IL-12 and T-lymphocyte dependant mucosal immune responses
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Chapter I

T-lymphocyte dependent responses:
Introduction

General

The immune system reacts to pathogens or other foreign antigens with two main types of response. A first line of defence, named the innate immune response is orchestrated by phagocytic cells that in a non-specific manner bind, internalise and eliminate microorganisms. The second type, called the adaptive immune response, is represented by the antigen-specific response mediated by B and T cells. The adaptive immune response has two amazing capabilities: It is able to recognise myriads of different antigens (diversity), but does not react with “self” antigens (tolerance). The mechanism underlying the first capability has been largely solved, and in large part is a result of both somatic mutation and clonal selection (lymphocytes are genetically programmed to recognise only one particular antigen). Lymphocytes that have encountered that specific antigen divide, express new receptors, secrete cytokines and differentiate into memory cells. Hence, specificity and memory are the two main features of the adaptive immune response (1). The other feature, immune tolerance, is less well understood. Clearly, immune tolerance is important, because several diseases are known to result from an immune response to self-components (autoimmunity), by mounting a response to non-pathogenic antigens or by causing an exaggerated immune response (hypersensitivity). Several autoimmune diseases such as multiple sclerosis (2, 3), rheumatoid arthritis (4), thyroiditis (5) and uveitis (6) are caused by T cells that are reactive against known self-components of the body. Although the specific aetiology of Crohn’s disease remains unknown, an abnormal T cell activation plays a central role in the pathogenesis of this inflammatory bowel disease.

1. Pathophysiology of Crohn’s disease

Crohn’s disease is a chronic inflammatory disease of the gastrointestinal tract often accompanied by intra- and extra-intestinal manifestations including fistulae, perforations, uveitis, arthritis, ankylosing spondylitis and erythema nodosum (7). Aphthous ulcers are the characteristic early lesions of Crohn’s disease often present in the absence of clinical symptoms (8). The intestinal chronic lesion is histologically characterized by transmural infiltration of high numbers of macrophages and T- and B-lymphocytes (9, 10). Inflammation may often result in extensive fibrosis and strictures that require surgery (11).
The aetiology of Crohn's disease is unknown. Specific pathogens such as *Mycobacterium paratuberculosis* (12, 13), *Listeria monocytogenes* (14) or measles virus (15) have been implemented in Crohn's disease, but these observations have not been confirmed (16). Nevertheless, luminal antigens are essential to trigger immune-mediated mucosal inflammation in susceptible individuals and these antigens most likely originate from the normal intestinal flora (17, 18). Lamina propria T lymphocytes from Crohn's disease patients show high proliferative responses to microbial antigens (19), display early activation antigens (20-22) and have a phenotype of memory cells (23, 24). Moreover, T cells from Crohn's disease patients secrete large amounts of IL-2 (25), the key cytokine in inducing lymphocyte activation and proliferation, as well as of pro-inflammatory cytokines such as TNF-α (26-28) and IFN-γ (29, 30).

2. *Initiation of a Th-1 cell mediated response*

In 1986 two distinct subsets of mouse CD4+ T cell clones capable of different pattern of cytokine production were identified (31). Th1 CD4+ T cell clones produce IL-2, IFN-γ and TNF-α and participate in cell-mediated responses such as delayed-type hypersensitivity (DTH) and macrophage activation, whereas Th2 clones produce IL-4 and IL-5 and induce B cell to secrete IgG and IgE antibodies implicated in humoral response and allergy (32-34). Although many factors may influence the differentiation toward either Th1 or Th2 subsets, the cytokine environment is one of the main driving forces (35). IL-12 induces the differentiation towards the Th1-subset (36, 37) and IL-4 and IL-6 drive the differentiation towards the Th2-subset (38). Th1 and Th2 cytokines can mutually reverse the ongoing differentiation of T lymphocytes. For example, Th2 cells express the IFN-γ receptor and IFN-γ is known to reverse initiation of a Th-2 response during ongoing Th1 cell-mediated response (39).

