Immunopathogenic aspects of resolving and progressing appendicitis

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Att våga är att förlora fotfästet en stund.
Att inte våga är att förlora sig själv
S. Kirkegaard

To my family; past, present and future
Abstract

Background Appendicitis is one of the most common diseases requiring emergency surgical intervention. There are several indications that the diagnosis appendicitis harbours two different entities, one progressing to gangrene and perforation (advanced) and one that resolves spontaneously (phlegmonous). An immunologically driven pathogenesis in appendicitis has been suggested on the basis of an inverse relationship between appendicitis and ulcerative colitis, a positive association with Crohn’s disease, and a decreased incidence during pregnancy, generating the hypothesis that the immunopathogenesis in advanced appendicitis is characterized by a Th1 inflammatory response. The aim of this thesis was to test this hypothesis and investigate the immune response in advanced and phlegmonous appendicitis.

Material and Methods The immunologic response was investigated in appendicitis tissue and compared to the immunological response in peripheral blood, analysed by enzyme-linked immunospot assay (ELISPOT). The response pattern was also investigated in patients with an actual appendicitis in the peripheral plasma and peripheral serum before surgery, analysed with Luminex. The immunological response pattern was investigated in peripheral blood several months to years after an appendectomy using ELISPOT and enzyme-linked immunosorbent assay (ELISA).

Results The local immune response in the appendiceal tissue in appendicitis was similar to the response in peripheral blood. Patients with actual advanced appendicitis had increased levels of IL-6, CCL20, CCL2, TGF-β, IL-17, IFN-γ, IL-12p70, IL-10, IL-1ra, IL-4, MMP-8, MMP-9 and MPO compared with those with phlegmonous appendicitis. Sex, age or duration of symptoms could not explain the differences between the groups. Individuals with a history of advanced appendicitis had increased secretion of IFN-γ months to years after the appendectomy compared with individuals with a history of phlegmonous appendicitis.

Conclusions The local immune response in the appendiceal tissue is mirrored in the blood, which justifies the use of peripheral blood in studies on appendicitis. The immunological response pattern in peripheral blood suggests Th1/Th17-induced inflammation in advanced appendicitis that is present at an early stage. Individuals with a history of advanced appendicitis have stronger Th1 responses than individuals with a history of phlegmonous appendicitis. This may reflect constitutional differences between patients with different outcomes of appendicitis. The increased inflammatory response observed early in advanced appendicitis suggests a more violent inflammation and supports the hypothesis of different immune pathogeneses, where excessive induction of Th1/Th17 immunity and/or deficiencies in down-regulatory feedback mechanisms may explain the excessive inflammation in advanced appendicitis, where the inflammation eventuates in gangrene and perforation.
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Specific aims

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Original publications

I. Marie Rubér, Anna Berg, Christina Ekerfelt, Gunnar Olaison and Roland E. Andersson
Different cytokine profiles in patients with a history of gangrenous or phlegmonous appendicitis
Clinical and Experimental Immunology, 143:117–124, 2006

II: Marie Rubér, Manne Andersson, B. Fredrik Petersson, Gunnar Olaison, Roland E Andersson and Christina Ekerfelt
Systemic Th17-like cytokine pattern in gangrenous appendicitis but not in phlegmonous appendicitis
Surgery 147: 366-372, 2010

III. Marie Rubér, Manne Andersson, Gunnar Olaison, Roland E Andersson and Christina Ekerfelt
Dysregulated Th1/Th17 response in advanced appendicitis
Manuscript Submitted

IV. Marie Rubér, Roland E Andersson, Christina Ekerfelt and Gunnar Olaison
Local and systemic cytokine secretion in advanced and phlegmonous appendicitis
Manuscript Submitted
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMC</td>
<td>Appendix mononuclear cell</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T cells</td>
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<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Enzyme-linked immunospot assay</td>
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<tr>
<td>FAE</td>
<td>Follicle-associated epithelium</td>
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<td>Foxp3</td>
<td>Forkhead box protein3</td>
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<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<td>IEC</td>
<td>Intestinal epithelial cells</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
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<td>LT</td>
<td>Lymphotoxin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PP</td>
<td>Payer’s patches</td>
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<tr>
<td>PPD</td>
<td>Purified protein derivate of Mycobacterium tuberculosis</td>
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<tr>
<td>TCM</td>
<td>T cell culture medium</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TT</td>
<td>Tetanus toxoid</td>
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<td>WBC</td>
<td>White blood cell</td>
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Introduction

Appendix vermiformis

Anatomy and physiology

The appendix vermiformis is an approximately 2- to 20-cm long (average 9 cm in adults) and 0.5- to 1-cm-wide blind-ended tubular sac, extending from the caecum, just distal to the ileocaecal junction, of the human large intestine (Figure 1). Vermiform is Latin and means “in the shape of a worm”. Appendix-like structures are only found in humans, apes (Fisher, 2000; Scott, 1980; Smith et al., 2009), rodents (Smith et al., 2009), monotremes and marsupials.

The appendix is composed of a substantial amount of lymphoid tissue (Neiburger et al., 1976; Spencer et al., 1985) and its general structure resembles the rest of the large intestine. In some mammals, the appendix is involved in prolonged digestion of cellulose. However, the function of the human appendix has not been established. One suggested function is as a sentinel sampling organ. Under this hypothesis, the position immediately after the ileocaecal valve is strategic because the sampling material empties after the small intestine digestion (Bazar et al., 2004). Another proposed function of the appendix is acting as a “safe house” for commensal bacteria, facilitating re-inoculation of the colon in circumstances when the intestinal contents have been purged following exposure to a pathogen (Randal Bollinger et al., 2007).

The lymphoid tissue in the appendix develops within the first year of life. During the teens some atrophy is seen, but the appendix has immunological function throughout life, albeit with gradually declining activity (Dasso et al., 2000).
Introduction

Appendicitis

Overview
Appendicitis is one of the most common diseases requiring emergency surgical intervention. Every year, about 12,500 appendectomies are performed in Sweden (Fenyö, 1995), where the lifetime risk of appendicitis ranges from 6% to 9% (Addiss et al., 1990).

Historical
In 1886, Reginald Fitz suggested that the appendix is the primary cause of most inflammatory diseases of the right lower quadrant (Williams, 1983). Fitz coined the term appendicitis, described its clinical features and recommended its early surgical treatment. In 1889, Charles McBurney described the clinical signs with characteristic migratory pain and localization of the pain along an oblique line, including the elective point of tenderness that bears his name (Keyzer and Gevenois, 2011).
Diagnosis

The decision to investigate a patient with suspected appendicitis is based mainly on the disease history and the findings at physical examination. The typical presentation begins with periumbilical pain, followed by anorexia and nausea. Initially, the pain is visceral and localized to the epigastrium, between the ribs. The pain then localizes to the right lower quadrant, as the progression of the inflammatory process involves the peritoneum, overlying the appendix. This migratory pain is the classical pattern and the most reliable symptom of acute appendicitis. Associated gastrointestinal symptoms with occasional vomiting and generalized malaise may also be present.

Patients with acute appendicitis typically look ill and lie still in bed, as movement and coughing intensify the pain. Low-grade fever is a common feature (≈38 °C). Diminished bowel sounds and focal tenderness, with guarding (voluntary muscle contraction) are usually found upon examination of the abdomen. The maximal site of tenderness is said to lie over McBurney’s point, between the umbilicus and the iliac crest. Peritoneal irritation can be elicited on physical examination by the findings of guarding, rigidity (involuntary muscle contraction), rebound tenderness and indirect tenderness. Further examination findings have been suggested to aid in the diagnosis of appendicitis. Rovsing’s sign is said to be present when palpation in the left iliac fossa results in pain in the right iliac fossa. The clinical presentation is seldom typical and diagnostic errors are common (Andersson et al., 1992; Wagner et al., 1996). Pelvic examination in women and rectal examination in all patients are carried out to exclude other diseases, and are a mandatory part of the physical examination.

The diagnosis can be strengthened by adding laboratory investigations. Inflammatory variables (temperature, leukocyte and differential white blood cell (WBC) counts, and C-reactive protein (CRP)) have been shown to be as important as clinical findings (direct and rebound abdominal tenderness and guarding), especially in cases with advanced appendicitis (Andersson et al., 1999; Andersson et al., 2000).

In cases of a perforated appendix, the abdominal pain intensifies and is often of a more diffuse character, with probable development of rigidity, tachycardia and elevation of temperature above 39 °C. The pain may occasionally improve somewhat after rupture of the appendix because of relief of visceral distension, but it does not disappear.
Introduction

Pathogenesis

Pathological sub classification
Appendicitis can be histopathologically divided according to different degrees of inflammation as follows:

Catarrhal
Inflammation, with accumulation of neutrophils, is limited to the mucosa. The clinical significance of these findings is however controversial as they are also frequently seen also in asymptomatic patients subjected to appendectomy “en passant” with other, most often gynaecological, operations.

Phlegmonous
The inflammation, with accumulation of neutrophils, is transmural, including all layers of the appendix from mucosa and submucosa to muscularis propria. Phlegmonous comes from the word phlegmon, which means “purulent inflammation and infiltration of connective tissue”.

Gangrenous
The inflammation, with accumulation of neutrophils, is transmural, including all layers of the appendix from mucosa and submucosa to muscularis propria, but has additional areas with necrosis. Gangrenous comes from the word gangrene, which means “local death of soft tissue due to loss of blood supply”.

Perforated
The inflammation, with accumulation of neutrophils, is transmural, including all layers of the appendix from mucosa and submucosa to muscularis propria, but has additional areas with necrosis and perforation.

Non-perforated
A name for phlegmonous and gangrenous appendicitis, where the appendix has not perforated.

Advanced
A name for gangrenous and perforated appendicitis. For the purpose of this study, the term “advanced appendicitis” is used for gangrenous and perforated appendicitis.

Aetiology
The aetiology of appendicitis is not well known, it is believed to be caused by a number of pathogenic pathways (Carr, 2000), where obstruction of the lumen is believed to be the major cause of acute appendicitis, but is seen only in a few cases (Carr, 2000; Prystowsky et al., 2005). The obstruction leads to bacterial overgrowth and, with continued secretion of mucus, will result in increased wall
pressure. Mucosal ischemia also follows owing to the decreased lymphatic and venous drainage, which, altogether, promotes a localized inflammatory process that may progress to gangrene and perforation. Fecaliths, lymphoid hyperplasia, parasites or tumors may be the cause of the obstruction (Prystowsky et al., 2005).

Age
Appendicitis occurs at all ages, but is rare before the age of five years; however, it has a sharp peak in adolescence (Andersson et al., 1994).

Inflammatory bowel disease
The development and underlying causes of inflammatory bowel disease (IBD) are thought to comprise several factors including genetic factors, intestinal microbiota, other environmental factors and the host immune system (Kaser et al., 2010). The two major forms of IBD are Crohn’s disease and ulcerative colitis. Appendicitis (Andersson et al., 2001) or appendectomy (Duggan et al., 1998; Radford-Smith et al., 2002) has shown to reduce the risk of developing ulcerative colitis, especially if the appendicitis occurred in childhood or adolescence (Frisch et al., 2009). In contrast, an increased risk of developing Crohn’s disease has been shown after appendicitis (Andersson et al., 2003; Kaplan et al., 2008) or appendectomy (Frisch and Gridley, 2002; Frisch et al., 2001; Koutroubakis et al., 1999; Kurina et al., 2002), but conflicting results have also been published (Radford-Smith et al., 2002). A meta-analysis has shown that the risk of Crohn’s disease in appendectomy patients dropped and was insignificant after 5 years, and that the overall increased occurrence in these patients appears to be explained by a diagnostic bias (Kaplan et al., 2008).

Pregnancy
There was found to be a decreased incidence of appendicitis during pregnancy, especially in the third trimester, compared with that in controls (Andersson and Lambe, 2001). This result suggests a protective effect of pregnancy against the development of appendicitis. During pregnancy a range of physiological changes occur, which may influence the pathogenesis of appendicitis.

Different outcomes
Appendicitis does not always progress to perforation
The general view is that untreated appendicitis is a ticking bomb and will progress to perforation, which has been shown to be associated with increase in morbidity and mortality in these patients. In recent years, studies of appendicitis progressing to perforation have provided increasing evidence suggesting that not
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all patients with the diagnosis of appendicitis will progress to perforation. The influence of a restrained attitude to explore patients with appendicitis has resulted in the performance of 50% fewer negative appendectomies (Howie, 1964). Further research on the influence of this restrained approach, but in which appendicitis was defined as transmural inflammation, has shown that it results in the diagnosis of fewer patients with non-perforated appendicitis, while resulting in a low incidence rate of negative appendectomies and not affecting the incidence rate of perforated appendicitis (Andersson et al., 1994). In a randomized study focusing on different diagnostic techniques, and which included patients with acute abdominal pain, the differences between early diagnostic laparoscopy and the use of conventional management in the diagnosis of appendicitis were evaluated (Decadt et al., 1999; Morino et al., 2006). The group using conventional management detected fewer patients with appendicitis. Altogether, these results suggested that a restrained attitude to exploration results in a smaller number of patients being diagnosed with appendicitis. Moreover these findings suggest that appendicitis may spontaneously resolve without being diagnosed.

