi Blue (Biocare Medical) as chromogen. Quantification of APRIL-expressing cells was performed on at least five HPF (high power field) on sections double stained for APRIL and CD11c. Immunostained sections were photographed using the DP-70 Olympus digital camera mounted on the Olympus BX60

microscope, and the digital pictures (each corresponding to 0.036 $mm^2$) were used for cell count.

For BLyS experiments sections were stained with anti-BLyS (Immunological Science), anti-IL-17 (Santa Cruz) and anti-INF-$\gamma$ (Santa Cruz), using the same protocol used for APRIL.

Values were expressed as the mean ± SD.

## 10 Statistical analysis

Data were reported as mean values ± SD. Statistical significance was calculated by unpaired Student's t-test. A probability (p) of less than 0.05 was considered significant and was indicated with *, a $p < 0.01$ was indicated with ** and a $p < 0.001$ was indicated with ***.

One-way Anova were used for statistical analysis of the differences between experimental groups. p values less than or equal to 0.05 were considered significant.

# Results and discussion

## 1 Gastric MALT Lymphoma

*H. pylori* colonizes the human gastric mucosa and triggers a strong local inflammatory response . Chronic inflammation due to the persistence of *H. pylori* infection can give rise to organized lymphoid tissue in the gastric mucosa, the so-called MALT that, in a small subset of individuals, can ultimately progress to "low-grade gastric B-cell lymphoma of MALT type" [66]. The current model of MALT lymphoma genesis assumes that one or more neoplastic clones, displaying characteristics of marginal zone B cells, originate from the organized MALT, colonize and replace the original follicles and eventually destroy the gastric glands to form lympho-epithelial lesions [7]. Although MALT lymphoma usually grows slowly and has a low propensity to spread, a small percentage of cases undergo high-grade transformation. It is generally accepted that, in early stages of gastric lymphoma development, neoplastic growth is both antigen-driven and dependent of the helper activity of T cells specific for *H. pylori* [68]. The fact that the eradication of the bacterium leads to regression of the lymphoma, especially in its early stages, is consistent with such a postulate [7]. However, less is known about the role of the host immune response in MALT lymphoma pathogenesis.

APRIL is one of the most recently cloned members of the TNF family expressed by a variety of immune cells including neutrophils, monocyte, macrophages, dendritic cells and T-cells, but also epithelial cells and tumour cells [11]. By binding to its BCMA and TACI receptors, APRIL promotes survival and proliferation of B cells as well as their differentiation to plasma cells. As recently reported, APRIL-transgenic mice develops lymphoid malignancies originating from peritoneal B-1 B cells [62]. In addition, not only APRIL is expressed in a wide array of B-cell malignancies, including Hodgkin lymphomas, but high levels of APRIL in the serum of these patients correlate with poor prognosis [122]. Consistently, *in vitro* experiments have clearly shown that APRIL promotes cell survival and proliferation of neoplastic B-cells [77]. Interestingly, APRIL expression in human non-Hodgkin B-cell lymphomas is found in neoplastic cells as well as in associated inflammatory cells, predominantly neutrophils.

The correlation between *H.pylori* infection and MALT lymphoma is now established: indeed, the eradication the bacterium lead to a complete remission of the cancer in more that 80% of cases. Thus, since the cytokine or soluble factor that may promote the rising and the progression of MALT lymphoma are currently unknown we decide to investigate if APRIL could be play a role in MALT lymphoma induction and progression, but most importantly, if *H. pylori* might be the trigger for APRIL secretion. This finding will add an new, undiscovered piece of information about *H. pylori* induced lymphomagenesis.

## 1.1 *H. pylori*-associated gastritis and gastric MALT lymphoma are enriched in APRIL-containing tumour associated macrophages

We initially evaluated by immunohistochemistry whether APRIL might be expressed in gastric MALT lymphomas. To this end, nine cases of *H. pylori*-associated WI-5 (WI, Wotherspoon index) cases lacking the API2/MALT1 translocation, as detected by FISH analysis (not shown), were used. Compared to normal gastric mucosa, in which APRIL reactivity was scanty (fig. 1A), we found numerous cells reacting to anti-APRIL in all WI-5 cases (fig. 1B). The large proportion of APRIL+ cells showed a macrophage-like morphology and were admixed with CD20+ neoplastic B-cells (inset of fig. 1B) and to surrounding plasma cells (not shown).

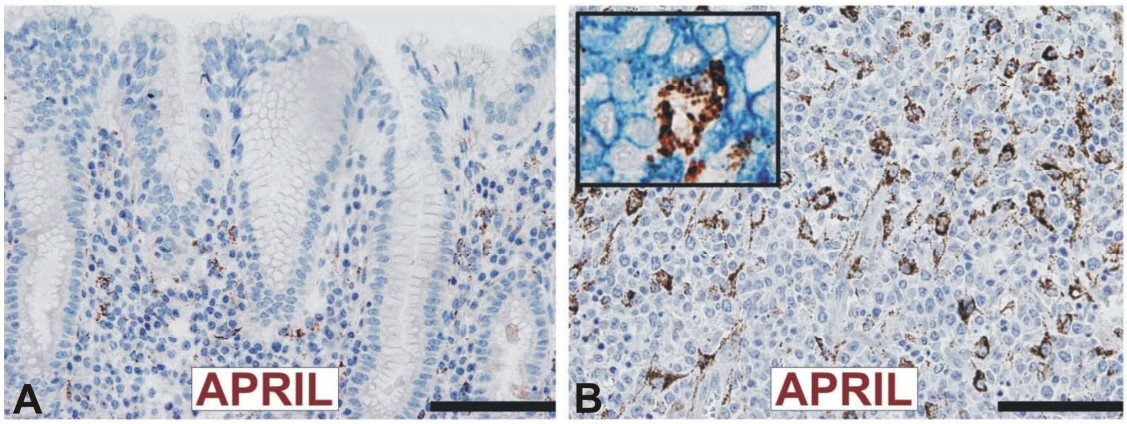


**Figure 1**: Sections are from normal mucosa (A) and MALT lymphoma WI 5 (B); Compared to normal gastric mucosa where APRIL reactivity is observed in rare large cells of the lamina propria (A), numerous APRIL+ cells are found surrounding CD20+ neoplastic cells in MALT lymphoma (B). Scale bar: 100 μm (A, B).

By double immunohistochemistry of five representative cases, APRIL+ cells were negative for lymphoid markers CD20 and CD3 (inset of fig. 1B and not shown) but

strongly reacting to CD11c, CD68 and CD163 but not to CD1a, thus confirming their macrophage identity (fig. 2A-C and data not shown). In addition to macrophages, a very small fraction of CD15+ polymorphonuclear cells also reacted to APRIL (not shown), while, in all cases, no reactivity for APRIL was observed in the large majority of neoplastic cells (fig. 1B, 2A-C ).