IL-12 is produced by activated macrophages, dendritic cells and to a lesser extent by granulocytes. The active form of IL-12 is a disulfuric heterodimer protein that consists of p35-kDa and p40-kDa subunits. p40 homodimers (p40)2 antagonise IL-12 heterodimer by binding and competing with the IL-12 receptor, which consists of two subunits (β1 and β2 chains), respectively important for binding and signalling of IL-12 (40). However, agonistic effects of p40 homodimers have been shown on CD8+ T cells, that were induced to produce IFN-γ (41, 42) and on macrophages that respond by recruitment to the site of inflammation (43, 44).
IL-12 is a central cytokine for the initiation of T helper 1 response during infection (primary response) with intracellular protozoal, bacterial or fungal pathogens (45-47). IL-12 is also involved in the initiation of autoimmune Th1-type mediated models of experimental autoimmune encephalomyelitis (EAE) (48), diabetes in the nonobese mice (NOD) (49) and experimental colitis (50, 51). IL-12 may induce the upregulation of the receptor for IL-18 - the cytokine firstly identified as IFN-γ inducing factor - on resting T cell and Th1 cells but not on Th2 cells (52). Thus IL-12 and IL-18 are synergistic in polarising towards the Th1-response. IL-18 is produced by activated macrophages (53) and requires processing of pro-IL-18 by the IL-1 converting enzyme (ICE) for activity (54-56). Apart from its primary role in inducing IFN-γ production from natural killer and T cells, IL-18 may directly induce IL-1β and TNF-α production by peripheral mononuclear cells (57, 58). IL-18 is a pro-inflammatory cytokine because it also enhances the production of both IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and potentiates T cell proliferation (59). Moreover, IL-18 induces the Fas-ligand-mediated cytotoxic activity by natural killer (60) and T cells (61), a mechanism involved in killing of intracellular microbial organisms during innate immune responses (62). Hence, IL-18 is involved in innate responses against infections but also importantly contributes to T cell mediated responses. The production of both IL-12 (63, 64) and IL-18 (65, 66) is increased in the intestinal mucosa of patients with active Crohn's disease.

3. Mucosal T lymphocyte trafficking

Naïve T cells circulate continuously through the peripheral lymph nodes and this increases the likelihood that antigen-specific T lymphocytes encounter the appropriate antigen (67). When luminal antigens enter either the Peyer’s patches in the ileum or the organised mucosal lymphoid tissue in the colon under the epithelium they are taken up and presented by antigen presenting cells (APC: M cells, mucosal dendritic cells and macrophages) to the naïve T cells that express high levels of the adhesion molecule L-selectin (68). Naïve T cells differentiate into memory T cells that express high levels of the CD44 adhesion molecule and migrate to the regional lymph nodes (69, 70). The gut associated lymphoid tissue (GALT) is somewhat different from the systemic immune system because lymphocytes primed in the intestinal mucosal pass via regional lymph nodes to the blood stream and then return to the intestinal lamina propria (71).
Homing molecules are important for this selective recruitment of primed GALT-derived T cells to the lamina propria. Memory T cells enter the lamina propria of the intestine by expressing the gut homing $\alpha_4\beta_7$ integrin and do not require L-selectin (72).

**Figure 1.** Schematic distribution of T lymphocytes in the intestinal mucosa. Naive CD62L (L-selectin) positive T lymphocytes circulate through the Peyer’s patches in the ileum or the organised lymphoid tissue in the colon. Memory CD44<sup>high</sup> T lymphocytes cells migrate to the regional lymph node and then enter the thoracic duct and the blood stream. Gut-homing $\alpha_4\beta_7$ T lymphocytes return to the intestinal lamina propria from the blood stream. Abbreviations: follicular associated epithelium (FAE), follicular dendritic cell (FDC), high endothelial venule (HEV), macrophage (MF). The natural ligand of $\alpha_4\beta_7$ is the mucosal addressin cell adhesion molecule 1 (MAdCAM 1) expressed on the post-capillary venules of the lamina propria (see Fig. 1) (73, 74).