Resolving appendicitis
A history of recurrent appendicitis, including at least 6.5% of those who have had an inflamed appendix removed, may be explained as a consequence of spontaneous resolution (Barber et al., 1997). Furthermore, resolving symptoms have been reported in patients with appendicitis verified by computed tomography (CT) or ultrasound and not operated (Cobben et al., 2000; Heller and Skolnick, 1993; Jeffrey et al., 1988; Kirshenbaum et al., 2003; Migraine et al., 1997; Ooms et al., 1991). Regression of acute appendicitis has been histologically identified by cell clusters being scattered throughout the muscularis propria and subserosa rather than the “normal” inflammatory distribution, which demonstrates a more diffuse pattern (Ciani and Chuaqui, 2000). The cells dominating in numbers in resolving appendicitis were lymphocytes and eosinophils and only a few or no neutrophils could be identified.

Negative explorations and perforation
A common measure of the quality of management of patients with suspected appendicitis is the proportion of perforations and that of negative appendectomies. An important goal is therefore to maintain an appropriate balance between preventing perforated appendices and avoiding an increase in the number of negative appendectomies, where a negative appendectomy is not always beneficial as a negative appendectomy can have detrimental effects. A follow-up study of deaths within 30 days after all appendectomies in Sweden, during the period from 1987 to 1996, was carried out by analysing register
linkage. Different discharge diagnoses were compared according to the case fatality rate and the standardized mortality ratio. The study showed that, compared rate in the background population, the excess rate of deaths after an appendectomy operation for non-perforated appendicitis was 3.5-fold, that after perforated appendicitis was 6.5-fold and that after a negative appendectomy, with the discharge diagnosis of non-specific abdominal pain, was 9.1-fold (Blomqvist et al., 2001). A high mortality after negative appendectomy has also been reported in other studies (Andersson and Andersson, 2011; Faiz et al., 2008; Flum et al., 2001).

**Proportions of perforations**

Population-based studies have demonstrated that age has a great impact on the incidence rate of non-perforated appendicitis, but not on perforated appendicitis, which shows almost constant incidence rates (Andersson et al., 1994; Luckmann, 1989). The relationship between pre-hospital delay and in-hospital delay has been studied, with an association of pre-hospital delay and perforation being identified, whereas no connection was seen with in-hospital delay (Maroju et al., 2004; Temple et al., 1995; Williams and Bello, 1998; Yardeni et al., 2004). This suggests that the majority of perforations occur before the patients arrive at hospital.

**Natural history of appendicitis**

Many studies have shown an increase in the proportion of cases of perforations with an increasing duration of symptoms of appendicitis. Two different models have been proposed to explain this association. In these two models the proportions of cases with perforation are identical at each moment in time. In the traditional model the increase is explained by a steady progression of the inflammation, from phlegmonous via gangrenous to perforation. It is believed that all inflamed appendices will eventually progress to a perforated appendicitis. The only way to stop this progression is treatment by an early operation. The alternative model gives another view, suggesting that most of the perforations occur at an early stage and that spontaneous resolution of appendicitis is common (Andersson, 2007). The continued increase in the proportion of perforated appendicitis with time is explained by selection due to the spontaneous resolution of simple appendicitis. The second model suggests that only a few perforations can be prevented by an early operation, after arrival at hospital.
Introduction

Immunology

Overview of the innate and adaptive immune systems

The immune system has evolved to protect the host from a wide range of pathogenic microbes, and it uses a complex set-up of protective mechanisms to control and usually eliminate these organisms and toxins. A characteristic feature of the immune system is that it recognizes structural patterns of pathogens or toxins that mark them out as being distinct from the host’s cells. There are two general categories that account for this recognition: the innate response, which is a hard-wired response where genes encoded in the host’s germ line, recognize molecular patterns shared by the infectious agents and the adaptive response, which is encoded by gene elements that somatically rearrange and put together antigen-binding molecules which have a unique specificity for different foreign structures.

The major components of the innate immunity are as follows (Abbas et al., 2012; Turvey and Broide, 2010):

1. Physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces.
2. Phagocytic cells (neutrophils, macrophages) and NK (natural killer) cells.
4. Cytokines, proteins which coordinate cell activities.

The adaptive immune response can be divided into two different categories.

1. Humoral immunity which comprises antibodies, molecules in the blood and mucosal secretions, produced by B cells, which recognizes, neutralize and target microbes for elimination.
2. Cell-mediated immunity, which is mediated by T cells that protect against intracellular microbes.

Neutrophils

One of the body’s primary lines of defence is neutrophils which act against invading pathogens such as bacteria (Wright et al., 2010). These cells are classically characterized by their ability to act as phagocytic cells, to release lytic enzymes from their granules and to produce reactive oxygen intermediates with antimicrobial potential (Mantovani et al., 2011). The activation of neutrophils is a two-stage process, where resting neutrophils become primed by infectious agents such as bacterial products and pro-inflammatory cytokines or chemokines. It has also been shown that neutrophils can migrate to lymph nodes following antigen capture at peripheral organs or tissues, similar to dendritic cells (DC).
Granules are the hallmark of granulocytes (eosinophils, basophils and neutrophils), which contain stores of proteins able to kill microbes and digest tissues. The presence of characteristic granular proteins classifies the neutrophil granules into three subsets: primary (azurophil) granules (containing myeloperoxidase (MPO)), secondary (specific) granules (containing lactoferrin, gelatinase and collagenase), and tertiary (gelatinase granules) (containing gelatinase e.g. matrix metalloproteinase (MMP-9)) (Borregaard, 2010). It has also become apparent that neutrophils are important mediators of the T helper 17 (Th17)-controlled pathway and in the resolution of inflammation (Mantovani et al., 2011). The neutrophils are an important cell type in appendicitis, as shown by the histological confirmation of this disease.

**Cytokines produced by antigen presenting cells**

Antigen-presenting cells (APC) such as DC and mononuclear phagocytes produce cytokines, which are effective in generating a potent innate immune response and provide important signals initiating and specifying the nature of the adaptive immune response. Cytokines predominately produced by APCs include tumor necrosis factor (TNF), interleukin (IL)-1, IL-6 and CXCL8, as well as IL-12, IL-15, IL-23 and IL-27 (Commins et al., 2010).

**Immune regulation**

**T helper cell subpopulations and cytokines**

Over 20 years ago, Mosmann and Coffman demonstrated that effector T helper cells can be categorized into two distinct subsets, Th1 and Th2, based on their characteristic cytokine profiles (Mosmann et al., 1986). Th1 cells produce interferon (IFN)-γ and lymphotoxin (LT), whereas Th2 cells produce IL-4, IL-5 and IL-13. Th1 cell cytokines drive cell-mediated responses, activating mononuclear phagocytes, NK cells and cytotoxic T cells (CTL) for the killing of intracellular microbes and virally infected targets (Bonilla and Oettgen, 2010) (Figure 2, Figure 3, Table 1). Th2 cytokines, in contrast, enhances antibody production, particularly that of IgE and IgG4 isotypes, which are involved in hypersensitivity and parasite-induced immune responses. The relationship between Th1 and Th2 cells has been viewed as a Yin-Yang paradigm, where the immune response to an immunologically mediated disease or specific pathogen has been considered as primarily Th1 or Th2 mediated. An expansion of this model occurred in 1995 with the discovery of regulatory T cells (Treg; (Sakaguchi et al., 1995)). In contrast to Th1 and Th2 cells, which are generated in the periphery or in secondary lymphoid organs and require T cell activation, most Treg mature in the thymus, which are referred to as natural (n) Treg. However, another type of Treg which differentiate in the periphery has also been found and is referred to as inducible (i)Treg (Vignali et al., 2008). Both types are
important in regulating and dampening immune cell activation, and are associated with the expression of transforming growth factor (TGF)-β, IL-10 and IL-35. iTreg which expresses Foxp3 are induced in the periphery by immune suppressive cytokines such as TGF-β after T cell receptor stimulation (Chen et al., 2011). Type 1 regulatory (Tr1) and Th3 cells are other types of iTregs. Tr1 cells are believed to be activated in the presence of IL-10 and to secrete both IL-10 and TGF-β, and have the ability to suppress antigen-specific effector T-cell responses via a cytokine-dependent mechanism (Roncarolo et al., 2006). The intestinal mucosa is often the location for Tr1 cells and presently no specific surface marker for Tr1 cells has been identified (Chen et al., 2011). Th3 cells have shown to be connected with oral tolerance and express TGF-β, and may also express Foxp3.

A further change in Th biology, away from the original Th1/Th2 paradigm occurred with the discovery of a fourth T helper cell subset, Th17 which is characterised by secretion of IL-17 (A), IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F (Bettelli et al., 2006; Harrington et al., 2006; Park et al., 2005; Veldhoen et al., 2006). In vitro and in vivo, IL-17 may act as a potent inflammatory cytokine with pleiotropic activity (Kolls and Linden, 2004). IL-17 coordinates tissue inflammation through the induction of pro-inflammatory cytokines (IL-6 and TNF), chemokines (CCL2) and matrix metalloproteases, which mediate tissue infiltration and tissue destruction. Th17 cell-derived cytokines (such as IL-17, CXCL8, IFN-γ, TNF and granulocyte macrophage colony stimulating factor (GM-CSF)) favour recruitment, activation and prolonged survival of neutrophils at inflammatory sites (Pelletier et al., 2010). The Th17 cells are found at epithelial barriers and a proposed function for them is in epithelial barrier immunity fighting extracellular bacteria and fungi (Zygmunt and Veldhoen, 2011).

The magnitude and duration of innate immune responses are regulated by a variety of feedback inhibition mechanisms that limit potential damage of tissues. IL-1 receptor antagonist (ra) is a member of the IL-1 family and has antagonizing effect to both IL-1α and IL-1β, by blocking the binding of IL-1 to cell surface receptors (Dinarello, 1996). IL-6 and TNF-α have shown to induce IL-1ra, as a possible negative feedback in response to the pro-inflammatory cytokines released (Gabay et al., 1997; Tilg et al., 1994). IL-4 down regulates antibody-dependent cellular cytotoxicity by mononuclear phagocytes, and down regulates the production of nitric oxide, IL-1, IL-6, and TNF-α while stimulating production of IL-1ra and IL-10, have opposite effects to IFN-γ (Commins et al., 2010; Hart et al., 1989; Lee et al., 1990; Steinke and Borish, 2006). IL-4 is the major stimulus of Th2-cell development (Mosmann and Sad, 1996), but it also
suppresses Th1-cell development, and could in the context of Th1-inflammation be considered as anti-inflammatory.

**Figure 2.** A simplified view of the cytokines produced and cytokines needed for lineage specification of different T helper cell subsets. RA=retinoic acid
**Introduction**

**Chemokines**
Chemokines are a group of molecules able to induce chemotaxis in a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes (Borish and Steinke, 2003). Although chemotaxis stands as the hallmark feature of chemokines, their physiological role is more complex than originally described, and new functions continue to be identified. Chemokines are small proteins with four conserved cysteines forming two essential disulphide bonds (Cys1-Cys3 and Cys2-Cys4) (Baggiolini, 2001) (Figure 3, Table 2). The position of the first two cysteines distinguishes the CC and CXC chemokines from each other, which are either adjacent (CC) or separated by one amino acid (CXC). There is remarkable homology in this family, despite the fact that it includes many members. The chemokines are mostly produced during pathological conditions by tissue cells and infiltrating leukocytes, but some also have housekeeping functions. Examples of their functions are involvement in leukocyte maturation in the bone marrow, the trafficking and homing of lymphocytes and mechanisms associated with the renewal of circulating leukocytes.