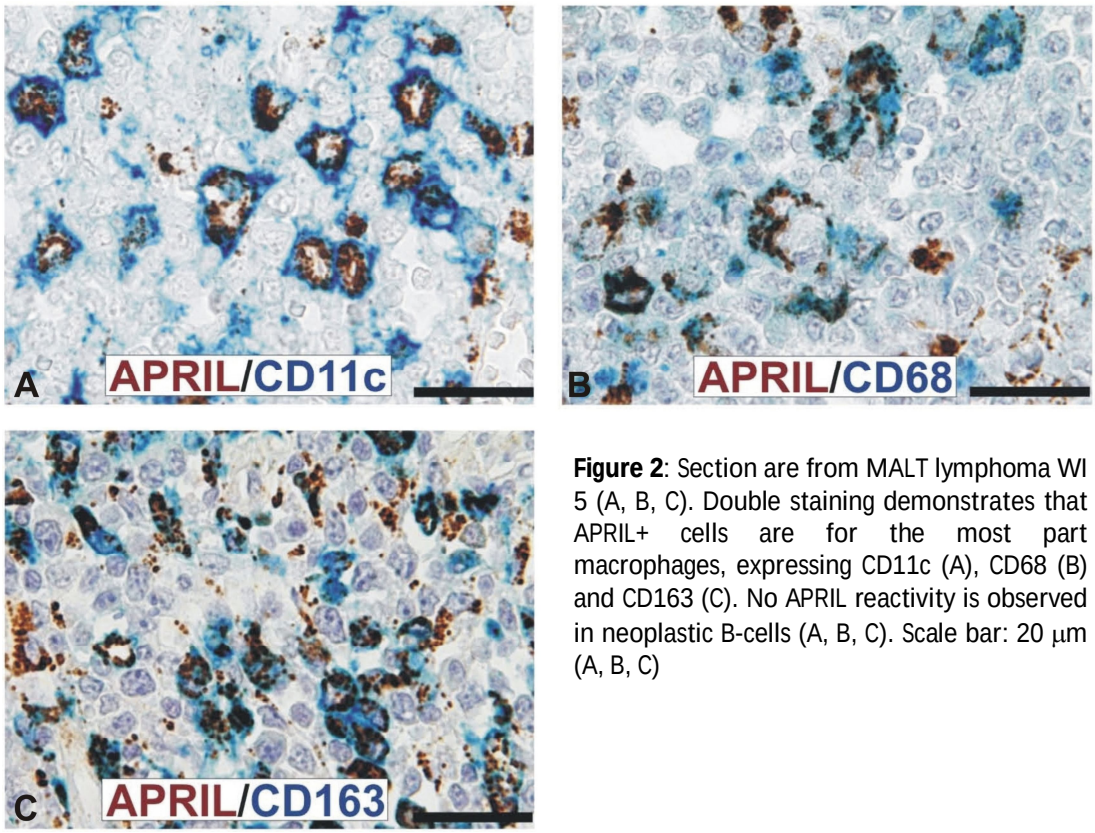


**Figure 2**: Section are from MALT lymphoma WI 5 (A, B, C). Double staining demonstrates that APRIL+ cells are for the most part macrophages, expressing CD11c (A), CD68 (B) and CD163 (C). No APRIL reactivity is observed in neoplastic B-cells (A, B, C). Scale bar: 20 μm (A, B, C)

A further characterization of APRIL reactivity revealed numerous APRIL+ macrophages also in *H. pylori*-associated WI grade 1 to 4 (fig. 3). Of note, the number of APRIL-producing macrophages was significantly higher in WI lesions as compared to normal gastric mucosa and *H. pylori*-negative gastritis, being the mean number of CD11c+ APRIL+ cells respectively 22.2 [WI-1], 22.6 [WI-3/4] and 28.5 [WI-5] *vs* 7.2 [normal mucosa] and 9.2 [*H. pylori*-negative gastritis] ($p<0.01$); on the contrary, differences among WI groups were not significant (p: 0.07). Remarkably, in matched biopsies obtained from six MALT lymphoma patients post eradication treatment, the number of CD11c+APRIL+ macrophages was dramatically reduced (19.6 vs 4.1; $p<0.01$) in WI-5 cases which underwent remission following *H. pylori*-eradication treatment (fig. 3). Notably, local production of APRIL was not exclusive of MALT lymphomas, since

numerous CD11c+APRIL+ cells were observed also in cases of *H. pylori* negative gastric diffuse large B-cells lymphomas (not shown).

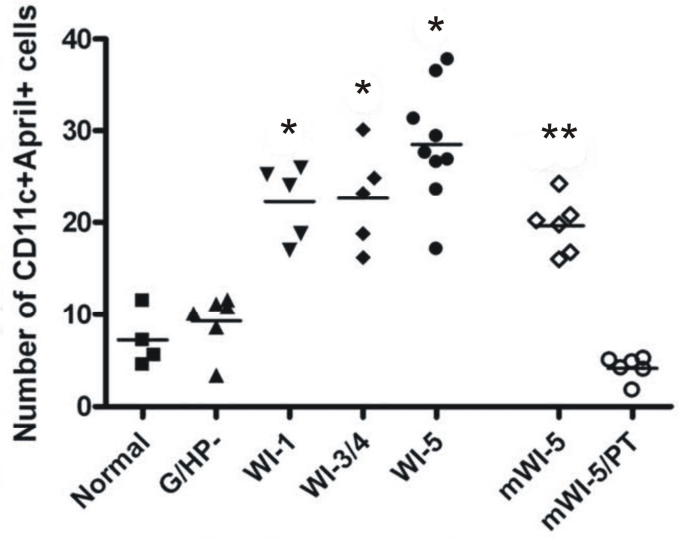


**Figure 3**: APRIL expressing macrophages (positive for both CD11 and APRIL) were counted at different stages of MALT lymphoma progression. Abbreviations: G/HP-, HP-negative gastritis; mWI-5, matched WI-5; mWI-5/PT, matched WI-5 post-eradication treatment. Red hashes indicate significance versus normal and G/HP- (*, p< 0.01) or versus mWI-5/PT (**, p< 0.01)

Altogether, these results strongly support the notion that macrophages represent a relevant source of APRIL available from the early phases of MALT lymphoma progression.

## 1.2 *H. pylori*-infected macrophages produce APRIL in gastric mucosa

It was recently reported that cells containing *H. pylori*-derived products can be found in the lamina propria of patients with gastric cancer and chronic gastritis, confirming early and more recent studies proving that mucosal invasion by *H. pylori* might occur in *H. pylori*-dependent pathologies. By using the same antibody (recognizing an *H. pylori*-specific form of LPS), we were able to confirm this finding on cases of WI grade 1 densely colonized by *H. pylori*. In particular, in addition to its classical localization in the gastric surface epithelium or in the spaces between mucous-secreting cells, we detected anti-*H. pylori* reactivity within the cytoplasm of macrophages localized in multiple foci in the gastric lamina propria (fig. 4A,4B).

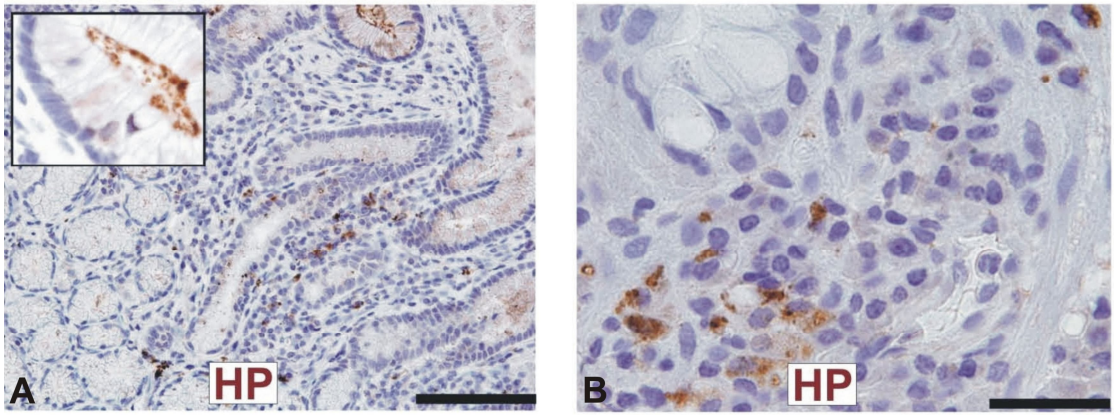


**Figure 4**: Section are from gastric mucosa WI 1; *H. pylori* reactivity is observed in the cytoplasm of numerous cells in the gastric lamina propria (A, B). The anti *H. pylori* antibody recognizes bacilli also in the gastric mucous layer (B, inset). Scale bar: 100 μm (A) and 20 μm (B)

Remarkably, *H. pylori*-containing macrophages were positive for CD11c, CD163 and CD68 (fig. 5A-C), as well as intensely stained by the anti-APRIL antibody (fig. 5D).