4. **Maintenance of chronic Th1-cell response: role for IL-12, TNF-$\alpha$ and IFN-$\gamma$**

IL-12 plays a role in maintaining the Th1-mediated response in models of autoimmune diseases (75) and experimental colitis (50, 51). From these experimental data we know that IL-12 promotes Th1 cell proliferation, directly or by upregulating the IL-2 receptor (76), and
by rescuing T cells from activation-induced cell death (77). Furthermore, IL-12 increases the production of TNF-α, a potent pro-inflammatory cytokine (78, 79).

TNF-α is a type II membrane-anchored protein, which is released in a secreted form by the metalloproteinase TNF-α converting enzyme (TACE) (80). Secreted TNF-α is active as a trimer that binds one of two plasma membrane receptors, the 55- or 75-kDa TNFR. p75 TNFR signalling has been implicated in lymphocyte proliferation while the p55-kDa receptor mediates TNF-induced cytotoxicity, apoptosis and NF-kB activation (81-83). TNF-α can importantly contribute to local inflammation by activating the vascular endothelium and the blood coagulation system (84), by inducing the production of free radicals and tissue degrading enzymes such as metalloproteinases (85, 86).

IFN-γ is a prototype Th1-type cytokine that was initially discovered as a potent antiviral factor (87). Twenty years later the same factor produced by antigen-stimulated T cells was called macrophage activating factor (88). NK cells and T cells are the main source of IFN-γ after stimulation with IL-12 and IL-18 (89, 56). There are also some recent data on IFN-γ production by activated macrophages via IFN-γ itself (90) and after IL-12 and IL-18 stimulation (91, 92). Once secreted IFN-γ binds to two α-chains of the IFN-γ receptor expressed on the cell membrane (93). The ultimate formation of the receptor complex (which is composed of two β-chains plus the IFN-γ-α-chains) leads to the transphosphorylation and reciprocal activation of Janus kinases (JAK) (94). This is followed by phosphorylation of the transcription factor STAT-1α (signal transducer and activators of transcription) that from the cytosol is translocated to the nucleus to induce transcription of specific target genes (95, 96). After signaling, the IFN-γ (ligand)-receptor α-chain complex is internalised and dissociated, and the uncoupled α-chain is recycled back to the cell membrane (97).

IFN-γ is a prominent activator of macrophages by inducing the upregulation of the class II molecule of the major histocompatibility complex (MHC) that is necessary for antigen presentation to CD4⁺ T cells (98). IFN-γ has a primary function in host defence against microorganisms (99). Children with either IFN-γ receptor (ligand) α-chain (IFN-γR1) or and IFN-γ receptor (signaling) β-chain (IFN-γR2) deficiency develop severe disseminated Mycobacterium tuberculosis infection (100, 101). Patients lacking the IL-12 receptor β1 (102,
have impaired IFN-γ secretion and are affected by disseminated *Salmonella enteriditis* infection (104).

A role for IFN-γ in autoimmunity has been long hypothesised and indeed administration of exogenous IFN-γ exacerbated multiple sclerosis (105). In the 1990s newly identified techniques offered the possibility for studying the role of endogenous cytokines by deleting specific target genes in mice. Surprisingly, endogenous IFN-γ was not essential for the induction of a T cell-mediated autoimmune disease. Indeed, IFN-γ and IFN-γ receptor α-chain-deficient mice developed more severe collagen-induced arthritis (CIA) (106), uveitis (107), thyroiditis (108) and allergic encephalomyelitis (EAE) (109-111). In view of these overall findings, immunologists started to re-examine the anti-inflammatory proprieties of IFN-γ in controlling disease activity.

5. **Regulatory mechanisms of the T-cell immune response**

A mechanism important in controlling T-cell responses is programmed cell death or apoptosis. Apoptosis is instrumental in the maintenance of homeostasis in antigen-rich environments such as the intestinal mucosa (112). Without apoptosis within one week a single T cell could multiply to almost $1 \times 10^{12}$ cells, doubling the total number of T cells in the body (113). Prolonged survival of T lymphocytes may lead to uncontrolled inflammation. A defect in T cell apoptosis was reported in chronic atopic dermatitis (114), rheumatoid arthritis (115, 116) and asthma (117). Lamina propria T lymphocytes from patients with Crohn’s disease were found to have defective apoptosis induced by IL-2 deprivation, FAS receptor binding and exposure to nitric oxide (NO) (118).