**Matrix metalloproteinases (MMP)**
The matrix metalloproteinases (MMP) are a major group of enzymes that regulate cell-matrix composition, and the degrading and remodelling of the extracellular matrix. Most of the MMP are secreted as latent, inactive pro-enzymes by various cell types, including mesenchymal cells, T cells, monocytes, macrophages, neutrophils, keratinocytes and tumour cells (Pender and MacDonald, 2004) (Table 3). Their transformation into an active enzyme usually occurs in the pericellular or extracellular space (Parks et al., 2004). To be classified as an MMP, a protein needs to have at least the conserved pro-domain, which contains a zinc ion in the active site, and a catalytic domain. MMP are structurally related but can be divided into subclasses according to their primary substrate specificities: collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10 and -11), elastase (MMP-12) and membrane types (MMP-14, -15, -16, -17, -24 and -25) (Pender and MacDonald, 2004). MMPs work together to form an activation cascade; once one MMP, is activated it can catalyse the conversion of other pro-enzymatic MMPs to their active forms. Inhibitors of MMP are tissue inhibitors of metalloproteinase (TIMP), which are produced by the same cells that produce MMP, and act by forming a 1:1 complex with activated catalytic zinc in MMP. Most cells synthesize and immediately secrete MMP into the extracellular matrix, while inflammatory cells, in contrast, store these proteases (MMP-8 and MMP-9) (Chakraborti et al., 2003).
Introduction

Gut-associated lymphoid tissue (GALT)
The intestinal tract, with an area of approximately 200 m², is the largest surface of the body (Abbas et al., 2012; Artis, 2008). The barrier between the gut lumen and the host connective tissue is lined by only a single layer of columnar epithelial cells. The adult human intestine is home to an estimated of $10^{14}$ commensal bacteria. The organized lymphoid tissue dealing with this antigenic challenge in the gut is known as the gut-associated lymphoid tissue (GALT), which is part of a mucosal site system called mucosal-associated lymphoid tissue (MALT). The most common GALT structures are Peyer’s patches, found mainly in the distal ileum, as smaller aggregates of lymphoid follicles or isolated follicles in the appendix and colon. The GALT structure is not encapsulated compared with lymph nodes. The Peyer’s patches are formed by multiple distinct lymphoid follicles, where each patch consists of a large number of B cell follicles with germinal centres, follicular Th cells, follicular DC and macrophages. Every follicle has an important role, being the site for the generation of antigen-specific IgA precursors.
Figure 3. Produced cytokines and chemokines analysed in paper I-IV, divided among the different cell types, which might be the major source of them.
### Table 1. The major function of, and cells producing the cytokines included in paper I-IV (Akdis et al., 2011; Commins et al., 2010).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mainly produced by</th>
<th>Major function</th>
<th>Cell targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Macrophages, endothelial cells, neutrophils and others</td>
<td>Induction of pro inflammatory proteins, haematopoiesis, differentiation of Th17 cells. Activating T cells, by enhancing production of IL-2 and expression of IL-2 receptor. Interaction with the central nervous system to produce fever, lethargy, sleep and anorexia.</td>
<td>T cells, epithelial and endothelial cells.</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Macrophages, endothelial cells, neutrophils and others</td>
<td>Antagonistic function, to bind the IL-1 receptor. Secreted in inflammatory processes in response to many cytokines, including IL-4, IL-6, IL-13 and TGF-β.</td>
<td>T cells, epithelial and endothelial cells.</td>
</tr>
<tr>
<td>IL-2</td>
<td>CD4 and CD8 activated T cells, DCs, NK and NKT cells.</td>
<td>Proliferation of effector T and B cells. Involved in the generation and maintenance of Treg and growth factor for B cells.</td>
<td>CD4 and CD8 T cells, NK and B cells.</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells, basophils, eosinophils, mast cells, NKT cells and γ/δ T cells.</td>
<td>Promotes Th2 differentiation, IgE class switch, up regulation of MHC class II expression on B cells, survival factor for B and T cells, role in tissue adhesion and inflammation.</td>
<td>T and B cells.</td>
</tr>
<tr>
<td>IL-5</td>
<td>Th2 cells, activated eosinophils and mast cells, NK and NKT cells, γ/δ T cells and others.</td>
<td>Stimulates eosinophil production and release from the bone marrow, chemotactic for eosinophils and activated mature eosinophils, inducing eosinophil secretion and enhanced cytotoxicity.</td>
<td>Eosinophils, basophils and mast cells.</td>
</tr>
<tr>
<td>IL-10</td>
<td>T and B cells, monocytes, macrophages and DCs.</td>
<td>Immunosuppression, stimulates humoral and cytotoxic responses</td>
<td>Macrophages, monocytes, T, B, NK and mast cells, DC and granulocytes.</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>Monocytes, macrophages, neutrophils, microglia, DCs and B cells.</td>
<td>Induces Th1-differentiation, stimulates IFN-γ production and activates and induces proliferation, cytotoxicity, and cytokine production of NK cells.</td>
<td>Th1 and NK cells.</td>
</tr>
<tr>
<td>IL-15</td>
<td>Monocytes, activated CD4+ T cells and others.</td>
<td>Similar to IL-2, is a T-cell growth factor and is chemotactic for T cells. Involved in the activation of NK cells. Necessary for maintaining the survival of CD8 memory T cells.</td>
<td>T, NK and NKT cells.</td>
</tr>
<tr>
<td><strong>Cytokine</strong></td>
<td><strong>Cell Types and Functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-17A</strong></td>
<td>T cell-mediated responses to extracellular pathogens. Induces expression of a variety of cytokines and chemokines, including IL-6, IL-11, GM-CSF, CXC/IL-8, CXCL10 and TGF-β1, and metalloproteinases. Induction of cytokines responsible for polymorphonuclear cells recruitment and activation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td>Neutrophils, T, NK and mast cells and endothelium. Interacts with endothelial cells to induce adhesion molecules, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin which facilitates granulocytes reaching the inflammatory site. Activator of neutrophils, mediating adherence, chemotaxis, degranulation, and the respiratory burst.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Th1, NK and NKT cells, macrophages, CTL and B cells. Most important cytokine responsible for cell-mediated immunity. Signature cytokine produced by Th1 cells. Mediates increased MHC class I and II expression and stimulates antigen presentation and cytokine production by APC. Stimulates mononuclear phagocytic functions and accumulation of macrophages to the site of inflammation. Stimulates killing by NK cells and neutrophils. Inhibitor of Th2 response.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TGF-β</strong></td>
<td>T cells especially Treg, monocytes and neutrophils. Stimulant of fibrosis, inducing formation of the extracellular matrix and promoting wound healing and scar formation. Inhibitory for B and Th/cytotoxic cells. Inhibits proliferation and induces apoptosis. Inhibits cytotoxicity of mononuclear phagocytes and NK cells. Immune suppression by Treg. Production by mucosal (Th3) cells support isotype switch and secretory IgA production by B cells and important tolerance. Central in the differentiation of Th17 cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>Th2 cells, fibroblasts, endothelial cells, monocytes/macrophages, mast cells, neutrophils and eosinophils. Maturation of DCs, neutrophils and macrophages. Synergizes with other colony-stimulating factors to support the production of platelets and erythrocytes. Activation factor for mature granulocytes and mononuclear phagocytic cells. Prolongs the survival and contributes to the activity of eosinophils.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. The major function of, and cells producing the cytokines included in papers I-IV

<table>
<thead>
<tr>
<th>Chemokine and inflammatory marker</th>
<th>Mainly produced by</th>
<th>Major function</th>
<th>Cell targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2 (MCP-1)</td>
<td>Endothelial cells, macrophages and fibroblasts</td>
<td>Attracts monocytes and memory T cells. Pro-inflammatory, multiple pleiotropic roles in acute inflammatory response.</td>
<td>Monocytes and memory T cells.</td>
<td>(Curfs et al., 1997)</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>T and B cells, monocytes, mast cells, fibroblasts and neutrophils.</td>
<td>Chemotactic for monocytes (inducing activation of IL-1, IL-6 and TNF), neutrophils, eosinophils and T-cells. Stimulates adhesion of T-cells to endothelial cells.</td>
<td>Monocytes.</td>
<td>(Curfs et al., 1997)</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>Monocytes, macrophages, neutrophils, lymphocytes, endothelial cells, epithelial cells and others</td>
<td>Chemoattractant for neutrophils, NK and T cells, eosinophils and basophils.</td>
<td>Neutrophils, NK and T cells, basophils, eosinophils, endothelial cells.</td>
<td>(Akdis et al., 2011; Commins et al., 2010)</td>
</tr>
<tr>
<td>CCL20 (MIP-3α, LARC)</td>
<td>Epithelial cells, follicle-associated epithelial, that covers intestinal lymphoid aggregates.</td>
<td>Mucosal immune response, is the ligand for CCR6 which is expressed by Th17 cells.</td>
<td>Th17 cells.</td>
<td>(Williams, 2006)</td>
</tr>
</tbody>
</table>
Table 3. The substrates and activators of the matrix metalloproteinases (MMP) included in papers I-IV (Chakraborti et al., 2003; Parks et al., 2004).

<table>
<thead>
<tr>
<th>Name</th>
<th>Common name</th>
<th>Category</th>
<th>Extracellular substrates</th>
<th>Non extracellular substrates</th>
<th>Activated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1, ColA, ColB</td>
<td>Collagenase</td>
<td>Collagen, gelatin, proteoglycan and more</td>
<td>IL1-β, pro-TNF, MMP-2, MMP-9</td>
<td>MMP-3, -10</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Gelatinase</td>
<td>Collagen, gelatin, and more</td>
<td>IL1-β, MMP-1, -9, CCL7, CXCL12</td>
<td>MMP-1, -7</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1</td>
<td>Stromelysin</td>
<td>Collagen, gelatin, aggrecan and more</td>
<td>Latent TGF-β1, substance P, MMP-1, MMP-2/TIMP-2 complex, MMP-7, -8, -9</td>
<td>Plasmin, kallikrein, chymase tryptase</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin-1, PUMP1</td>
<td>Stromelysin</td>
<td>Collagen, gelatin, aggrecan and more</td>
<td>FAS ligand, latent TNF, MMP-1, -2, -9, MMP-9/TIMP-1 complex</td>
<td>MMP-3, -10</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-A, neutrophil collagenase; PMNL collagenase</td>
<td>Collagenase</td>
<td>Collagen, gelatin, aggrecan and more</td>
<td>MMP-9</td>
<td>MMP-2, -3, -10</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Gelatinase</td>
<td>Collagen, gelatin, aggrecan and more</td>
<td>α1-Antiproteinase, latent TGF-β1</td>
<td>MMP-2, -3</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase, macrophage proteinase, macrophage elastase</td>
<td>Stromelysin</td>
<td>Collagen IV, gelatin, elastin and more</td>
<td>ProMMP-9, latent TNF</td>
<td>Not determined</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Collagenase</td>
<td>Collagen, gelatin, aggrecan and more</td>
<td>MMP-9</td>
<td>MMP-2, -3</td>
</tr>
</tbody>
</table>

Introduction
Appendicitis as an immunological issue

Lymphocyte subsets recruited to the inflamed appendix
Peripheral lymphopenia and increases of lymphocytes in the appendix, have been shown in patients with inflamed appendices compared with those in normal appendices (Soo et al., 1995). Patients with appendicitis showed a reduction of the CD45RO+ (memory) T cells in peripheral blood and an increase in the inflamed appendix, accompanied by an inverse relationship in the CD45RA+ (naïve) T cell subsets. These patients also showed increases in cells expressing the T cell activation marker CD25 in peripheral blood and the appendix.

Expression of cell surface markers in inflamed appendix
Two studies have analysed the local expression of cell surface markers in appendicitis. The first study investigated T cells (CD3), helper T cells (CD4), cytotoxic T cells (CD8), NK cells (CD57), monocytes (CD14) and activation marker CD25 in the lamina propria of appendix specimens and showed that all of these cell types were increased in phlegmonous appendicitis compared with the levels in normal appendices (Tsuji et al., 1993). The total plasma cell isotypes (IgG + IgA + IgM) were also more abundant in acute appendicitis than in normal appendices. The second study analysed the lymphocyte surface markers of T helper cells (CD4), B cells (CD19), NK cells (CD56) and cytotoxic T cells (CD8) in appendiceal specimens and peripheral blood (Kuga et al., 2000). Perforated appendicitis was associated with increased numbers of infiltrating CD8+ T cells and NK cells in the appendix specimens compared with those in non-perforated appendicitis. The patients with perforated appendicitis also showed a decreased number of NK cells in the blood.

Inflammatory bowel disease
Ulcerative colitis and Crohn’s disease are the two most common chronic inflammatory bowel diseases. Ulcerative colitis involves chronic inflammation of the large bowel, most often limited to the mucosa and submucosa. In Crohn’s disease inflammation may affect the whole of the gastrointestinal tract, although the distal ileum and the colon are the two most prominent localizations. The intestinal inflammation in Crohn’s disease is most often transmural. The immune response in ulcerative colitis has been associated with an atypical Th2-like inflammation, with production of IL-5 and IL-13 (Fuss et al., 2004; Heller et al., 2005; Strober and Fuss, 2011). In contrast, Crohn’s disease has predominately been associated with a Th1-mediated response, with increased production of IL-12 (Hart et al., 2005; Monteleone et al., 1997; Parronchi et al., 1997) and IFN-γ (Fuss et al., 2004). Identification of the involvement of Th17 cells has revised
this paradigm, where particularly Crohn’s disease has shown involvement of Th17 cells in the intestinal inflammation (Brand, 2009; Fujino et al., 2003).

**Pregnancy**
During pregnancy, a range of physiological changes occur, which may influence the pathogenesis of appendicitis; one example is that the immune system shifts toward Th2-like inflammation (Marzi et al., 1996; Saito et al., 1999). Interestingly, appendicitis has been reported to be less common during pregnancy (Andersson and Lambe, 2001).

**Population-based studies on inflammatory bowel disease**
An immunologically driven pathogenesis in appendicitis has been suggested on the basis of an inverse relationship between appendicitis and ulcerative colitis (Andersson et al., 2001), a positive association with Crohn’s disease (Andersson et al., 2003), and a decreased incidence during pregnancy (Andersson and Lambe, 2001). This is the basis of the studies described below. This work attempts to answer the following questions. What are the differences between an acute and chronic inflammatory disorder in the intestine? Are some people more prone to develop appendicitis, depending on differences in their immune defence? Are there differences in the type of inflammation between different types of appendicitis? Are there different types or entities of appendicitis?
Aims and hypothesis

Hypothesis
The diagnosis appendicitis harbours two different entities with different immunopathogeneses, one progressing to gangrene and perforation (advanced) and one that resolves spontaneous (phlegmonous). The progression to gangrene and perforation is caused by an excessive inflammation of Th1 type.

General aim
To investigate the immune response in advanced and phlegmonous appendicitis.

Specific aims
To investigate

- the immune response in patients with advanced and phlegmonous appendicitis in regard to Th1, Th2, Th17 and innate immunity.

- the local immune response in appendiceal tissue in appendicitis and it’s relationship to the systemic immune response.