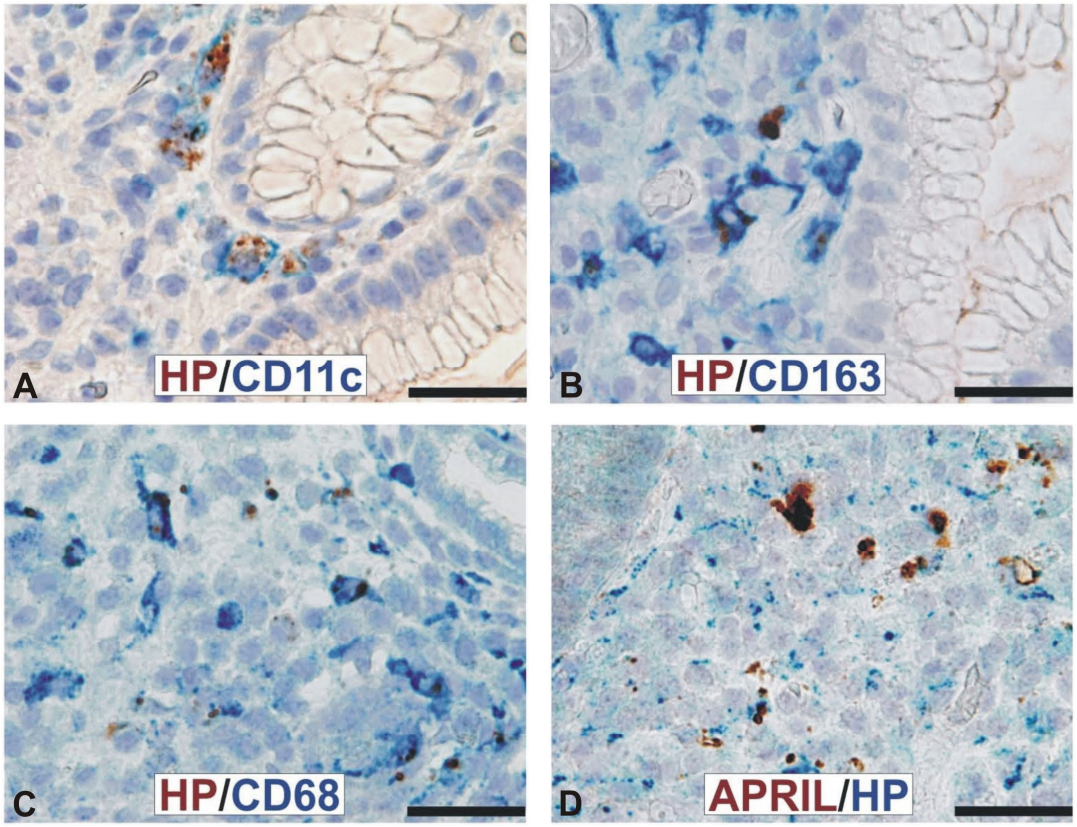


**Figure 5:** Sections are from gastric mucosa WI 1. Double stain demonstrates that *H. pylori*-containing cells are represented by macrophages co-expressing CD11c (A), CD68 (B) and CD163 (C) and some of them produce APRIL (D). Scale bar: 20 μm (A-D)

These results confirm that *H. pylori* is able to cross gastric epithelium and, most importantly, that macrophages infiltrating gastric mucosa since the first stage of progression of MALT lymphoma (i.e. chronic gastric) engulf bacteria. The presence of *H. pylori* correlates with the presence of APRIL in macrophages suggesting a

correlation between the engulfment of bacteria and the production of APRIL during MALT lymphoma progression.

## 1.3 *H. pylori* triggers APRIL secretion in macrophages

Based on the data from tissue sample evaluations, we subsequently addressed whether *H. pylori* could trigger, directly or indirectly, APRIL production by macrophages *in vitro*. Given the peculiar mechanisms of APRIL production and secretion, we decided to monitor if there is any protein accumulation during the differentiation of monocytes into macrophages following M-CSF exposure. APRIL is barely detectable in freshly purified monocytes, but it is actively produced at mRNA and protein level during monocytes differentiation to macrophages (fig. 6A and 6B); however, as quantified by ELISA of the culture supernatant (not shown), APRIL was not significantly released at any time point during the culture.

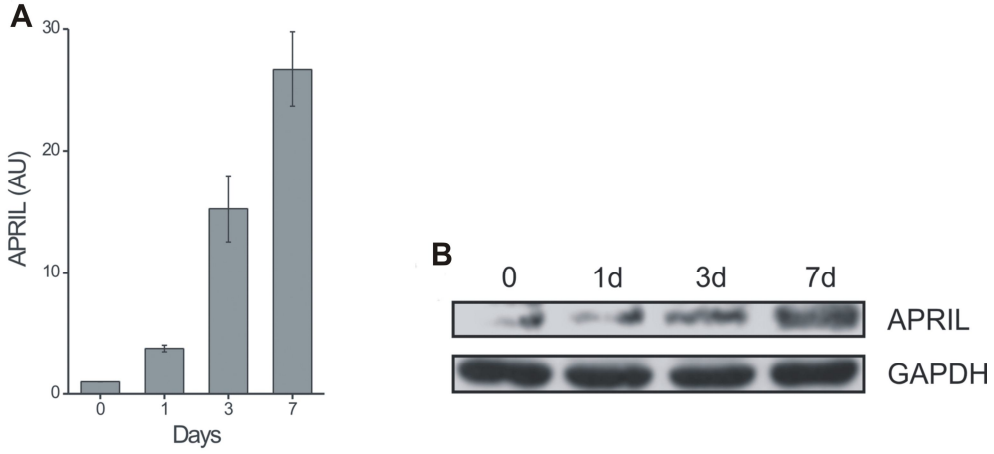


**Figure 6:** Monocytes exposed to M-CSF were harvested at the indicated time points and APRIL mRNA was quantified by Real time PCR (A). In parallel the APRIL protein cell content was determined by western blot. GAPDH was used as marker of equal loading (B).

*H. pylori* exposure of M-CSF-differentiated human macrophages (MDM) induces a time-dependent expression of APRIL mRNA (fig. 7A) in parallel with the secretion of mature protein in culture supernatants (fig. 7B). Addition of *H. pylori* to MDM also triggered a time-dependent accumulation of intracellular APRIL (fig. 7C).

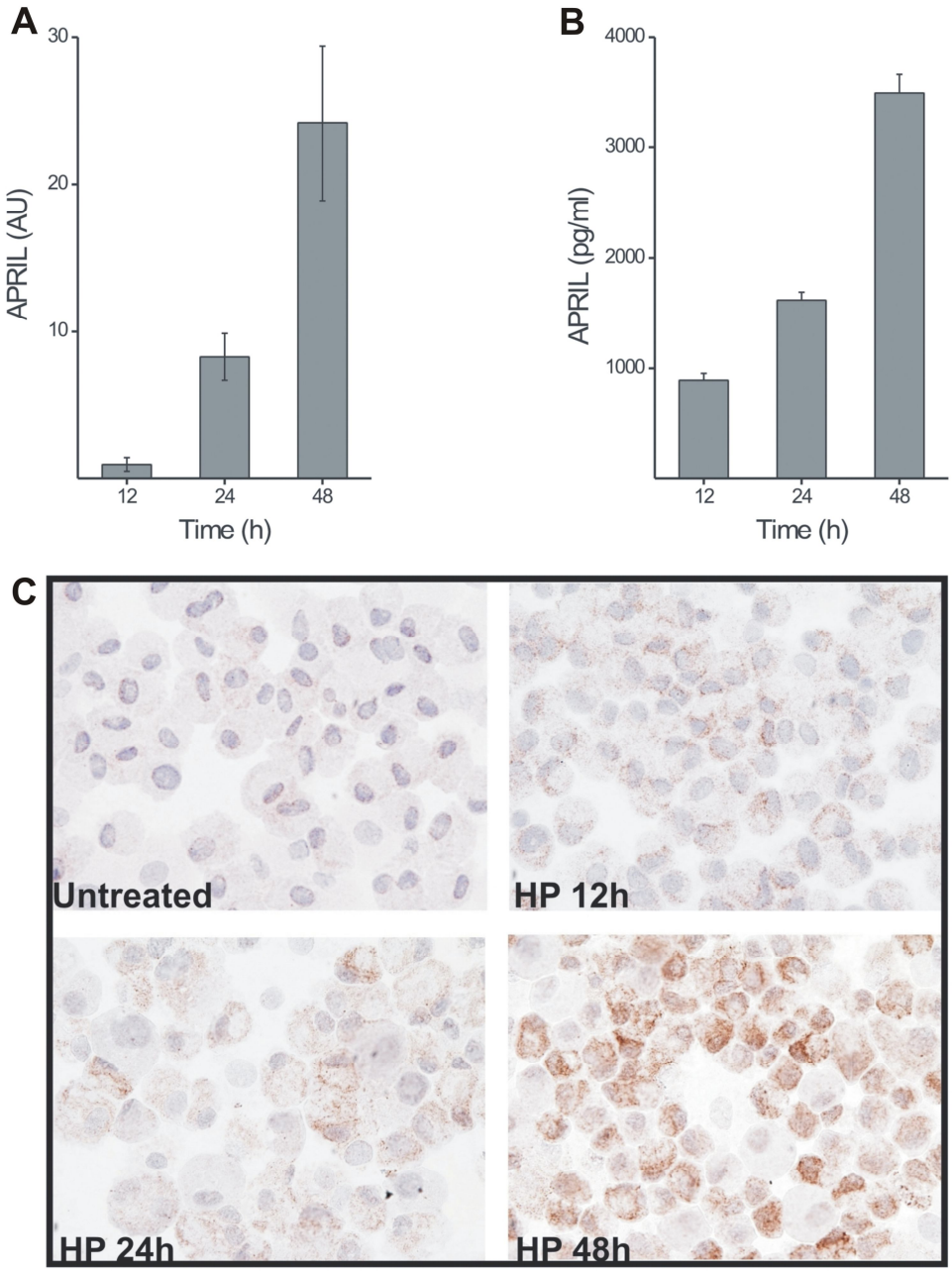


**Figure 7**: M-CSF-differentiated macrophages (MDM) were exposed to *H. pylori* ($5 \times 10^5$ CFU/ml). At the indicated time-points cells were harvested for mRNA evaluation (A) and culture supernatant collected for APRIL content determination (B); in parallel, MDM cytospins were labelled for their intracellular APRIL content (C).