Other regulatory mechanisms of the T-cell response comprise the mutual control of Th1 and Th2 cytokines and the presence of a subset of regulatory T cells (38). Th2 cytokines including IL-4 (119), IL-10 (120) and TGF-β (121) inhibit the production of IFN-γ by monocytes. On the other hand, IFN-γ inhibits the proliferation of Th2 cells (122). Inhibition of the secretion of IL-12 from macrophages can indirectly down regulate the Th1-response. IL-10 and IL-4 mediate this effect and interestingly IL-12 induces IL-10 production by macrophages as negative feedback of its own activity (123). IL-10 is produced by T cell and monocytes and is a key regulatory cytokine in the immune system (124). Exposure of T cells to high-dose IL-10 during antigen-priming results in generation of a specific subset of T regulatory lymphocytes (Tr-1). Tr-1 cells secrete IL-5, IL-10 and TGF-β but no IL-4 and
downregulate antigen-dependent T-cell activation in experimental colitis. At present, Tr1 cells are considered a main class of regulatory T-cells in the intestinal immune system (125, 126).

6. Anti-inflammatory properties of IFN-γ

Despite its role in promoting cellular immunity, IFN-γ may counter-regulate inflammation. For example, it can inhibit the production of metalloproteases that are responsible of tissue damage during inflammatory processes (127) and activates a subset of suppressor T cells that may eliminate autoreactive T cells (128). Recent studies showed that IFN-γ can de-activate macrophages (129) and can inhibit the proliferation of T cells (130).

There is evidence that IFN-γ induces apoptosis in keratinocytes (131, 132). Activated murine macrophages (133) and monocytes infected by the intracellular pathogen Coxiella burnetii were also found in vivo to undergo IFN-γ-dependent apoptosis (134). Interestingly, killing of Coxiella burnetii and death of infected monocytes, although both mediated by IFN-γ, were dependent on TNF, indicating a synergism between TNF-α and IFN-γ already known in promoting inflammation (135, 136). This observation was subsequently confirmed at the cell-signaling level as it was found that STAT-1α-deficient cells failed to undergo TNF-α induced apoptosis (137).

7. NO in T-cell response

From the first report on nitric oxide (NO), which was based on the finding of a large amount of nitrate in the urine of a febrile patient in 1818, many studies on NO have been published. Among many other biological activities, NO is considered to be a regulator of T-cell responses (138). NO derives from L-arginine catabolism via the constitutive (endothelial and neuronal) and the inducible forms of the NO synthase (iNOS). The latter can be induced in macrophages via IFN-γ, IFN-α/β and TNF-α (139, 140). NO regulates many physiological activities such as blood vessel relaxation (141), inhibition of platelet aggregation and adhesion (142, 143), neurotransmission (144) and host defence (145). High tissue concentrations of iNOS have been implicated in the pathogenesis of inflammatory arthritis (146, 147), type I diabetes (148) and inflammatory bowel disease (149). But more recently NO was shown to control T cell activation by its ability to reduce IL-12 production from activated macrophages (as auto negative feed back) and to inhibit T cell proliferation (150, 151). Moreover, NO can be pro-apoptotic depending on its concentration and the target cell (152). High levels of NO
may induce T cell apoptosis, but low concentrations of NO rescue NK cells from cell death (153). Circulating monocytes are sensitive to NO-induced apoptosis (154, 155) whereas activation-induced apoptosis of tissue macrophages seems to be NO independent (133, 156). Hence, NO is produced in inflammatory responses but its activity is tightly regulated, in part by its own biological activity.

7. Experimental models of T cell mediated colitis

Experimental models of colitis are very important for understanding the pathophysiology of Crohn's disease and have helped to design novel intervention strategies. Three main models of experimental colitis in mice are characterized by a dysregulated T helper 1 response: 1) Mice with selective deletion of the IL-10 gene (IL-10-/− mice); 2) mice with altered T cells populations such as the transfer model of CD45RB<sup>hi</sup>CD4<sup>+</sup> T cells into immunodeficient SCID mice and 3) administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) that results in a Th1-mediated colitis.