- the response of mononuclear blood cells to stimuli in patients previously appendicectomized for gangrenous and phlegmonous appendicitis.

- the systemic immune response in patients with actual advanced and phlegmonous appendicitis.

- the systemic immune response over time in patients with an actual advanced and phlegmonous appendicitis, and to clarify the relationship of observed differences with the duration of symptoms.
Material and Methods

Subjects
All studies were approved by the local ethics committee at Linköping University. The characteristic of all subjects included in papers I-IV, is shown in Table 4.

Paper I
Blood was obtained from 20 healthy patients who had previously been appendicectomy for gangrenous appendicitis (n=7), phlegmonous appendicitis (n=8), or those without appendicitis (n=5), a so-called negative appendectomy. The patients were identified from the computerised register at Linköping University Hospital.

Papers II and III
Patients admitted during 2003-2005 for suspected appendicitis to the emergency departments at Linköping University Hospital and County Hospital Ryhov in Jönköping, Sweden were included. In total 500 patients were included. For paper II we selected the first 20 with a discharge diagnosis of advanced appendicitis, 20 patients with phlegmonous appendicitis and 40 patients with a discharge diagnosis of non-specific abdominal pain. Non-operated patients who were treated with antibiotics were excluded as this may have masked the true diagnosis of appendicitis. After the histopathologic re-examination there were 21 patients with phlegmonous appendicitis, 16 patients with advanced appendicitis and 42 patients with non-specific abdominal pain included in paper II. The age and sex distributions were the same between the groups.

In paper III, all patients operated by appendectomy and having appendicitis according to the pathology report were included. After the histopathological classification, there were 108 patients with phlegmonous appendicitis and 61 patients with advanced appendicitis. The sex distribution between the groups were even (p=0.517), but the patients with advanced appendicitis were older (p=0.015) and had a longer duration of symptoms at blood sampling (p=0.0001), based on the interval from when the patients first felt pain to when the blood sample was taken. There were no significant differences between the times from sampling to operation between the groups.
Paper IV

Here, the included patients were admitted during 2005-2008 for surgery with suspected appendicitis at Linköping University Hospital and County Hospital Ryhov in Jönköping, Sweden. Blood samples were taken during the initiation of anaesthesia. After the appendectomy, approximately half of the appendix, divided transversally, was taken for routine pathology and the other half was put on ice for analysis of cytokine secretion. After the histopathological classification, seven patients with phlegmonous appendicitis and ten patients with advanced appendicitis were included.

Table 4. Characteristics of the different patient groups in papers I-IV, the patients in paper II were in also included in paper III.

<table>
<thead>
<tr>
<th></th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>PA</td>
<td>NA</td>
<td>NSAP</td>
</tr>
<tr>
<td>Number of patients</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>3/4</td>
<td>3/5</td>
<td>1/4</td>
<td>22/20</td>
</tr>
<tr>
<td>Age, median (range)</td>
<td>16 (14-17)</td>
<td>17 (15-20)</td>
<td>16 (11-27)</td>
<td>21 (13-44)</td>
</tr>
<tr>
<td>Time after surgery in months, median (range)</td>
<td>78 (48-101)</td>
<td>54 (4-52)</td>
<td>32 (5-92)</td>
<td>38.0 (8-239)</td>
</tr>
<tr>
<td>Duration of symptoms at sampling (h), median (range)</td>
<td>38.0 (8-239)</td>
<td>25.5 (9-79)</td>
<td>25.0 (11-73)</td>
<td>23 (1-144)</td>
</tr>
</tbody>
</table>

Advanced appendicitis (AA), phlegmonous appendicitis (PA), negative appendectomy (NA) and non-specific abdominal pain (NSAP)
Cell separation

Separation of peripheral blood mononuclear cells (papers I and IV)

Peripheral blood was obtained in vacutainer tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were separated within two/three hours on Lymphoprep (Medinor AB, Lidingö, Sweden). Lymphoprep is a gradient solution with a density of 1.077 g/mL, consisting of sodium diatrizoate (also known as Hypaque; 9.1% w/v) and polysaccharide (5.5% w/v). The blood was diluted with Hank’s balanced salt solution (HBSS, Invitrogen, Paisley, Scotland; UK) and layered on top of Lymphoprep, during centrifugation, the erythrocytes aggregated and the lymphocytes and monocytes were trapped in the interphase between Lymphoprep and a mixture of plasma and HBSS (Figure 4). Erythrocytes were depleted as they formed a pellet at the tube bottom. The high osmolarity of the Lymphoprep solution affected the granulocytes by making them shrink and sediment with the erythrocytes. To discard plasma and the remainder of Lymphoprep, cells were washed in HBSS.

Figure 4. Separation of mononuclear cells from blood by density gradient centrifugation.

For culturing, cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂. Cells were resuspended in T cell culture medium (TCM) consisting of Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with L-glutamine (292 mg/mL, Flow Lab, Irvine, Scotland), sodium bicarbonate (3.024 g/l), penicillin (50 IE/ml, Flow Lab), streptomycin (50 µg/ml Flow Lab or
Cambrex, New Jersey, USA), 100x non-essential amino acids (10 mL/L, Gibco BRL) and 5% heat-inactivated foetal calf serum (Sigma-Aldrich, St. Louis, MO, USA). The cells were then counted by phase contrast microscopy in a Bürker Chamber and the cell density was adjusted to $1.0 \times 10^6$ mononuclear cells/ml in paper I and $0.5 \times 10^6$ mononuclear cells/ml in paper IV.

**Separation of appendix mononuclear cells (paper IV)**

After surgical removal, the appendix was placed in a tube containing HBSS, without Ca$^{2+}$ and Mg$^{2+}$, (Invitrogen) and brought on ice to the laboratory. Preparation of the tissue was carried out by washing with HBSS, without Ca$^{2+}$ and Mg$^{2+}$, removing additional coagulated blood remnants and surrounding fat, followed by cutting the tissue into smaller pieces with sterile scissors in a Petri bowl. The pieces were minced through a strainer with the help of a piston, into a clean Petri bowl containing HBSS without Ca$^{2+}$ and Mg$^{2+}$, and finally filtered through a 100 µm cell strainer (BD Biosciences, San Jose, CA, USA). The cell suspension was further washed by centrifugation. The cell pellet was resuspended in HBSS without Ca$^{2+}$ and Mg$^{2+}$ and placed on top of Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and appendix mononuclear cells (AMC) were acquired as described for PBMC above and adjusted to a cell density of $0.5 \times 10^6$/mL. Ficoll-Paque Plus has long been used for separation of tissue at our laboratory as, to the best of our knowledge, Ficoll-Paque Plus and Lymphoprep are comparable reagents.

**Antigens and mitogens**

In paper I, cells were stimulated with purified protein derivate (PPD) of *Mycobacterium tuberculosis*, tetanus toxoid (TT) from *Clostridium tetani* and lipopolysaccharide (LPS). All children in Sweden born before 1975 were vaccinated against tuberculosis and most adults should therefore have an immunological memory of PPD. Tuberculosis is caused by an intracellular bacterium and the immunological response is usually of Th1-type (Del Prete et al., 1991; elGhazali et al., 1993). LPS is found in the outer cell wall of Gram-negative bacteria. Tetanus toxoid is known to induce a strong humoral immune response in humans after vaccination and most of the TT-specific T cells secrete Th1 cytokines such as IFN-γ but also IL-4 (elGhazali et al., 1993; Mayer et al., 2002). Phytohaemagglutinin A (PHA) is a plant lectin which acts as a mitogen that activates T-cells polyclonally (O’Flynn et al., 1985). In papers I and IV, PHA was used as a positive control, but in paper IV, it was further used to elucidate the capability of activation.
Analysis of cytokine production

Enzyme-linked immuno spot assay (papers I and IV)

Enzyme-linked immuno spot assay (ELISPOT) is a highly sensitive method for in vitro detection of cell-secreted cytokines, at the single cell level. It was originally developed for detection of anti-body-secreting cells (Czerkinsky et al., 1983; Sedgwick and Holt, 1983), but has been modified for detection of antigen-secreting cells (Czerkinsky et al., 1984) and later adapted for measurement of cytokine-secreting cells (Czerkinsky et al., 1988). Czerkinsky and colleagues coined the term ELISPOT (Czerkinsky et al., 1983). Its principle can be summarized as follows: antibodies for the cytokine of interest are coated on a membrane and cells and culture media are added to the plate, which enables the secreted cytokines to be immediately captured by the antibodies at the bottom of the wells (Figure 5). Following cell removal, to visualize the imprint of the secreted cytokine, a secondary antibody conjugated with biotin is added. The small size of the biotin molecule enables a larger number of molecules to fit the antibody. Biotin binds irreversibly to avidin. The next substance to be added is an enzyme-conjugated streptavidin, which in turn binds to the biotin molecules. A coloured precipitate is formed by the enzyme substrate, which under a microscope can be identified as a “spot”. Each spot corresponds to one cytokine-producing cell.

In papers I and IV, nitrocellulose-bottomed 96-well MAHAN 4550 microtiter plates were used (Millipore, Bedford, MA, USA). These plates were coated with mouse anti-human IFN-γ, mouse anti-human IL-4, mouse anti-human IL-10 or mouse anti-human IL-12-I monoclonal antibody (all purchased from Mabtech AB, Stockholm, Sweden) in papers I and IV. In addition, they were coated with mouse anti-human IL-17 and recombinant human TGF-β sRII/Fc chimera (both R&D Systems, Abingdon, UK) in paper IV. Cultures were set in ELISPOT plates for analysis of the spontaneous and antigen/mitogen-induced (in paper I, TT and PPD for all cytokines except IL-12, where TT was exchanged for LPS; in paper IV, PHA) cytokine secretion. As a negative control TCM alone was used without adding cells. As a positive control, cells were stimulated with PHA. After incubation with cells, plates were emptied and incubated with biotinylated mouse anti-human IFN-γ, IL-4, IL-10 and IL-12 (all from Mabtech) for paper I and in addition for paper IV, anti-human TGF-β1 and IL-17 (both R&D Systems) detection antibodies. This was followed by incubation with streptavidin-alkaline phosphatase (AP) conjugate (Mabtech), then after washing, the wells were incubated with alkaline phosphatase substrate BCIP-NBT (Bio-Rad, Solna, Sweden). The spots were counted using the ELISPOT reader system Transtec 1300 (Autoimmune Diagnostica GmbH, Straßburg, Germany).
Material and Methods


Enzyme-linked immunosorbent assay (paper I)
The enzyme-linked immunosorbent assay (ELISA) technique was originally developed by Engvall and colleagues (Engvall et al., 1971; Engvall and Perlmann, 1971). The technique was originally developed for quantification of the amount of IgG, but has been modified also to allow detection of antigens (Kemeny, 1992). Its principle can be summarized as follows: antibodies for the cytokine of interest are coated onto the wells of polystyrene microtiter plates, unbound binding sites are blocked and cell-free media including bovine serum albumin are added to the plate. When adding cells, secreted cytokine is captured by the antibodies bound in the wells. In parallel, a standard curve of different concentrations of recombinant cytokines is also incubated on the same plate. To visualize the imprint of the secreted cytokine, a secondary antibody conjugated
with biotin is added followed by the addition of poly-horseradish-peroxidase (HRP) (enzyme)-conjugated streptavidin. When the substrate 3,3',5,5'-tetramethylbenzidine (TMB), is added to the wells, it reacts with the enzyme following the substrate to form a yellow, soluble product, proportional to the amount of cytokine bound to the primary antibody. The colour intensity is then detected by spectrophotometry and the amount of cytokine in the sample is determined by comparison with the standard curve.

For the collection of supernatants, cells were incubated with or without antigen/mitogen at the same time as the ELISPOT for paper I, where 1 x 10^6 mononuclear cell/mL were diluted ½ with mitogen/antigen, PHA PPD and TT. The cells for spontaneous secretion were only diluted with TCM and the negative control consisted of medium only. PHA-stimulated cells were incubated for two days at 37 °C in a humid atmosphere with 5% CO₂, while TT- and PPD-stimulated cells were incubated for seven days. The optimal incubation times had previously been tested (Jonsson et al., 2005). The cells were then centrifuged and the supernatants were collected, frozen and stored at -70 °C until use.