Interestingly, neither monocytes nor neutrophils showed any increased expression of APRIL or secretion of the mature cytokine, following *H. pylori* infection. The same results were obtained with dendritic cells (DCs), another kind of antigen-presenting cell, very abundant in the infiltrates occurring during *H. pylori* infection (data not shown).

Our results pointed up macrophages as the only source of this B-cell activating factor during the development of gastric MALT lymphoma.

## 1.4 *H. pylori*-specific T helper (Th) clones stimulate APRIL production from autologous macrophages

Proliferation of neoplastic B cells in MALT lymphoma is sustained by both the antigen and the tumour-infiltrating helper T-cells. To test the hypothesis that *H. pylori*-specific T-cells might sustain macrophage-derived APRIL production, we studied the helper function of clones of *ex-vivo* freshly purified T cells obtained from antral biopsies of five patients with uncomplicated *H. pylori* chronic gastritis and five *H. pylori*–associated gastric low-grade MALT lymphoma (see Material and Methods for details). By measuring the [3H]TdR uptake, we screened our T-cell clones for *H. pylori*-induced proliferation in MHC-restricted conditions and obtained seventy-one *H. pylori*-specific T helper (Th) clones from patients with *H. pylori*-associated low grade MALT lymphoma and fifty *H. pylori*-specific T helper clones from patients with *H. pylori* chronic gastritis without MALT lymphoma. To test the helper ability of *H. pylori*-specific T-cells for APRIL production, *H. pylori*-specific T-cell clones were co-cultured for 16 h with autologous monocyte-derived macrophages in the presence of *H. pylori* antigen. Remarkably, as demonstrated by ELISA, *H. pylori*-specific Th clones from chronic gastritis and, even more strongly, those purified from MALT lymphoma cases were able to stimulate the production of variable amounts of APRIL protein in an antigen-specific manner.

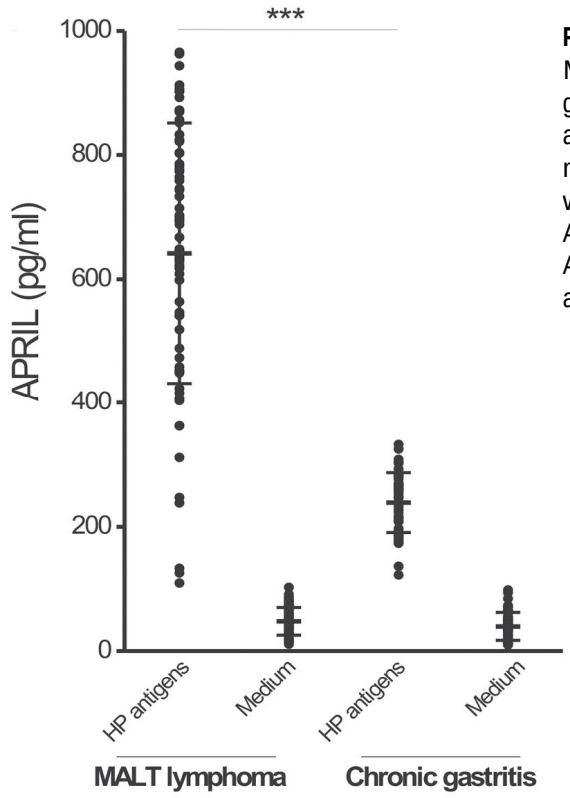


**Figure 8**: *H. pylori*-specific Th cells isolated from MALT lymphoma patients and from chronic gastritis patients were co-cultured with autologous macrophages in the presence of medium or *H. pylori* and the APRIL production was assessed by ELISA (F). Results represent the APRIL levels induced by T cell clones over the APRIL production in cultures of macrophages alone. ***, $p<0.01$

In this study, we have demonstrated that *H. pylori* infection in the gastric mucosa might sustain the local production of APRIL, a tumor necrosis factor superfamily member known to be important for B-cell development, maturation and survival. In particular, numerous APRIL-producing macrophages are found in *H. pylori*-infected chronic gastritis and MALT lymphoma biopsies, while they are very rare in normal mucosa. Notably, a fraction of APRIL-positive macrophages contain *H. pylori*-derived products; moreover, *in vitro H. pylori* infection induces expression of APRIL in monocyte-derived macrophages, thus indicating a primary role of the bacterium in promoting monocyte-derived cells to produce APRIL in infected gastric mucosa. A large body of experimental and clinical evidence suggests that APRIL sustains B-cell transformation and progression of B-cell lymphomas. Different forms of B-cell lymphoma were found to contain APRIL-positive cells, that were represented either by neoplastic B-cells and by tumour associated immune cells, including macrophages [122]. In keeping with these observations we have documented that APRIL+ macrophages are also abundant in gastric diffuse large B-cell lymphomas devoid of *H. pylori* infection. These observations indicate that the induction of APRIL in lymphomas partially reflect the local activation of tumor-associated macrophages likely mediated by different mechanisms. In fact, Craxton et al. reported that human macrophages but

not monocytes or monocyte-derived dendritic cells constitutively express high levels of APRIL [123]. Furthermore, the expression of APRIL has been detected in macrophage-like cell lines, monocyte-derived macrophages and inflammatory macrophages. Stimuli mediating APRIL release by macrophages are largely unknown. We found that APRIL is gradually induced but not released by macrophages during M-CSF-dependent differentiation from monocytes. However, we provide evidence that APRIL release by macrophages depends on *H. pylori,* either following a direct stimulation by the bacteria, or by *H. pylori*-specific T cells, especially those purified from overt MALT B-cell lymphoma.

The role of *H. pylori* in gastric MALT lymphoma is well established, considering that chronic *H. pylori* antigen stimulation is essential for their progression [65]. Survival and proliferation of transformed B-cells in gastric MALT lymphoma require signals from tumour-associated T-cells or direct auto-antigen stimulation of neoplastic cells [7]. Our data suggest that tumour associated macrophages might sustain progression of MALT lymphoma by releasing APRIL. We propose that *H. pylori*-driven gastric inflammation leads to the generation of a local pool of macrophages that upon *H. pylori* infection release large amount of APRIL. This functional loop can be further amplified and perpetuated by *H. pylori*-specific T-cells. Remarkably, as proof of principle of this postulate, APRIL-producing macrophages are dramatically reduced upon lymphoma remission induced by *H. pylori*-eradication.

Our results provide a new piece of information to *H. pylori*-dependent MALT lymphomagenesis pointing for tumour-associated macrophages as contributors of tumour progression *via* APRIL release. Thus, based on these findings, we suggest that, among biological therapies targeting B cells, blockade of APRIL might represent a novel additional therapeutic strategy for gastric MALT lymphoma, supporting the notion that the targeting tumour-associated macrophages and their products can be particularly relevant in lymphoid malignancies.

# 2 B-Lymphocyte stimulator (BLyS) in the pathogenesis of *H. pylori*-induced AIG

Persistent bacterial infections can trigger an autoimmune disease in genetically susceptible individuals. A well-known example is represented by the bacterium *B.burgdorferi*, the etiological agent responsible for Lyme arthritis. It has been demonstrated that the development of Lyme arthritis is driven by the molecular mimicry between an epitope of *B.burdorferi* of the outer membrane protein OspA and a particular T lymphocyte population that resides specifically in the joints of these patients. Lyme arthritis may eventually evolve into an autoimmune form of synovitis [46].

Similarly to what happens with *B. burgorferi*, it has been demonstrated that chronic infection by *H. pylori* can lead to, or accelerate the development of, AIG in genetically susceptible individuals. Indeed, 20-30% of *H. pylori* - infected individuals develop autoantibodies which recognize the $H^+$ $K^+$-ATPase gastric pump. Interestingly, the presence of these autoantibodies is positively related to the severity of gastric inflammation and increased corpus atrophy [102].

So far, the Th1 response in *H. pylori* infection has been intensively studied, and it is established that most part of $H^+$ $K^+$-ATPase-specific T cell clones are Th1; less attention has been devoted to another T helper cell subset, which is as important as the Th1 in *H. pylori* infection, the Th17. Notably, a study carried out in mice by Stummvoll and colleagues [110] demonstrated that Th1, Th2 and Th17 cells are all able to induce AIG, but the Th17 cells exert the most destructive activity.