Deletion of the IL-10 gene, a potent downregulator of T helper 1 cytokines, in mice resulted in a chronic inflammatory bowel disease that affected the entire intestine. The disease likely results from an enhanced T helper 1 response to antigens of the bacterial flora secondary to the lack of IL-10. In the absence of IL-10, a chronic inflammation develops via continuous overproduction of cytokines such as TNF-α, IL-1 and IFN-γ (157, 158). Recent studies in IL-10-/− mice have established different roles of IFN-γ during the onset of disease and during progression and chronicity of the intestinal inflammation. Treatment of neonatal IL-10-deficient mice with anti-IFN-γ delayed the onset of disease and reduced the severity of the inflammation (51). No effects of anti-IFN-γ were seen when disease had already established in adult mice whereas anti-IL-12 could abrogate colitis in both young and aged mice. Therefore, IL-12 seems to be required for sustaining chronic colitis.

The second model results from adoptive transfer of a subset of CD4<sup>+</sup> T cells, expressing high levels of the surface molecule CD4<sup>+</sup>CD45RB<sup>hi</sup> into immunodeficient SCID mice which results in a moderate to severe colitis (159, 160). CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells are considered to be a Th1 precursor population and activate resident macrophages to produce high levels of TNF-α (161). The occurrence of colitis can be prevented by the co-transfer of the regulatory CD4<sup>+</sup>CD45RB<sup>lo</sup> subpopulation, which contains T cells capable of secreting anti-inflammatory cytokines.
Finally, in mice and rats intrarectal administration of the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) results in a chronic inflammation of the colon (162, 163). During the initiation of colitis, activated macrophages present hapten-modified self-antigens to naïve CD4⁺ T lymphocytes that produce Th1 cytokines such as IL-2, IFN-γ and TNF-α. Repeated administration of TNBS causes a chronic Th1-mediated colitis that mimics chronic relapsing Crohn's disease. Successful treatment of already established colitis could be obtained by systemic administration of antibodies to IL-12 and by intraperitoneal injection of antibodies to TNF-α (50, 164).

Aims of this thesis
The main aim of this thesis was to investigate the intestinal T-lymphocyte-mediated immune response, which plays a central role in the pathogenesis of Crohn's disease. In chapter 2, the expression of IFN-γ and IL-4 cytokines, respectively considered the prototypic Th1 and Th2 cytokines, was analysed in intestinal specimens from patients with Crohn's disease, ulcerative colitis and from controls.

In chapter 3, the colitis model induced by administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) was used to study the mucosal T-cell response. To obtain more insight in T-cell trafficking during colitis, the expression of the adhesion molecule CD44 and the gut homing α4β7 on T cells was studied in the early and chronic stages of the disease.

IL-12 is considered a major cytokine in driving the Th1-cell response. In chapter 4, development of TNBS-colitis was examined in mice deficient for either the p35 subunit or the p40 subunit of IL-12 and in mice lacking the IL-12 β1(binding)-chain receptor. The T-cell response was investigated in the total absence of IL-12 and in the presence of endogenous p40 (IL-12p35-deficient mice still produce p40). Moreover, IFN-γ and IL-18 production in the absence of either IL-12 or endogenous IL-12p40 was studied.

IFN-γ is a pro-inflammatory cytokine involved in Crohn's disease. In chapter 5, mice lacking the α-chain of the IFN-γ receptor (IFN-γR1-deficient mice) and therefore unable to respond to IFN-γ were studied after induction of TNBS-colitis. The pattern of cytokine production was analysed. In chapter 6, the anti-inflammatory properties of IFN-γ were re-examined in mice lacking either IFN-γ or IFN-γ receptor, with particular focus on the expression of iNOS and
the induction of apoptosis in the absence of IFN-\(\gamma\). In chapter 7, the summary and the concluding remarks of this study are presented.

References


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