For paper I the production of IFN-γ, IL-10 and IL-5 was detected by ELISA. Costar 3690 plates (Costar Inc., Corning, NY, USA) were coated with mouse anti-human- IFN-γ (Sanquin Reagents, Amsterdam, Netherlands), mouse anti-human- IL-10 (Sanquin Reagents) or purified rat anti-human IL-5 (BD Pharmingen, San Diego, CA, USA). For the standard curve different dilutions in TCM of IFN-γ (Sanquin Reagents, range 3.9 – 250 pg/mL), IL-10 (Sanquin Reagents, range 3.1 – 100 pg/mL) or recombinant human IL-5 standard, (BD Pharmingen, range 3.9 – 250 pg/mL) were used. Samples, standards and a blank were added in duplicate. TCM was used as a blank. Following a washing step, a biotin-conjugated rabbit anti-human polyclonal IFN-γ antibody (Sanquin Reagents), mouse antihuman monoclonal IL-10 antibody (Sanquin Reagents) or a rat anti-human IL-5 monoclonal antibody (BD Pharmingen) was diluted with a high performance ELISA buffer (HPE, Sanquin Reagents) and added to the plate. The plates were washed and streptavidin Poly-HRP (Sanquin Reagents) diluted with HPE (Sanquin Reagents) was added to the plate followed by washing and the addition of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich); the reaction was stopped by adding 1.8 M H₂SO₄. The optical density (OD) for the amount of substrate converted to product was thereafter detected at 450 nm, with a wavelength correction made at 600 nm, in a Multiscan Ascent ELISA reader (Thermo Labsystems, Helsinki, Finland). Values are expressed as pg/mL calculated from the OD of the standard curve after subtracting the blanks and the wavelength correction.
Material and Methods

Multiplex bead assay (Luminex) (papers II-III)
The multiplexed microsphere-based flow cytometric assays use polystyrene microspheres, 5.6 µm in diameter, which are internally dyed with two different fluorochromes (Dunbar, 2006; Vignali, 2000). Using precise amounts of each of these fluorochromes, red and infrared fluorescent dyes, an array is created consisting of 100 different microsphere sets with specific spectral addresses (using the “Luminex 100” instrument); in this way, different fingerprints are formed. To each set of microspheres antibodies against the inflammatory analyte of interest, are coupled. This bead set can be mixed with several other bead sets, to which antibodies against other inflammatory markers have been coupled, enabling detection of several analytes in a single sample. Beads with bound analytes are then incubated with detection antibodies conjugated with biotin together with streptavidin bound to a green light emitting fluorochrome, forming a complex (Figure 6). The green mean fluorescence intensity (MFI) is proportional to the amount of bound analyte, which gives information about the concentration of analyte. This requires use of a standard curve with known concentrations of the analytes.

In paper II, IL-1ra, IL-1β, IL-2, IL-6, CXCL8, IL-10, IL-12p70, IL-15, IL-17, IFN-γ, TNF, CCL2 and CCL3 (Linco Research, St. Charles, MO, USA) were analysed in plasma and MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13 (R & D Systems, Minneapolis, MN, USA) in serum. The assay detected the MMP proteins in pro-mature and TIMP-1-complexed form. In paper III, IFN-γ, IL-12p70, IL-4, IL-5, IL-17, IL-6, IL-1β, CXCL8, CCL2, myeloperoxidase (MPO), granulocyte macrophage-colony stimulating factor (GM-CSF) (all from LINCOplex, Millipore Corporation, Billerica, MA, USA) and CCL20 (BioLegend, Inc., San Diego, CA, USA) were analysed. The analyses were carried out according to the manufacturers’ instructions, but for the standard in paper III, the curves for IFN-γ, IL-12p70, IL-4, IL-5, IL-17, IL-6, IL-1β, CXCL8, CCL2 and GM-CSF were extended with two extra standard points below the lowest. Single samples were analysed. The plates were read using the Luminex®100TM system. For acquisition and analysis of data, for paper II, the software program StarStation 2.0 (Applied Cytometry Systems, Sheffield, UK) was used; for paper III, StarStation 2.3 for IFN-γ, IL-12p70, IL-4, IL-5, IL-17, IL-6, IL-1β, CXCL8, CCL2, GM-CSF and MPO and StarStation 3.0 for CCL20 were used. Values below the detection limit were given half the value of the detection limit.
Material and Methods

Different properties of enzyme-linked immunospot assay, enzyme-linked immunosorbent assay and Luminex

ELISPOT, ELISA and Luminex use the same immunochemical “sandwich” principle, but they differ in two ways. ELISA and Luminex answer the question, “How much is secreted?” by measuring the real concentration, whereas ELISPOT answers the question, “What is the frequency of secreting cells?” by measuring the number of cells. The other difference is that ELISA and Luminex are mostly used for measurements in cell-free media, plasma or serum and are defined as immunoassays whereas ELISPOT measures secretion from cultured live cells directly in the ELISPOT plate, thus combining a bioassay with an immunoassay. ELISPOT appears to be 200 times more sensitive than ELISA, with ELISPOT detecting 10-100 cells per well, whereas results from ELISA were below the detection limit for less than $10^4$ cytokine-releasing cells (Tanguay and Killion, 1994). Luminex has a wide dynamic range, where fewer dilutions are required for many analytes; in analyses for many analytes, it is possible to obtain quantitative estimates for a test sample analysed in a single dilution (Krishhan et al., 2009). The ELISA format normally requires numerous sample dilutions to avoid misleading results. Detection of spontaneous cytokine secretion from resting cells can be difficult, as in the case of IL-4, whereas ELISPOT has been shown to provide this sensitivity (Ekerfelt et al., 2002; Ewen and Baca-Estrada, 2001; Tanguay and Killion, 1994). In theory, when a large number of cells are secreting a small amount of cytokine, their combined action produces a high concentration; however, this might fail to be detected with ELISPOT, but there might be a detectable signal by Luminex or ELISA. ELISPOT is not preferable when detecting high levels of cytokine, for which
ELISA provides more precise measurements. In Luminex each microsphere can be thought of as an ELISA well, and therefore a sample containing N number of beads is considered N number of independent ELISA measurement. The advantages of Luminex are the small sample volume needed, multiplexing in the same sample well and a wide dynamic range.

**Statistics**

In papers I-III, Kruskal-Wallis test was used for comparisons between the three groups, and P<0.05 was considered statistically significant. When the Kruskal-Wallis test indicated significant differences between the three diagnostic groups, the Mann-Whitney U-test was used as a further post hoc test, for analyses of non-normally distributed variables.

In paper II, the Kolmogorov-Smirnov test was used to test for a normal distribution of each variable and for diagnoses. Fisher’s exact test with the Freeman-Halton extension for date of two-rows by three-columns was used for analysing differences in the proportion of patients with detectable markers when the median was below the detectable limit. As this is a hypothesis-generating study, we did not perform any correction for multiple comparisons beside the limit of posthoc analyses for factors where Kruskal-Wallis test indicated differences between the groups.

In paper III, Fisher’s exact test was used for variables where >30% of the samples showed non-detectable levels, values below the lowest standard point were assigned as undetectable and the variables were analysed as dichotomized proportions (non-detectable/detectable). Multiple logistic regression was used to adjust for differences in confounding factors, like duration of symptoms, sex and age.

In paper IV, the Mann-Whitney U-test was used to analyse differences between groups. Wilcoxon’s test was used to analyse the differences in cytokine secretion between blood and appendix. A p-value below 0.05 was considered statistically significant. A p-value below 0.10 was considered to indicate a trend in view of the small numbers, and is also reported.

All statistical analyses were performed using SPSS 11.5 for Windows® (SPSS, Chicago, IL, USA, paper I), SPSS 14.0 for Windows (paper II) or SPSS 16.0 (papers III-IV). VassarStats (http://faculty.vassar.edu/luny/VassarStats.html, accessed July 5, 2007) was used for the Freeman-Halton extension of the Fisher’s exact test to allow inferences on two by three contingency tables (paper III).
Results & Discussion

Systemic response in blood four months or more after surgery (paper I)

As the antigen in appendicitis is unknown, even though several bacteria have been characterized during its surgery (Bennion et al., 1990; Gladman et al., 2004), it is hard to conclude which bacteria is the “bad guy” because of the resemblance with the normal flora, and to determine if a bacterium, virus or something else is the aetiological factor in appendicitis. In paper I, different recall antigens used in vaccinations (TT and PPD), antigens for Gram-negative bacteria (LPS) were used instead of the unknown antigen in appendicitis, to investigate the memory response in patients with phlegmonous and advanced appendicitis. PHA does not generate a memory response; instead, it produces a general activation. The spontaneous secretion was also analysed to study if the patient samples responded in the same way to the harsh treatment involving non-physiological condition, that is removing blood, purifying PBMC and incubating these cells in a 37 °C humidified atmosphere. Two different methods were used to enable analyses of both the number of cells secreting cytokines (ELISPOT) and the amount of cytokine secreted (ELISA). The cytokines analysed were IFN-γ, IL-4, IL-10 and IL-12p70 with ELISPOT and IFN-γ, IL-5 and IL-10 using ELISA. As IL-4 and IL-12p70 have been shown to be difficult to detect using ELISA, these cytokines were detected with ELISPOT, which has been shown to be a sensitive method (Ekerfelt et al., 2002; Ewen and Baca-Estrada, 2001; Tanguay and Killian, 1994). As both IL-4 and IL-5 belong to the Th2 response, IL-5 was used in the ELISA instead of IL-4 as a marker for a Th2 response. IFN-γ and IL-12p70 were used as markers for a Th1 response (Bonilla and Oettgen, 2010) and IL-10 was mainly used for an anti-inflammatory response. IL-10 is secreted mainly by Treg (Sakaguchi et al., 1995; Vignali et al., 2008), but also by Th1, Th2 and Th17 cells. (Cope et al., 2011; Ouyang et al., 2011). IL-10 is mostly considered to have anti-inflammatory activity with a down regulating effect on Th1, Th2 and Th17, but the effect on NK cells is more of a stimulatory nature with increased cytotoxicity (Mocellin et al., 2003).

TT is an antigen known to induce strong CD4 T cell- specific immunity in humans after vaccination and has been shown to induce a mixed Th1/Th2 response with production of IFN-γ, IL-4 and IL-5 (elGhazali et al., 1993; Rivino et al., 2004; Rowe et al., 2001). PPD on the other hand has after vaccination been shown to induce a strong IFN-γ secretion, as Mycobacterium tuberculosis is an intracellular bacteria and the response is a typical Th1- response (Del Prete et al., 1991; elGhazali et al., 1993).
In this unique study, patients with advanced appendicitis showed increased levels of tetanus toxoid-induced secretion of both IFN-γ and IL-10, accompanied by an increased number of cells spontaneously secreting IL-10 compared with the case in phlegmonous appendicitis (Figure 7); no other study on appendicitis has covered this before. The TT-induced IFN-γ implies that the patients suffering from advanced appendicitis had a response that deviated more towards Th1, which is in line with our hypothesis. However, this finding has to be considered cautiously in view of the low number of patients. The increased number of cells spontaneously secreting IL-10 may depend on the fact that the cells from the advanced compared with phlegmonous appendicitis patients responded more easily to the harsh treatment to purify and culture them. This may also indicate that the advanced patients are more prone to secrete IL-10 in response to various stimuli than patients with a history of phlegmonous appendicitis. The increase in the amount of IL-10 induced by TT is in line with this, although the differences between the groups were smaller. However, we did not observe any differences between the groups regarding the number of TT-induced IFN-γ and IL-10-secreting cells. This may indicate that the cells from the advanced patients secreted more cytokine per cell than those of patients with phlegmonous appendicitis.

When comparing advanced and phlegmonous appendicitis a control group with negative appendectomy was used for comparison. This negative appendectomy group is heterogeneous; we do not know what the cause of the abdominal pain was, if it was spontaneously resolved appendicitis, nonspecific abdominal pain or some other inflammatory condition causing right iliac pain. Regression of acute appendicitis has been histologically identified as involving cell clusters being scattered throughout the muscularis propria and subserosa, which contrasts with the “normal” inflammatory distribution that demonstrates a more diffuse pattern (Ciani and Chuaqui, 2000). The dominant cells in resolving appendicitis have been identified as lymphocytes and eosinophils, with only a few or no neutrophils. In this study, the focus of the routine pathologist examining the appendix was not to find any signs of resolving appendicitis. Furthermore, we do not know what type of appendicitis these patients would develop if they would suffer from this illness later on. The observed differences in the control group compared with the appendicitis group are therefore difficult to interpret. The phlegmonous appendicitis group may also be a heterogeneous group owing to the fact that it may include patients who could have spontaneously resolving appendicitis or patients who would have developed advanced appendicitis over time. We studied the patients from 4 up to 117 months after the appendicitis; they were healthy at the time of sampling, but the presence of a subclinical infection cannot be completely ruled out.
No differences in the PPD-induced stimulation of any cytokine were observed. This may have been due to the fact that our patients (with median ages of 16, 17 and 16 years for negative appendectomy and advanced and phlegmonous appendicitis, respectively) are too young to have been included in the earlier vaccination program: Sweden stopped vaccination against tuberculosis for all children born after 1975.