As described previously, BLyS is involved in several autoimmune disorders such as Systemic lupus erithematous (SLE), arthritis, and psoriasis. The cytokine is up-regulated in these patients and usually high serum levels of BLyS are positively related with a poor prognosis [2].

BLyS potently activates auto-reactive B-cells (which, in turn, up-regulate the receptor for BLyS) that start proliferating and producing large amount of autoantibodies.

Interestingly, it has been recently suggested a role for BlyS not only on self-reactive B-cells, but also on the development of a Th17 response in autoimmune diseases.

Indeed, Lam Kwan Lai and colleagues demonstrated that knocking down BLyS specifically in the joint of mice with collagen induced arthritis (CIA), results in a better outcome of the disease, due to the turning off of the Th17 response [120].
Moreover, Zhou and collaborators demonstrated that BLyS is able to promote a Th17 profile that aggravates the outcome of experimental autoimmune encephalomyelitis[121].

Based on these premises, we decided to evaluate whether BLyS could play a role in the Th17 response induced by *H. pylori* and, eventually, if the Th17 response might be important for *H. pylori*-induced AIG development.
Our hypothesis is that an axis between BLyS and the Th17 response exists and that it could lead, or exacerbate, AIG in susceptible patients infected by *H. pylori.*

## 2.1 *H.pylori* infection are characterized by a Th17 response

Chronic gastritis is an inflammatory state that might precede the development of AIG in genetically susceptible individuals [101]. Indeed, in humans, the strong inflammation induced by *H. pylori* in chronic gastritis is sufficient to partially breakdown gastric mucosal tolerance of $H^+$ $K^+$-ATPase-specific T-cell; therefore, a possible evolution of chronic gastritis in AIG is likely [104].

In the first set of experiments, we evaluated the expression of IL-17 in gastric biopsies from a group of 15 patients with *H. pylori*-related chronic gastritis before and after the eradication of the bacterium. In particular, we evaluated the expression of IL-17 in the biopsies in terms of both mRNA (by real time PCR) and protein (by immunohistochemistry) (fig. 9).

According to already published data, we found that in *H. pylori*-driven chronic gastritis a IL-17 was present. Interestingly, after *H. pylori* eradication the expression of IL-17 dropped down (fig. 9A), confirming that *H. pylori* is crucial for the induction of a Th17 profile.

In order to confirm the relevance of the bacterium for the induction of the Th17 response, we compared the expression of IL-17 in *H. pylori*-induced gastritis with that of gastritis induced by Non-Steroidal Anti-Inflammatory Drugs (NSAID). Although the latter had histological features similar to those of *H. pylori*-induced gastritis, it showed a weaker expression of IL-17, with respect to *H. pylori* infected patient, both at mRNA (fig. 9A) and protein levels (fig. 9E). Quantification of IL-17-expressing cells was performed on at least ten HPF (high power field) on sections stained for IL-$17^+$ (fig. 9B-E). In normal tissues (fig. 9B), NSAID gastritis (fig. 9E), and after the eradication of the bacterium (fig. 9D), few cells were positive for IL-17 0.5 ± 0.7 and 0.3 ± 0.5 respectively). On the contrary, in *H. pylori*-driven gastritis an average of 1.4 ± 1.1 cells were positive in each HPF considered (fig. 9C). Despite the high difference in terms of mRNA expression, only weak differences were detected in immunohistochemistry for IL-17 expressing cells. This difference could be explained by the fact that IL-17 is a secreted cytokine, so it would be difficult to found it inside the cells.

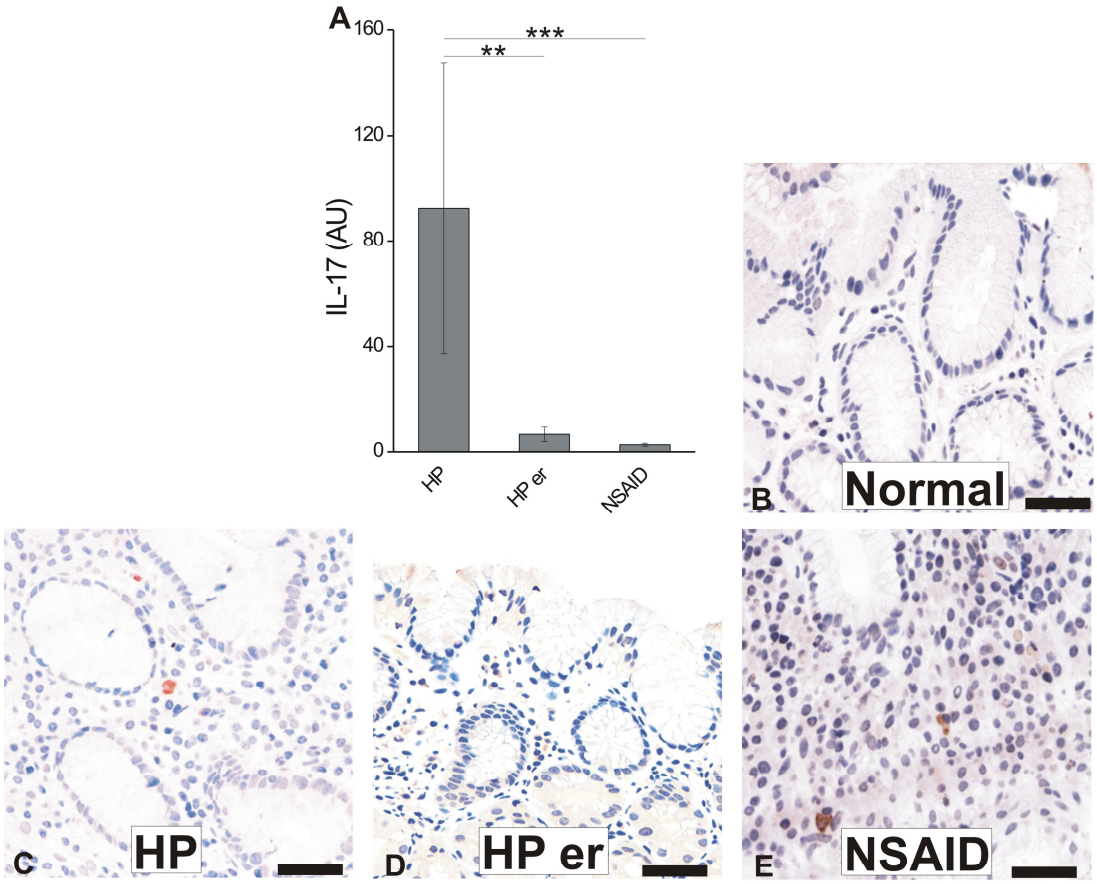


**Figure 9:** IL-17 expression is evaluated by real time analysis on mRNA from gastric biopses of patients with *H. pylori*-induced chronic gastritis (n=15) pre- and post- treatment for *H. pylori*, and patients with NSAID-driven gastritis (n=15) (A). Values are normalized to GADPH. Data are expressed as n-fold of IL-17 expression in normal tissues. Biopsies are from normal individuals (B), *H. pylori*-driven chronic gastris (C), same group of patients of C after eradication of *H. pylori* (D), NSAID-driven gastritis (E). Sections are stained with an anti-IL-17 antibody and revealed with DAB. Scale bar: 100 μm.

Interestingly, all the mRNAs encoding for the cytokines involved in the Th17 differentiation were up-regulated in the same biopsies in presence of *H. pylori* (fig. 10A-E).

On the contrary NSAID-induced chronic gastritis showed a faint increase of IL-6, TGF-β and IL-23 expression; among the pro-th17 cytokines the only exception was represented by IL-1β whose the expression was higher than that of *H. pylori*-positive patients (fig. 9B). The over-expression of IL-1β is not surprising because it is a pleiotropic pro-inflammatory cytokine, which is up-regulated in many inflammatory contexts.

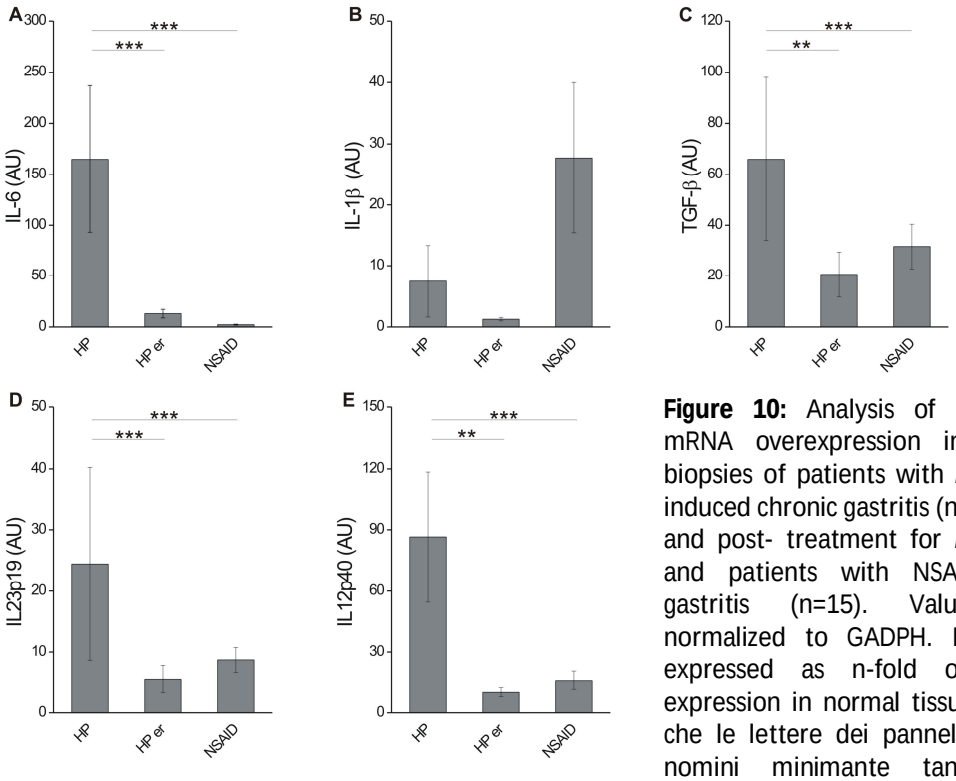


**Figure 10:** Analysis of cytokines mRNA overexpression in gastric biopsies of patients with *H. pylori*-induced chronic gastritis (n=15) pre- and post- treatment for *H. pylori*, and patients with NSAID-driven gastritis (n=15). Values are normalized to GADPH. Data are expressed as n-fold of genes expression in normal tissues. Visto che le lettere dei pannelli non le nomini minimante tanto vale cavarle.

## 2.2 BLyS is up-regulated in *H.pylori* infection

Considering that several studies suggested that the cytokine BLyS might be involved in the induction of the Th17 profile in autoimmune disease, we moved to evaluate the expression of BLyS in biopsies from chronic gastritis of *H. pylori*-infected patients, being gastritis the pathological substrate for AIG development.

We found that the expression of BLyS was up regulated in chronic gastritis associated to *H. pylori* (fig. 11A) and, notably, BLyS expression dropped down after the eradication of the bacterium (fig. 11A). Accordingly, in biopsies from subject treated for bacterium (fig. 11D), few cells were positive for BLyS (7.9 ± 1.4), while the staining was impressive before the antibiotic treatment (20.6 ± 1.8) (fig. 11C). As expected, in normal mucosa only 1.6 ± 0.5 cells were positive in each HPF (fig. 11B). Collectively, these observations indicate that BLyS expression in gastric mucosa is strictly related to *H. pylori* infection. In case of NSAID-induced gastritis BLyS, despite a slight expression in terms of mRNA, BLyS was clearly detectable by immunohistochemistry, although to a less extent than in *H. pylori* patients (14.5 ± 3.7) (fig. 11E).

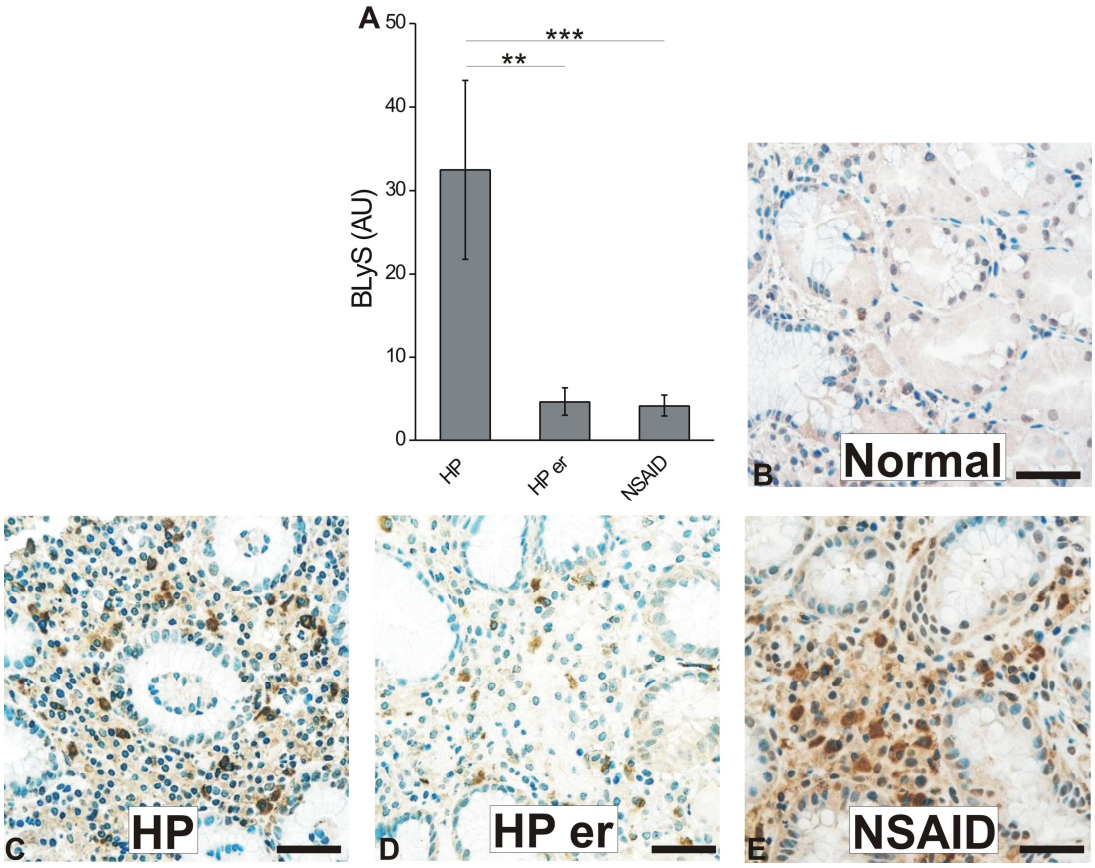


**Figure 11:** BLyS expression is evaluated by real time analysis on mRNA from gastric biopses of patients with *H. pylori*-induced chronic gastritis (n=15) pre- and post- treatment for *H. pylori*, and patients with NSAID-driven gastritis (n=15). Values are normalized to GADPH. Data are expressed as n-fold of BLyS expression in normal tissues (A). Biopsies are from normal individuals (B), *H. pylori*-driven chronic gastris (C), same group of patients of C after eradication of *H. pylori* (D), NSAID-driven gastritis (E). Sections are stained with an anti-BLyS antibody and revealed with DAB. Scale bar: 100 μm.

Taken together these data suggest that BLyS might be involved in gastric inflammation elicited by *H. pylori* and open the possibility that such a cytokine may actively participate in inducing the Th17 profile as demonstrated in other pathological conditions [120, 121] .

## 2.3 BLyS triggers Th17 response through the intervention of innate immune cells

Even if it has been demonstrated the role of BLyS in the induction of the Th17 response in two models of autoimmune diseases in mice [120, 121], it remains unclear if such a relation exists also in humans. Moreover it remains unclear if BLyS acts directly on T lymphocytes promoting their differentiation towards the Th17 lineage or if it requires the participation of other cells or mediators. There is the evidence of a

direct role of BLyS in the generation of Th17 cells [121], however being BLyS secreted by, and acting on, almost all immune cells, it is conceivable that it may also have an indirect role on the Th17 cell differentiation, involving other immune cells.

Being monocytes and macrophages abundantly infiltrating the gastric mucosa of *H. pylori* infected patients, we assessed the effect of BLyS administration to these two type of cells, in terms of induction of pro-Th17 cytokines. mRNA and protein levels of pro-Th17 cytokines were determined after BLyS stimulation of monocytes isolated from healthy donors. Following stimulation, IL-1β mRNA was found to be up to 400 times higher (fig 12A-B); BLyS induced also IL-6 expression that peaked at 5 h. Furthermore, also TGF-β was found to be up-regulated in BLyS stimulated monocytes. Accordingly, high levels of the mature cytokines were detected in the cell supernatants after 24 h.

Finally, BLyS was able to induce the expression of both IL-12p40 and IL-23p19, the two subunits forming the mature cytokine IL-23 which is crucial for Th17 cells proliferation.