The major finding of this study was that the patients with advanced appendicitis showed a stronger Th1 response than those with phlegmonous appendicitis, accompanied by a stronger IL-10 response, which is in line with our hypothesis. This was a study on patients with a history of appendicitis. The rationale for this strategy was, in a healthy state, to investigate whether constitutional differences in the ability to mount a strong Th1- response, could explain the different outcomes between patients that had previously developed phlegmonous or advanced appendicitis. This reasoning is based on the assumption that the ability to establish Th1 or Th2 responses, respectively, most likely differs between individuals, and is stable and similar over time in a certain individual. Such differences have been proposed to be either inherited, depending on genetic differences, e. g. cytokine promoter polymorphisms (Song et al., 1996) or acquired (Robinson et al., 2003), preferentially during childhood (Rook and Stanford, 1998). Genetic and environmental factors influences on the risk of acute appendicitis have been shown in twins, along with a slightly higher genetic risk for women (Sadr Azodi et al., 2009). The combined genetic effects in men and women accounted for 30% of the variation in risk of appendicitis, while non-shared environmental effects accounted for 70%. As such, conclusions cannot be drawn regarding whether the appendicitis itself was the cause of patients tending to develop a more Th1 type of response or whether the patients were more prone to develop a Th1- response before the appendicitis. These findings led to further investigation of the immune response in ongoing advanced and phlegmonous appendicitis and to analysis of this response when the patients had an inflamed appendix.
Figure 7. The levels of tetanus toxoid induced secretion of IFN-γ and IL-10 and the number of cells spontaneously secreting IL-10 in 100,000 lymphocytes from peripheral blood from patient’s appendectomy for either advanced appendicitis, phlegmonous appendicitis or false positive appendicitis, a so called negative appendectomy. P-values from comparison of all three groups with Kruskal-Wallis test is shown on a line at the top of the diagram and p-values between two groups are shown below on lines with markers indicating the groups compared.
The systemic response in patients with appendicitis (papers II-IV)

In paper II, including 16 patients with phlegmonous and 21 patients with advanced appendicitis, we wanted to screen the inflammatory response from a wide perspective in terms of inflammatory markers and compare the results with a control group with nonspecific abdominal pain. The design of the study was explorative and initially included a small amount of material with a wide perspective to facilitate the identification of important markers, with it then progressing to include all patients who were the sources off the collected material from suspected appendicitis cases. The rationale for use of a control group with nonspecific abdominal pain was to enable comparison between all patients admitted for right iliac pain, and to see if we could identify a feature of advanced appendicitis that leads to a need for surgery and that can be used to separate cases of phlegmonous appendicitis and non-specific abdominal pain. Furthermore, we explored the possibility of finding an inflammatory marker for use in the diagnosis of appendicitis.

The major finding of paper II was increased levels of IL-6, IL-17 and CCL2 in advanced compared with phlegmonous appendicitis. This may suggest the involvement of a Th17 response in the development of advanced appendicitis. Th17 responses are considered highly inflammatory and are involved in controlling tissue inflammation (Bettelli et al., 2007; Brand, 2009; Kolls and Linden, 2004).

On the basis of these findings, we wanted to analyse further the inflammatory response in advanced and phlegmonous appendicitis from the perspective of Th1, Th2 and Th17 in a larger sample.

Findings on differences between phlegmonous and advanced appendicitis in Th1-type responses

Phagocytes (monocytes/macrophages and neutrophils) and dendritic cells are, in the initiation phase of response to pathogens, the main producers of an immune response involving IL-12 (bacteria, fungi, intracellular parasites and viruses) through Toll-like receptors (TLR) and other receptors (Gee et al., 2009; Trinchieri, 2003). Induction of IFN-γ production from NK cells and T cells, enhancement of cytotoxicity of NK and cytotoxic T cells, and differentiation of naïve T cells into Th1 effectors are the major effects of IL-12, suggesting a key role for IL-12 in the development of cell-mediated immunity (Trinchieri, 2003). IL-12 has been suggested as a bridge between innate resistance and adaptive immunity as it is produced early during the innate response to infections and has the ability to sustain Th1-adaptive responses (Lyakh et al., 2008). IL-12 is composed of two covalently linked subunits, IL-p35 and p40, and when these are expressed together; the bioactive IL-12p70 is formed.
In paper III, advanced appendicitis showed to be associated with an increase in levels of the Th1-inducing IL-12p70, as analysed by Luminex (Figure 8). Similarly, a trend for an increased number of cells spontaneously secreting IL-12p70 in advanced appendicitis was detected in paper IV. The levels of IFN-γ as measured by Luminex in papers II and III, were similar in the two groups. The increased number of cells spontaneously secreting IFN-γ was, however, increased in advanced compared with phlegmonous appendicitis in paper IV. In sharp contrast to our results, a previous study including 37 patients with phlegmonous and 19 with advanced appendicitis, which is one of the few studies with a similar focus on cytokines to this study, observed no differences for IL-12p70 and detected lower levels of IFN-γ in patients with advanced compared with phlegmonous appendicitis using ELISA (Rivera-Chavez et al., 2003). We detected a similar proportion of patients with advanced appendicitis in terms of the IL-12p70, but fewer of such patients with phlegmonous appendicitis. The opposite situation was observed in terms of IFN-γ, where we detected a similar proportion of patients with phlegmonous appendicitis but more with advanced appendicitis. The grouping of patients included similar criteria with advanced appendicitis showing signs of gangrene and/or necrosis, and a pathologist blinded to other clinical data confirmed the diagnosis. It is difficult to explain these differences in results.

Taken together our results from papers I, III and IV suggest an increased Th1 response in advanced compared with phlegmonous appendicitis, which supports our hypothesis.
Figure 8. Findings on differences between phlegmonous and advanced appendicitis in Th1-type responses. Circle diagrams showing the proportion of patients with detectable and undetectable levels of IL-12p70 in plasma, in patients with advanced and phlegmonous appendicitis. The p-value refers to comparison by Fisher’s exact test. The number of spontaneously IL-12p70 and IFN-γ-secreting cells in 50,000 peripheral blood mononuclear cells (PBMC) from patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test.
Th17

IL-6 has long been known to be associated with reactions in the acute phase of an immune response, and it is an important factor for the synthesis of C-reactive protein, for which the serum level is increased in acute and chronic inflammatory diseases (Gabay and Kushner, 1999). The regulation of T cell differentiation and activation has been shown to be associated with the action of IL-6. Furthermore, IL-6 has been recognized as a major regulator of the balance between Treg and effector Th17 cells. The Th17 cells are induced by a combination of IL-6 and TGF-β (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen and Stockinger, 2006), while Treg cells are inhibited by IL-6 (Neurath and Finotto, 2011). In addition, IL-6 has been shown to activate Th2 cytokine production in T cells via the transcription factor C/EBP (Rincon et al., 1997). Dysregulation or overproduction of IL-6 is associated with autoimmune diseases such as multiple sclerosis (Lock et al., 2002; Matusevicius et al., 1999) and rheumatoid arthritis (Chabaud et al., 1999; Ziółkowska et al., 2000), in which Th17 cells are considered to be the primary cause of pathology (Kimura and Kishimoto). IL-6 was increased in advanced compared with phlegmonous appendicitis, and this finding could be repeated reproduced in paper II and paper III (Figure 9). The patients in paper II are also included in the larger population studied in paper III. Increase in serum (Anielski et al., 2009; Paajanen et al., 2002; Sack et al., 2006) and plasma (Rivera-Chavez et al., 2004; Rivera-Chavez et al., 2003) levels of IL-6 is well described in appendicitis and our findings corroborate several previous studies demonstrating increased secretion in advanced appendicitis. Furthermore, serum IL-6 has been shown to be increased in perforated compared with non-perforated appendicitis in adults (Yıldırım et al., 2006; Yoon et al., 2002) and children (Kharbanda et al., 2011). In line with this, the finding of a polymorphism in the IL-6 gene associated with severity of appendicitis, suggests a genetically determined difference in the immune activation between advanced and phlegmonous appendicitis, and in the development of gangrene and perforation (Rivera-Chavez et al., 2004). This genetic study included a total of 134 patients with appendicitis, where 91 patients were diagnosed with phlegmonous and 43 patients with advanced appendicitis.

Th17 cells are characterized by their expression of IL-17(A) and IL-17F. A number of biological functions are exerted by Th17 cells on the intestinal barrier (Brand, 2009). Th17 cells secrete a number of pro-inflammatory cytokines for which receptors are expressed on intestinal epithelial cells (IEC). IEC in turn release an array of pro-inflammatory cytokines and chemokines, such as CXCL8, which attracts neutrophils, and CCL20, which is chemoactive for Th17 cells and DC, further exacerbating the intestinal inflammation which has been shown in Crohn’s disease (Brand, 2009; Brand et al., 2006; Fujino et al., 2003). IL-17 was found to be increased in advanced appendicitis in paper II, but the finding could
not be reproduced in paper III (Figure 9). However, in paper III, probable induction of the production of other inflammatory markers associated with Th17 from intestinal epithelial cells, was observed. In paper IV, IL-17 was observed to be secreted by more cells locally in advanced appendicitis than in blood (Figure 10). This indicates that IL-17-secreting cells are enriched in the appendiceal tissue during appendicitis and in turn affect the surrounding intestinal epithelial cells in advanced appendicitis.

Although Th17 lineage development is independent of that of Th1, these cells can often be found side-by-side, suggesting that the interplay between Th17 and Th1 is important. Cells secreting both IFN-γ and IL-17, and have CCR6 and CXCR3 expressed on their surfaces, have been characterized (Acosta-Rodriguez et al., 2007). In the presence of IL-12, human Th17 clones could change and start producing IFN-γ in addition to IL-17, but Th1 clones could not be induced to produce IL-17, which indicates that Th17 seems to be more plastic than Th1 cells.

Interestingly, the chemokine CCL20, which is a chemokine and the ligand for CCR6, was found to be increased in advanced compared with phlegmonous appendicitis in paper III. This chemokine has been reported to be a predominant marker for Th17 cells and can specifically recruit CCR6-expressing cells. Epithelial cells of various tissues, but in particular in the specialized follicle-associated epithelial (FAE) cells in the dome region of Payer’s patch (Nishimura et al., 2009), have been shown to express CCL20, and mRNA for CCL20 has been demonstrated in epithelial cells in human appendix (Tanaka et al., 1999). CCR6 expression is not required for the induction of Th17 cells, but is important for the trafficking of Th17 cells to the target tissue, which is followed by CCR6-independent recruitment of more effector (Th1 and Th17) T cells, as shown in experimental autoimmune encephalomyelitis and multiple sclerosis (Reboldi et al., 2009). Neutrophils have been shown to attract Th17 through the secretion of CCL2 and CCL20 (Pelletier et al., 2010). Intriguingly, both CCL2 and CCL20 were increased, especially when the mucosal barrier was breached by necrosis, as in the case of advanced appendicitis (Figure 9). On the other hand, CXCL10 and CCL2 have been shown to mediate the recruitment of Th1 cells (Pelletier et al., 2010). Unfortunately, CXCL10 was not analysed in any of our studies. The increase of CCL20 in advanced appendicitis suggests a more pronounced Th17 induction with increased trafficking of Th17 cells or possibly increased IL-17 secretion from innate cells promoting epithelial cells to secrete CCL20 for the recruitment of neutrophils.

Furthermore, TGF-β is most commonly considered an anti-inflammatory cytokine, but has also been shown to induce differentiation of Th17 cells (Veldhoen and Stockinger, 2006). The widely different actions of TGF-β could
Results & Discussion

depend on local versus systemic expression and on its concentration. In paper IV, an increase of cells secreting TGF-β was seen in advanced compared with phlegmonous appendicitis, giving further support to the notion of increased Th17 in advanced appendicitis (Figure 10).

Altogether, the findings in papers II, III and IV are compatible with increased Th17 inflammation in advanced appendicitis, which may orchestrate the destructive tissue inflammation leading to gangrene and perforation.
Figure 9. Findings on differences between phlegmonous and advanced appendicitis in Th17-type responses. Box-plots of the levels of inflammatory markers in plasma in patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test or *the Freeman-Halton extension of the Fisher’s exact test.
Results & Discussion

Figure 10. Findings on differences between phlegmonous and advanced appendicitis in Th17-type responses. The number of spontaneously TGF-β-secreting cells in 50,000 peripheral blood mononuclear cells (PBMC) from patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test. The number of spontaneously IL-17-secreting cells in 50,000 (PBMC) compared to 50,000 appendix mononuclear cells (AMC), divided in advanced and phlegmonous appendicitis. P-values refer to comparisons by Wilcoxon’s test.

Th2 and anti-inflammation

Along with the indications of increases of Th1 and Th17 responses in advanced appendicitis, we also detected increases in anti-inflammatory factors. Both Th1 and Th17 cells can, as a negative feedback mechanism secrete IL-10 in response to pro-inflammatory cytokines, but Treg, Th2, Th9 cells and CTL can also under certain conditions secrete this cytokine (Cope et al., 2011; Ouyang et al., 2011). IL-10 was in mice initially classified in 1989 as a Th2-derived cytokine (Fiorentino et al., 1989) but this view has changed, and IL-10 is now considered as an essential molecule in the mechanism underlying suppression mediated by Tr1 cells, but also by Th1, Th2 and Th17, B cells, monocytes and keratinocytes (Saraiva and O’Garra, 2010; Taylor et al., 2006). IL-10 has a key effect on the suppression of Th1 response, but it also enhances the production of IL-10 by Treg, promotes CTL and more. It was recently suggested that Th1 cells themselves are a dominant source of IL-10 that controls immune responses (O’Garra and Vieira, 2007). The Th1 cell activation can be regulated extrinsically by IL-10-secreting induced Tr1 Treg. Alternatively, it can be regulated intrinsically via IL-10 production by Th1 cells, as a negative feedback loop that requires IL-12 for induction.

IL-10 was increased in advanced compared with phlegmonous appendicitis, as reported in paper II, but was not studied in paper III (Figure 11). Furthermore, the number of PHA-induced IL-10-secreting cells was increased in advanced appendicitis in paper IV, and the number of spontaneously secreting and TT
induced IL-10-secreting cells showed similar increases in paper I. Altogether, this may indicate an increase of the anti-inflammatory IL-10 in response to the increased Th1/Th17-type inflammation in advanced appendicitis, compatible with feedback mechanisms in inflammation.