The effect of BLyS on the induction of pro-Th17 cytokines in monocytes was dose-dependent: indeed, we detected cytokines' expression and secretion, although to a less extent, even when the concentration of BLyS administrated to the cells was reduced by 10 times, from 50 ng/ml to 5 ng/ml (not shown). However, It is likely that *in vivo*, being infiltrating cells in close contact with each other, the local concentration of BLyS might be even higher that 50 ng/ml.

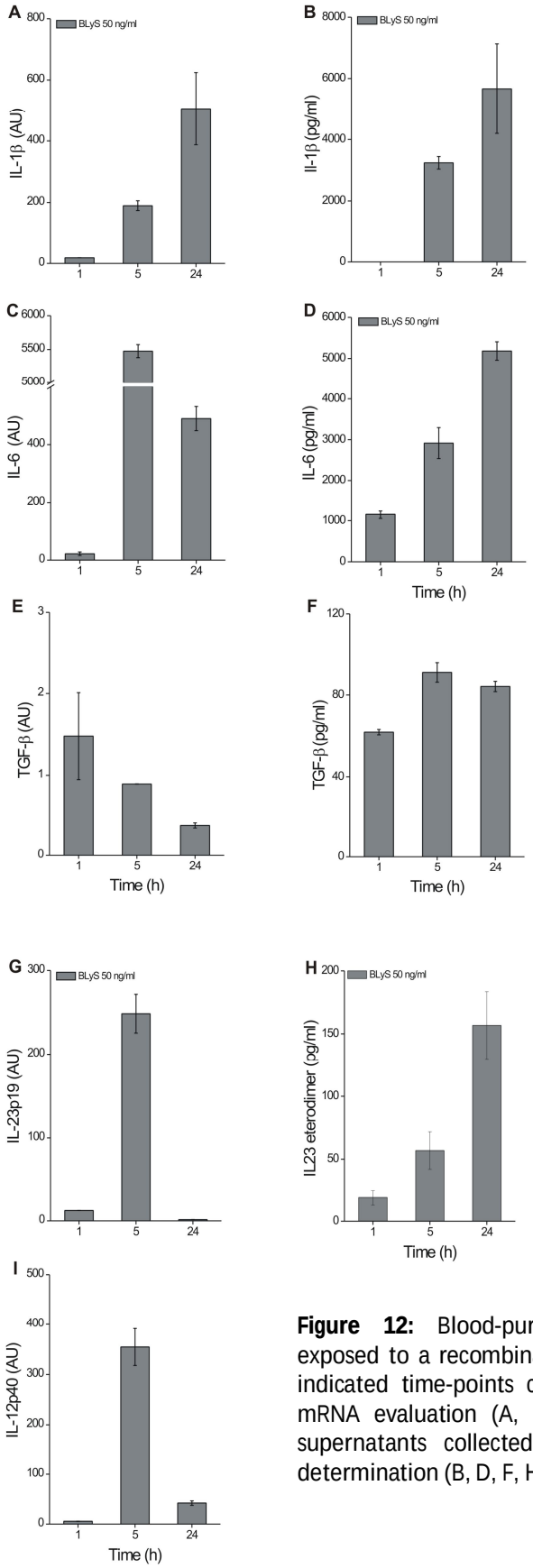


**Figure 12:** Blood-purified monocytes were exposed to a recombinant form of BLyS. At the indicated time-points cells were harvested for mRNA evaluation (A, C, E, G, I) and culture supernatants collected for cytokines content determination (B, D, F, H); AU, arbitrary units.

Our data indicated that BLyS increased the expression and secretion of all the cytokines that are involved in Th17 differentiation and proliferation; moreover, they confirm that BLyS is able to activate monocytes (probably upon TACI engagement, that is highly expressed by monocytes) and suggest the possibility that BLyS might exert an indirect role in inducing the Th17 response in humans. Obviously, the possibility that BLyS acts also directly on T cells exists, and we are currently addressing this possibility.

Since macrophages infiltrate abundantly gastric mucosa during *H. pylori* infection as well as monocytes, we decided to consider macrophages as other possible players in our scenario. Macrophages differentiate locally from circulating monocytes and they play a pivotal role in antigen presentation and activation of T lymphocytes.

BLyS might act locally in gastric mucosa for the induction of macrophages differentiation, as reported [124], but on the other hand it could also act as an activating stimulus for resident macrophages.

BLyS-exposed macrophages up-regulated the mRNAs for IL-6, IL-1β, TGF-β, IL23p19 and IL12p40 (fig13A, C, E, F, H), and they secreted the mature forms of the cytokines (fig. 13 B, D, G) although to an extent significantly lower than that detected for monocytes. This marked difference could be explained by the fact that TACI expression, which is very high in freshly isolated monocytes, might decreases during differentiation, probably due to M-CSF. Since it has been demonstrated that BLyS induces macrophages differentiation, it is possible that fully differentiated macrophages become anergic to BLyS. Our data suggest that macrophages may play only a marginal role in the induction of the Th17 response following BLyS treatment.

Despite this, there is the evidence (ref) “and our unpublished observation”), that *H. pylori*-infected macrophages polarize Th cells towards the Th17 profile, by secreting pro-Th17 cytokines following *H. pylori* stimulation. Therefore, during *H. pylori* infection, locally released BLyS would act on monocytes creating an environment enriched in pro-Th17 cytokines such as IL-6, IL-1β, TGF-β, IL23p19 and IL12p40. However, *H. pylori* by itself would be able to stimulate macrophages to release the same cytokines [111].

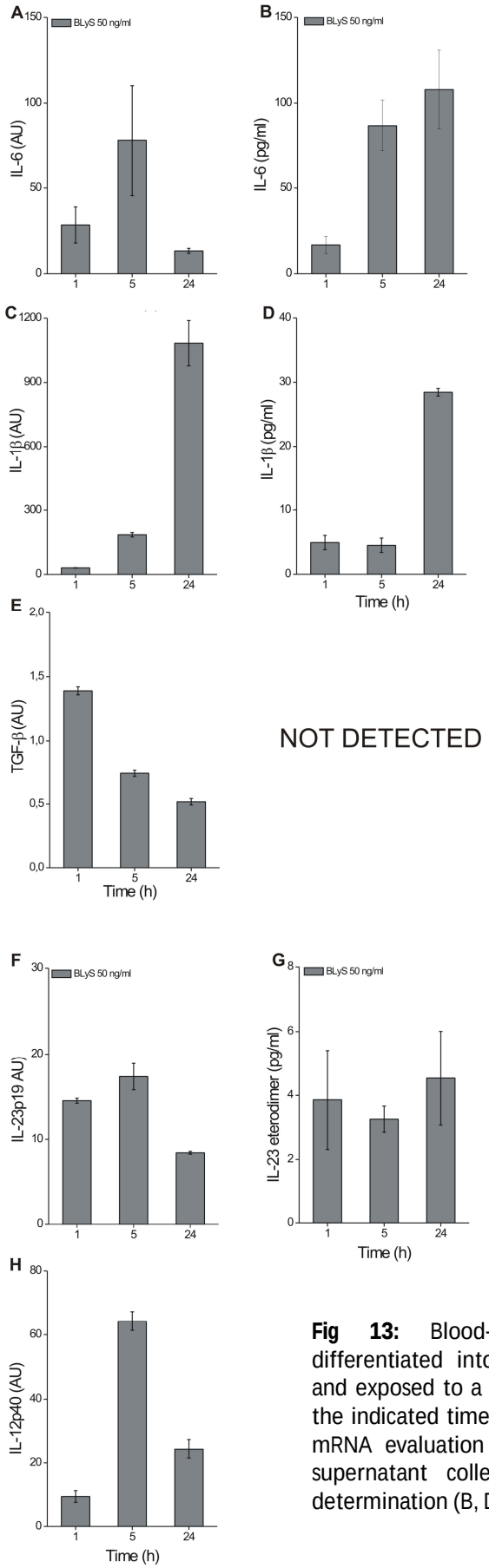


**Fig 13:** Blood-purified monocytes were differentiated into macrophages using M-CSF, and exposed to a recombinant form of BLyS. At the indicated time-points cells are harvested for mRNA evaluation (A, C, E, F, H) and culture supernatant collected for cytokines content determination (B, D, G); AU, arbitrary units.

## 2.4 Neutrophils represent a relevant source of BLyS when exposed to *H. pylori*

Although it appeared clear that the chronic gastritis induced by *H. pylori* was enriched in BLys, the source of this cytokine remained unknown.

Several studies reported that neutrophils are able to secrete a great amount of BLyS in different inflammatory context. For example, Cassatella and colleagues studied the dynamics of the inflammatory response and the effector functions of neutrophils in healthy individuals and in rheumatoid arthritis (RA) patients [5].

They demonstrate that BLyS production is induced in neutrophils by G-CSF, although its secretion occurred only following the administration of a pro-inflammatory stimulus (fig. 14) [5].

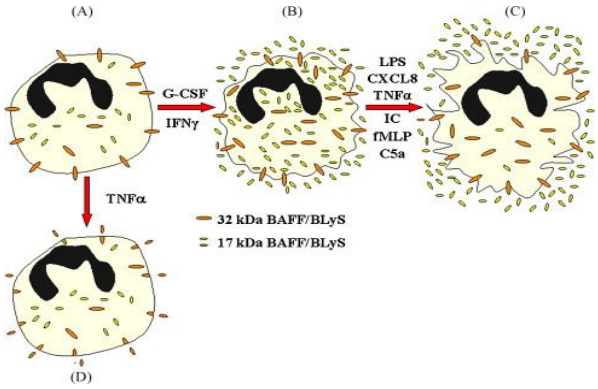


**Figure 14:** mechanism of BLyS secretion in human neutrophils. G-CSF or IFN-γ activate the synthesis of both BLyS and a partial release of it (B). Exposure of primed neutrophils to pro-inflammatory mediators, including TNFα, fMLP, LPS, triggers the secretion of BLyS (C). Alternatively, exposure of resting neutrophils to TNFα induces weak secretion of BLyS (D). Figure adapted from [5].

Furthermore, their experiments revealed that the intracellular concentration of BLyS in neutrophils from RA patients was higher than those found in healthy individuals [115].

Taken together, this evidence suggested that neutrophils are able to produce BLyS during an acute inflammatory response.

Chronic active gastritis is typically characterized by a massive infiltration of neutrophils besides monocytes/macrophages [9]. Notably, neutrophils do not express any BLyS receptor therefore we did not considered them as potential target for Blys but, conversely, we hypothesized that they might represent the main source of BLyS also in *H. pylori* infection.

For this purpose, neutrophils from healthy donors were exposed to *H. pylori* for different time points, with or without a preliminary 20h priming with G-CSF. Primed neutrophils had high intracellular concentration of BLyS and abundant cytokine was

secreted following *H. pylori* stimulation. On the contrary, in not primed neutrophils the total amount of BLyS (both intracellular and secreted) was significantly lower (fig. 15).

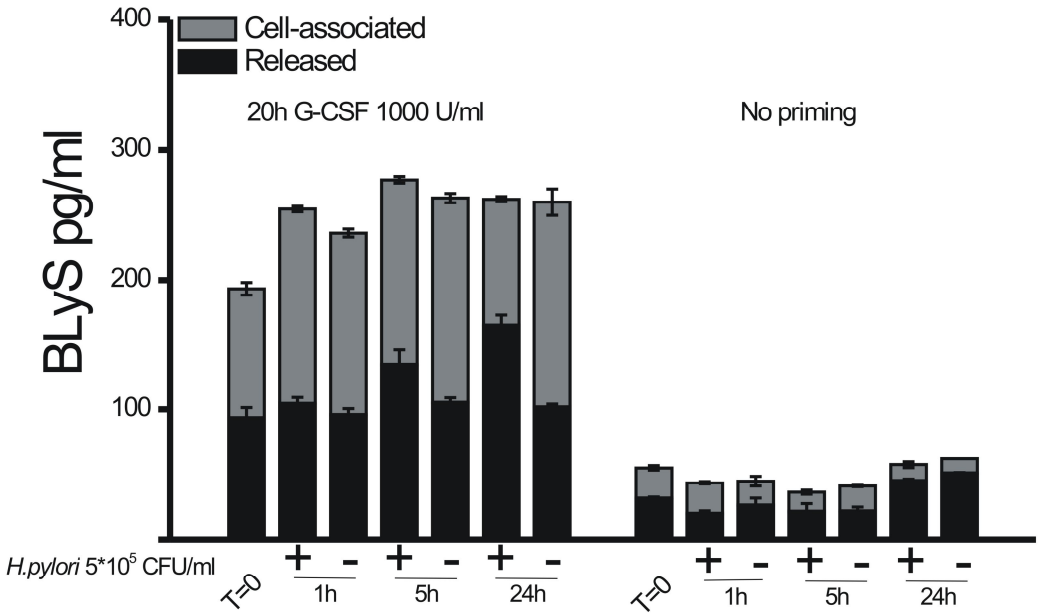


**Figure 15:** Blood-purified neutrophils were exposed to *H. pylori* $5*10^5$ CFU/ml with or without a 20h priming of G-CSF. At the indicated time-points cells were harvested for intracellular BLyS determination; culture supernatants were collected for determining the amount of BLyS released.

| | | % Released | % Cell Ass |
|---|---|---|---|
| | T0 | 48,4 | 51,6 |
| | | | |
| 1h | HP | 41,1 | 58,9 |
| | PBS | 45,6 | 54,4 |
| | | | |
| 5h | HP | 48,7 | 51,3 |
| | PBS | 40,1 | 59,9 |
| | | | |
| 24h | HP | 63,1 | 36,9 |
| | PBS | 39,3 | 60,7 |

**Table 1:** Amount of intracellular and released BLyS shown in fig 15 expressed as percentage of the total amount of the cytokine.

Interestingly, by expressing the data of fig. 15 as percentage of total BLys (Table 1) it appears more evident that the secretion of BLyS by G-CSF-primed neutrophils, stimulated with *H. pylori*, is time dependent (Table 1). These data suggest that neutrophils infiltrating the gastric mucosa could act as source of BLyS in this inflammatory context.

Importantly, *H. pylori* treatment increased by itself the production of BLyS in G-CSF-primed neutrophils (fig. 15). Accordingly, the expression of the mRNA encoding for BLyS was enhanced in these neutrophils following *H. pylori* stimulation (data not shown). On the contrary, *H. pylori* did not induce any synthesis or secretion of BLyS in not-primed neutrophils, confirming that both G-CSF and *H. pylori* are important for BLyS secretion. Notably, we got evidence that G-CSF is highly expressed in *H. pylori* associated chronic gastritis (data not shown) therefore it is tempting to speculate that *H. pylori*, through the induction of G-CSF, stimulates the synthesis of BLyS in neutrophils; the latter, in turn, would be induced by the bacterium to secrete the mature cytokine. Obviously, we cannot exclude that also monocytes/macrophages release BLyS following *H. pylori* infection and this possibility is under investigation. In parallel, we are currently verifying whether our in vitro data are confirmed in vivo. To this aim we are performing immunohistochemical evaluations, on specimens from biopsies of *H. pylori* patients, using antibodies against BLyS together with antibodies specific for neutrophils or monocytes/macrophages.

# Conclusions

APRIL and BLyS are two cytokines that act on B-cells increasing their survival, activation and maturation. Since their discovery several works have been published about their role in lymphomas and autoimmune diseases, anyway little is known about their role in infectious disease.

Herein we report new insights about the role of these two cytokines in *H. pylori*-associated diseases. In particular, we demonstrate that *H. pylori* is able to trigger the secretion of APRIL and BLyS by macrophages and neutrophils respectively.

APRIL is highly expressed in MALT lymphoma and its expression relies on the presence of the bacterium; moreover, there is a positive correlation between the amount of APRIL in the tissue and the stage of lymphoma. Finally, we report that *H. pylori* triggers APRIL secretion by macrophages in a direct manner, but its secretion can be also mediated by *H. pylori* specific T cells.

About BLyS we are at the beginning of the story, but we found that: i) its expression is abundant in chronic gastritis and it reflects the presence of *H. pylori*, since the eradication of the latter reduced the production of the cytokine; ii) such a behaviour is superimposable to that of IL-17. These data suggest the possibility that a BlyS/Th17 axis establishes in the stomach mucosa infected by *H. pylori*, similarly to what reported for at least two models of autoimmune diseases in mice. Neutrophils could be the main source of BLyS: indeed, not only they are abundantly represented in the inflammatory infiltrates typically associated to the *H. pylori* infection, but they also release BLyS once infected by the bacterium; however, to date, we cannot exclude that other cells, such as monocytes/macrophages, contribute to create a milieu enriched in BLyS.

It remains to elucidate if BLyS is really involved in inducing a Th17 response in *H. pylori* gastritis, as suggested for other pathological conditions: to this regard, we found that the exposure of monocytes/macrophages to BLyS results in the secretion of pro-Th17 cytokines.

The role of a Th17 response in the progression of the diseases associated to *H. pylori* remained an unexplored issue: the possibility exists that it might lead to the development of autoimmune gastritis, a possible sequel of chronic gastritis; however it

is also conceivable that the Th17 response might promote cancer development, as recently suggested. Both of these two possibilities will be investigated in the next future.

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