In paper I we considered the effect of IL-10 as promoting cytotoxic activity which is one of its effects. This was based on observations that IL-10 in combination with IL-2, which is a hallmark of T-cell activation most pronounced in Th1-cells, may enhance cytotoxicity (Groux et al., 1998). The cytotoxic effects of IL-10 has been demonstrated as increased numbers of CD8$^+$ cytotoxic T cells and natural killer (NK) cells (Borish and Steinke, 2003; Levings et al., 2002), which both are features of Th1-responses. Interestingly, an increase in the numbers of cytotoxic T lymphocytes and NK cells has been reported in appendices from patients with perforated compared to non-perforated appendicitis (Kuga et al., 2000; Tsuji et al., 1993). NK cells localize in certain tissues such as the appendix where they can be activated by certain cytokines such as IL-10. Soo et al (Soo et al., 1995) reported a systemic increase of naive T cells and selective recruitment of memory T cells to an inflamed appendix, an inflammatory response that is enhanced by IFN-$\gamma$ which in turn stimulates the secretion of IL-10, IL-12 and inhibits IL-4 mediated effects (Nakamura et al., 1997). Thus, it cannot be fully elucidated if the increase of IL-10 in advanced appendicitis represents an increased Th1-response or an increase of the anti-inflammatory response. Nevertheless, both alternatives are compatible with an excessive inflammation in advanced appendicitis.

Divergent results regarding IL-10 in appendicitis have been reported elsewhere. Increased levels of IL-10 in advanced compared with phlegmonous appendicitis have been reported (Rivera-Chavez et al., 2003), corroborating our results. In contrast, although the findings were difficult to compare to our results owing to the use of different groupings, the absence of differences between perforated and non-perforated appendicitis were also reported (Yoon et al., 2002).

IL-1ra is a member of the IL-1 family and has an antagonizing effect on both IL-1$\alpha$ and IL-1$\beta$, by blocking the binding of IL-1 to cell surface receptors (Dinarello, 1996). IL-6 and TNF-$\alpha$ have been shown to induce IL-1ra, as possible negative feedback in response to the pro-inflammatory cytokines released (Gabay et al., 1997; Tilg et al., 1994). Both IL-6 and IL-1ra (Figure 11) were increased in advanced compared with phlegmonous appendicitis in paper II. In contrast, another study failed to find any differences in IL-1ra levels between advanced and phlegmonous appendicitis (Rivera-Chavez et al., 2003). In the context of Th1 inflammation, IL-4, which shows strong antagonistic effects to IFN-$\gamma$, may be considered an anti-inflammatory cytokine (Paul and Zhu, 2010). In paper IV, the number of IL-4-secreting cells was increased in advanced compared with
phlegmonous appendicitis (Figure 11). In contrast, the absence of differences regarding IL-4 between phlegmonous and advanced appendicitis was previously reported (Rivera-Chavez et al., 2003).

Taken the findings together, an increase of IL-10 in advanced compared with phlegmonous appendicitis was consistently found in papers I, II and IV, which might be interpreted as an anti-inflammatory feedback mechanism induced by the similarly increased Th1 and Th17 immunity. Analysis of IL-1ra in paper II and IL-4 in paper IV similarly showed their increases in advanced appendicitis, and could in the context of Th1/Th17-type inflammation also be considered to mirror increased anti-inflammatory feedback mechanisms in response to excessive inflammation.
Figure 11. Findings on differences between phlegmonous and advanced appendicitis in Th2 and anti-inflammation. Box-plots of the levels of inflammatory markers in plasma in patients with advanced and phlegmonous appendicitis. The number of spontaneously IL-4 and phytohemagglutinin (PHA) induced IL-10-secreting cells in 50,000 peripheral blood mononuclear cells (PBMC) from patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test.
Innate immune system

At an initial stage during acute inflammation, neutrophils are predominant, but after 24 to 48 h, monocytic cells start to dominate (Kaplanski et al., 2003). In contrast chronic inflammation is histologically associated with the presence of mononuclear cells, such as macrophages and lymphocytes. Peripheral blood mononuclear cells and monocytes are recruited to the inflammatory site by the cytokines CXCL8 and CCL2, respectively. The production of CXCL8 usually occurs at an early stage and lasts for about 24 h, recruiting and activating more neutrophils locally. After stimulation with inflammatory cytokines for several hours, there is a change in neutrophil production to CCL2 and not CXCL8 (Yamashiro et al., 1999). Most nucleated cells express CCL2 in response to activation by pro-inflammatory cytokines or stimulation of innate immune receptors by a range of microbial molecules (Shi and Pamer, 2011). The effect of CCL2 in adaptive immunity depends on the context, in that during end-organ inflammation it seems to elicit effector cells that are already Th1-polarized, but in other contexts it has been shown to stimulate Th2 polarization (Daly and Rollins, 2003). In papers II and III, we observed an increase in CCL2 (Figure 9) but no differences regarding CXCL8 in advanced compared with phlegmonous appendicitis. One possible explanation regarding the lack of differences in expression of CXCL8 would be that both types of appendicitis have progressed for some time, as we performed measurements in blood just before surgery, and the neutrophils had already undergone the transition to produce CCL2. Other groups have reported divergent results regarding CXCL8 in advanced and phlegmonous appendicitis. In line with our findings, one study reported no differences in CXCL8 in sera when comparing similar patient groups (Paajanen et al., 2002). In contrast, increased IL-8 in serum in perforated compared with non-perforated appendicitis has been observed (Kharbanda et al., 2011; Yoon et al., 2002). In these latter two studies, the patients were divided in another way where a mixed group of phlegmonous and gangrenous appendicitis was compared to perforated appendicitis, making direct comparisons with the present work impossible. Furthermore, another study investigated the inflammatory gene expression in appendicitis (Murphy et al., 2008). This study used yet another way of grouping patients, according to appendicitis score, based on the product of the maximum number of neutrophils per high-power field and the proportion of tissue secretions infiltrated by neutrophils. This calculation results in a measure of the neutrophil content in the appendix, but it does not include the variable of how deep the neutrophils have migrated into the appendix. To test the score further, they investigated the correlation of the peripheral white blood cell count and the expression of CXCL8 mRNA within the appendix, which were found to be significantly correlated with each other. Results on mRNA expression may be hard to interpret, since mRNA expression does not necessarily result in the
secretion of the CXCL8 protein. No serum or plasma levels of CXCL8 were measured in this study. Furthermore, the study was carried out on children, whose immune system may respond differently to that of adults, which has been reported in Lyme disease, for example (Ekerfelt et al., 1997; Widhe et al., 2005). Murphy et al., studied 18 patients with appendicitis divided in cases of severe and mild disease, and 15,505 probes corresponding to human genes were analysed. Similar expression of Th1, Th2 or Th17 genes were reported for the groups, but as discussed above, it is not possible to compare their findings with the present work.

Neutrophils are a measure of appendicitis as phlegmonous appendicitis is histologically defined as transmural infiltration of neutrophils in the appendix. The neutrophils contain stores of proteins in their granules, enabling them to kill microbes and digest tissues, where MPO is found in the primary, MMP-8 is the secondary and MMP-9 in the tertiary granule (Borregaard and Cowland, 1997). The effect of MPO is closely related to sites where polymorph nuclear cells (PMN) accumulate (Arnhold and Flemmig, 2010). The infiltration of PMN into inflamed tissues is often determined by the activity of MPO, where a substantial proportion of MPO might be released into the extracellular matrix during activation of PMNs. Neutrophils undergoing necrosis also release MPO. We observed increased secretion of MPO in advanced appendicitis as a sign of increased neutrophil activity, severe inflammation and necrosis compared with those in phlegmonous appendicitis (Figure 12).

Upon stimulation of neutrophils in blood with pro-inflammatory cytokines or endotoxin, rapid release of MMP-8 and MMP-9 has been shown (Borregaard and Cowland, 1997; Claesson et al., 2002; Pugin et al., 1999). We reported an increase in MMP-8 and MMP-9 (Figure 12) in serum measured by Luminex in advanced compared with phlegmonous appendicitis, and no differences regarding MMP-1, MMP-2, MMP-3, MMP-7, MMP-12 and MMP-13, in paper II. In contrast, Solberg et al. could not find any differences in MMP-9 levels between gangrenous, perforated and phlegmonous appendicitis in biopsies (Solberg et al., 2008) or in plasma (Solberg et al., 2012) analysed using ELISA. Also contrary to our results, MMP-1 and MMP-2 in tissue biopsies was shown to be increased in gangrenous compared with phlegmonous appendicitis (Solberg et al., 2008). It is difficult to explain these differences in results; one explanation could be that we have measured serum that is, at the systemic level, using different method of Luminex, although this is similar to ELISA. Furthermore, the patient samples of MMP-9 were not at levels corresponding to the lower or higher parts of the standard cure, but increased, which could be less reliable and have affected the results. One results that was in line with our findings was that no differences
were found between the groups for MMP-3 in tissue biopsies (Solberg et al., 2008), which does not seem to have any impact in appendicitis.

IL-17 has been shown to increase signs of collective neutrophil activity locally, as judged by increased activity of MPO and MMP-9 after local administration of recombinant IL-17 protein in bronchoalveolar lavage in animal studies (Hoshino et al., 2000; Prause et al., 2004).

Thus, our finding of increased levels of MMP-8 and MMP-9 may indicate more attenuated neutrophil infiltration and activity in advanced appendicitis, with a possible action of the neutrophils to attract more Th17 cells.

We did not find any differences in TNF or IL-1β, which is in line with a previous observation (Rivera-Chavez et al., 2004). Furthermore, no differences were found in GM-CSF between the groups.
Figure 12. Findings on differences between phlegmonous and advanced appendicitis connected to the innate immune system. Box-plots of the levels of inflammatory markers in serum for MMP-8 and MMP-9 and in plasma for MPO in patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test.
Results & Discussion

Local response in appendiceal tissue after surgery

To the best of our knowledge, paper IV is the first study of the local cytokine-secreting cells in advanced and phlegmonous appendicitis. We found increases of spontaneously secreted IL-4 and IL-10 from appendix mononuclear cells and also trends for increases of IL-12p70 and TGF-β in advanced compared with phlegmonous appendicitis (Figure 13). This could be interpreted as increases of anti-inflammatory, Th1, Th2 and possibly Th17 immunity, compatible with increased Th1/Th17-type inflammation accompanied by a counterbalancing anti-inflammatory response. Blood is easy to collect and analyse. In this assay, we used appendix mononuclear cells, which cannot be stored for a long time and need to be analysed immediately. What is going on locally in the specific organ may not always be the same, as shown in serum or plasma. In this unique study, we measured the same cytokines locally in the appendix and in the blood at the same time, in the same patients. The cytokines secreted by increased numbers of cells in advanced appendicitis in the blood were IFN-γ, TGF-β and IL-4, and there were trends for increases in IL-12p70 and PHA-induced IL-10 compared with the case in phlegmonous appendicitis. We conclude that the overall expression in appendiceal tissue is mirrored in the blood. There are few reported studies on local cytokine expression in appendicitis utilizing immunohistochemistry, immunocytochemistry or analyses of peritoneal fluid (Kuga et al., 2000; Rivera-Chavez et al., 2003; Tsuji et al., 1993). Our finding of increased IL-10 in appendiceal tissue in advanced appendicitis corroborates the findings of another study (Rivera-Chavez et al., 2003). In contrast, no differences were found between the groups regarding IL-4, IL-12p70 and IFN-γ in peritoneal fluid (Rivera-Chavez et al., 2003). This study utilized ELISA, which may explain why IL-4 and IL-12p70 were hard to detect. Another possible explanation of the diverging results is that peritoneal fluid is another compartment, which may not completely correlate with the expression in the appendix.
Figure 13. Findings on differences between phlegmonous and advanced appendicitis connected to the local response in appendicular tissue after surgery. The number of spontaneously IL-4, IL-12p70, TGF-β and IL-10-secreting cells in 50,000 appendix mononuclear cells (AMC) from patients with advanced and phlegmonous appendicitis. P-values refer to comparisons by Mann-Whitney U-test.
Inflammatory bowel disease

Similarities between Crohn’s disease, ulcerative colitis and appendicitis are that they all affect the intestine, however, Crohn’s disease and ulcerative colitis are both chronic, while appendicitis is an acute inflammation. In Crohn’s disease, both Th1 (Fuss et al., 2004; Hart et al., 2005; Monteleone et al., 1997; Parronchi et al., 1997) and Th17 cytokines are up-regulated while Tregs are impaired (Brand, 2009). In contrast, in ulcerative colitis a Th2-like differentiation process is dominant, which results in expansion of natural killer T cells producing IL-13 (and perhaps IL-5) (Fuss et al., 2004; Heller et al., 2005; Strober and Fuss, 2011). CCL20 has been shown to be increased in mucosa from active Crohn’s disease and ulcerative colitis (Brand et al., 2006). Although appendicitis shows several similarities with Crohn’s disease, according to cytokine secretion and population-based studies, there are a lot of differences, such as in term of the pattern of genetic susceptibility (Brand, 2009). Besides the present work, there has been little research on appendicitis associated with Th17, compared with the extensive research on inflammatory bowel disease.
Influence of differences in duration of symptoms, sex and age on the results

It is not possible to study the initiation of inflammation in the appendix since we do not know which patients will develop appendicitis and when. The interval referred to as the duration of symptoms, measured from the initiation of abdominal pain, when the patients first felt pain to when the blood sample was obtained before surgery. In paper II, the distributions in age and duration of symptoms between the groups were the same, but in paper III, the patients with advanced appendicitis were older and had a longer duration of symptoms than the phlegmonous group. There were no differences between the time from sampling to operation between the groups in paper III. A large number of studies have also found that perforation occurs at an early stage, many hours before the patient arrives at hospital (Andersson, 2007). Our analyses are therefore based on a late stage in the process of gangrene development. The increased levels of IL-6, CCL2, CCL20, MPO and IL-12p70 could not be explained by differences in duration of symptoms, sex or age, as demonstrated by logistic regression in paper III (Figure 14). Furthermore, when comparing the levels of inflammatory markers within different intervals of symptom duration between advanced and phlegmonous appendicitis, IL-6, CCL2 and CCL20 were still increased in advanced appendicitis especially at the early and intermediate stages.

Some studies have focused on children and their similarities and differences with adults; an example is where CRP and leukocyte count have been shown to important to exclude acute appendicitis in adults, but in children, they were not effective (Gronroos, 2001) but opposing results are also found (Kharbanda et al., 2011; Sack et al., 2006). Another study has shown that WBC counts have higher predictive value in children than adults (Lee and Ho, 2006), where other studies have shown the importance of WBC in predicting appendicitis in children (Kharbanda et al., 2011; Sack et al., 2006). Children’s immune system may respond differently to that of adults, as previously mentioned. When comparing acute appendicitis and normal appendix no differences in serum between the groups in children regarding IL-10 and MMP-9 has been observed (Dalal et al., 2005) in contrast to the increase of both these markers in adults in paper II. IL-6 have also shown help to diagnose advanced stages of acute appendicitis in children, which is in line with our results from adults in paper II (Kharbanda et al., 2011; Sack et al., 2006) but opposing results have also been reported (Groselj-Grenc et al., 2007).
Figure 14. Findings on differences between phlegmonous and advanced appendicitis connected to duration of symptoms. Box-plots of the levels of inflammatory markers in plasma in patients with advanced and phlegmonous appendicitis subdivided on duration of symptoms: a)<24 hours, b)24-47 hours and c)>47 hours. P-values refer to comparisons by Mann-Whitney U-test.
Negative appendectomy and non-specific abdominal pain as controls

The inclusion of groups of patients with negative appendectomy and non-specific abdominal pain as controls, has both advantages and disadvantages, depending on the purpose of the study. In paper I, negative appendectomy was used to include a group of patients who experienced surgery to control for suspected appendicitis that turned out to be negative, not inflamed. For those with negative appendectomy, we do not know what caused the abdominal pain, and in these cases several months or years after the appendectomy, the patients were healthy at sampling but we do not know if the immune system was affected in some way. Un-inflamed appendix is supposed to reflect a normal state of view, and was used as a reference. In paper II, non-specific abdominal pain represented a group of patients who had suspected appendicitis, but where the discharge diagnosis was non-specific abdominal pain. Here, we also do not know what the cause of the abdominal pain was; they may have had other inflammatory conditions and are a very divergent group. However, if the interest of your study is diagnosis that is, attempting to separate patients with a specific entity of appendicitis that has to be treated by operation, this study composition is the right choice. A further comparison would have been to include healthy patients, to determine the extent to which they differ from those with abdominal pain and appendicitis.

Phlegmonous appendicitis as a group

Phlegmonous appendicitis is most likely not synonymous with resolving appendicitis, but consists of both types of appendicitis, that is, both the resolving type and the type progressing to gangrene and perforation. The true nature of the disease in patients with phlegmonous appendicitis can therefore not be known at the time of operation.
Histopathologic examination

The appendix specimens were examined by one pathologist, who was blinded to all clinical information and the result of the primary histopathologic examination, in papers II and III. The use of one person to analyse every appendix tissue in the same way is important, as several pathologists may differ slightly in their classification. It is impossible to know if the pathologists have looked at the exact same area in the saved material, but in this study between three and five sections of the appendices were taken for routine histopathologic examination, including the apex and resection margins in all patients. In paper II, we first used the routine histopathologic examination but after one pathologist made a structured histopathologic re-examination, the diagnosis was changed from advanced to phlegmonous appendicitis in six patients, from advanced to nonspecific abdominal pain in one patient, from phlegmonous to advanced appendicitis in three patients, and from phlegmonous to nonspecific abdominal pain in one patient. One female with the discharge diagnosis of phlegmonous appendicitis presented atypical inflammation with an abundance of eosinophils within the specimen, which could not be classified as either phlegmonous or gangrenous appendicitis; this patient was excluded from the study.
Significance of different compartments

When studying the immune defence in appendicitis, different compartments can be analysed. In this work, serum, plasma, peripheral blood mononuclear cells and appendix mononuclear cells were used. Furthermore appendix tissue and peritoneal fluid are also potential alternatives when analysing the local immune response in the appendix (Rivera-Chavez et al., 2003; Zeillemaker et al., 1996).

What are the differences between serum and plasma, in terms of the conclusions that can be drawn from studies on them? To obtain plasma, tubes including an anti-coagulant must be used, in papers II and III, ethylenediaminetetraacetic acid (EDTA) was used, and after centrifugation the top layer consisting of plasma could be collected. There are several other anti-coagulants that can be used, depending on the focus of study. However, these different analyses are not comparable, as the anti-coagulants can interfere with and disturb the results. This could have occurred for the matrix metalloproteinases in paper II which was why they were analysed in serum. To obtain serum, the blood is allowed to coagulate in the tube, and is then centrifuged, which results in a top layer consisting of serum. Allowing the blood to coagulate affects several factors in the blood, for example, cytokines. One study has analysed the cytokine profiles in terms of using different blood sampling tubes: serum, sodium heparin plasma, EDTA plasma and citrate plasma, from healthy volunteers (de Jager et al., 2009). The conclusion was that the use of various blood collection tubes significantly affects cytokine measurement, with sodium heparin tubes showing the most consistent cytokine recovery.

Working with human material, the collection and handling of tissues, as well as the separation of cells, are central to achieving high-quality research. In paper IV, appendix mononuclear cells were isolated. This harsh treatment of the cells, involving them being minced through a strainer with the help of a piston, and filtered through a 100 µm cell strainer followed by density centrifugation, may have triggered the cytokine production. Approximately half of the appendix was used in every assay, but the size of the pieces varied every time, however, as we minced the whole piece and put the same number of cell in every well the results should have been comparable between the patients.
Statistical considerations

In paper I, a larger sample size would have been preferable, and this study was designed as a pilot study. There is always the possibility of obtaining significant differences by chance, so-called mass significance, as 1 out of 20 statistical calculations will be due to chance when $p=0.05$ is considered significant. Kruskal-Wallis test was used as a pre-test followed by Mann-Whitney U-test as a post hoc test. Only when the Kruskal-Wallis test indicated significant differences between the groups were the groups further compared using the Mann-Whitney U-test.

Paper I and IV included quite a few patients and it would have been more appropriate to include more patients. The small sample was a consequence of difficulties in obtaining fresh appendicitis material, as most of the operations were carried out at night. We included significant results but also trends as we wanted to show the whole picture. Here, there is also the possibility of mass significance occurring. Therefore, it was decided only to express the opinion that the immune response in blood is mirrored in appendiceal tissue; the finding that many of the cytokines were increased made it impossible to draw any other conclusions.

In paper III, Kruskal-Wallis test and Mann-Whitney U-test were used in the same way as in paper I. Fisher’s exact test was used on dichotomized data when 30% or more of the patients were below the detection level. Power may have been lost due to the dichotomization of the data, this may be a better alternative than the Mann-Whitney U-test as the large proportion of patients with similar ranks due to the results below the detection level would have resulted in less reliable results.

Bonferroni correction was not used to adjust for the large number of tests as this post hoc test is too conservative which increases the risk of missing true differences (Bland and Altman, 1995). In the present study this would have meant that the significance level would had to be increased to $p=0.005$. Another possibility would have been to lower the level of statistical significance, to $p=0.01$, which would not have affected the material to any great extent.
Two different entities

It is generally thought that gangrenous appendicitis is a later stage of the inflammatory process in appendicitis, and the difference in the immune response is the result of the progression of the inflammation over time. This study sheds more light on the distinction between the two entities of appendicitis. Contrary to the hypothesis of advanced appendicitis being a result of the progression of inflammation that starts with phlegmonous appendicitis, no differences in inflammation associated with an increasing duration of symptoms. The most important differences found at the early and intermediate stages, in paper III. Furthermore, differences in symptom duration could not explain the increased levels of inflammatory markers in advanced appendicitis. The observations instead suggest dysregulated inflammation in advanced appendicitis that is present from the start or at least appears very early in the process. This is in line with previous reports on polymorphisms in the genes coding IL-6 and IL-8, associated with the severity of advanced appendicitis (Murphy et al., 2008; Rivera-Chavez et al., 2004). A genetic difference is supported by our findings of different responses in individuals with a history of different grades of appendicitis, with a phenotypically stronger Th1 response in patients with a history of gangrenous compared with phlegmonous appendicitis. Speculatively, the increased inflammatory response observed early in advanced appendicitis suggests a more violent inflammation, which supports the hypothesis of different immune pathogeneses, where excessive induction of Th1/Th17 immunity and/or deficiencies in down-regulatory feedback mechanisms may explain the excessive inflammation of the entity where the inflammation eventuates in gangrene and perforation. Further studies investigating the mechanisms between these connections are needed.
Conclusions

- The development of gangrene and perforation in appendicitis is related to a complex network of cytokines and chemokines (Figures 15 and 16).

- The local immune response in the appendiceal tissue in appendicitis is mirrored in the blood, which justifies the use of peripheral blood in studies on the immune response in appendicitis.

- Individuals with a history of advanced appendicitis have a stronger Th1 response orchestrated by increased secretion of IFN-γ than individuals with a history of phlegmonous appendicitis. This may reflect constitutional differences between patients with different outcomes of appendicitis.

- Advanced appendicitis is characterized by increased neutrophil activity with increases in MPO, MMP-8 and MMP-9.

- The increased levels of CCL20 and CCL2 in cases of advanced appendicitis, suggest a more pronounced Th17 induction with increased trafficking of Th17 cells.

- Patients with advanced appendicitis show increased levels of IL-6, CCL20, CCL2, TGF-β, IL-17, IFN-γ and IL-12p70 compared with those with phlegmonous appendicitis. The increases were not explained by the duration of symptoms and several of the differences were observed early in the disease course.

- In the context of Th1/Th17-type inflammation, an increase of IL10, IL-1ra and IL4 in advanced appendicitis may mirror an anti-inflammatory feedback mechanism induced by the similarly increased Th1 and Th17 immunity.

- Taken together, these observations suggest dysregulated inflammation in advanced appendicitis that is present from the start or very early in the progression of the disease. The increased inflammatory response suggests a more violent inflammation in advanced appendicitis and supports the hypothesis of different immune pathogeneses, where excessive induction of Th1/Th17 immunity and/or deficiencies in down-regulatory feed-back mechanisms may explain the aggressive inflammation that eventuates in gangrene and perforation.
Figure 15. Concluding remarks of the possible mechanisms in advanced appendicitis.
Figure 16. Concluding remarks of the possible mechanism in advanced and phlegmonous appendicitis.
Future perspectives

There are not many genetic studies on appendicitis, only two different studies have covered this area. The interplay between different aspects of the immune cells is a complex network of signalling molecules and cells. Therefore, it would be of great interest to investigate expression of the innate, Th1, Th2 and Th17 inflammatory genes in a microarray in advanced and phlegmonous appendicitis.

What cells that secrete the cytokines analysed in this thesis is not known beyond that they were secreted from peripheral or appendix mononuclear cells. We speculate that we analyse Th1, Th2 and Th17 cells, but we are not completely sure. It would therefore be interesting to analyse the expression of chemokine receptors, transcription factors and cell surface markers connected to Th1, Th2, Th17 and innate cells in the cytokine secreting cells. This could be done by flow cytometry. Chemokine receptor of interest to analyse for Th1 would be, CCR4- CXCR3+CCR6- (expressed on Th1 cells secreting IFN-γ and IL-17) and CCR4+ CXCR3+CCR6+ (Th1 cells secreting IFN-β), and for Th2 CCR6+CCR4+CXCR3- and Th17 CCR6-CCR4+CXCR3+. Transcription factors of interest would be TBX21, GATA3, RORC and Foxp3. It would also be interesting to expand the analysis of secreted e.g. for Th1 (IFN-γ, IL-12p70, IL-27), Th2 (IL-4), Th17 (IL-17, CCL20, IL-21, IL-23, TGF-β) and Treg (IL-35, IL-10, TGF-β).

The newly discovered innate lymphoid cells (ILC), which comprise a novel family of hematopoietic effector cells, which have protective roles in innate immune responses to infectious microorganisms and more, may also be of interest to study in appendicitis. The ILC family has been shown to secrete similar cytokines as in a Th1, Th2 and Th17 response, but show different CDs, chemokine receptors and transcription factors and most of the research has so far been done in mice. Are the ILC as numerous as the Th1, Th2 and Th17 cells, which are the important cells in appendicitis?

In the future a better understand of the interplay between immune cells in advanced and phlegmonous appendicitis could lead to the identification of inflammatory markers in the clinical diagnosis of appendicitis, to distinguish patients which are in need of a surgery from those with resolving appendicitis and non-specific abdominal pain. Using blood sample in addition to the physical examination could reduce the costs for the healthcare system and decrease the discomfort for the patients.
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