CD40 in MS
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Chapter 1

General introduction
The immune system

The immune response is a complex process that is designed to protect the body against pathogenic microorganisms. The immune system can respond in different ways to different threats, classified as acute or chronic inflammation [1, 2]. Infection or trauma can cause acute inflammation, which can be described by the cardinal signs heat, pain, redness, swelling and loss of function. The function of acute inflammation is to restore homeostasis by fighting the inflammatory stimulus [2]. In contrast to the protective role of acute inflammation, chronic inflammation is characterized by a prolonged, low-grade activation of the immune system that can promote the development or progression of diseases including the neurological, metabolic and vascular syndromes described in this thesis [1, 3-5].

The innate immune response is the first line of defense and the adaptive immune response remembers how to react to a pathogen that it has encountered before [6, 7]. The innate immune response is activated by microbial substances called pathogen-associated molecular patterns (PAMPs) or by endogenous signals called damage-associated-molecular patterns (DAMPs). PAMPs and DAMPs bind to the pattern recognition receptors (PRRs) on innate immune cells. Main cells of the innate response are macrophages, neutrophils and dendritic cells (DCs). Macrophages and neutrophils phagocytose and kill the microbes via the production of anti-microbial reactive oxygen-containing molecules within the phagocytes or the production and secretion of nitric oxide. Once they get activated, either by PAMPs or DAMPs, the phagocytes secrete cytokines and chemokines that recruit and activate other cells. This process is called inflammation [7]. Signals from the innate immune response are necessary to initiate the adaptive immune response, the T and B lymphocytes. In the draining lymphoid tissues activated antigen presenting cells (APCs) including macrophages, DCs and B-cells present antigens to lymphocytes. The proteins on the APCs that display the antigen are called the major histocompatibility complex (MHC) and antigens are recognized by the T-cell receptor (TCR) specific for the presented antigen. There are two types of MHC molecules: class I (MHCI) and class II (MHCII). MHCI presented antigens are recognized by CD8+ cytotoxic T-cells and MHCII associated antigens are recognized by CD4+ T-helper cells. After binding of the MHC to the TCR, T-lymphocytes become activated and start proliferating, resulting in clonal expansion [6]. In order to properly activate T-cells three signals are necessary. First, antigen is presented by an APC and recognized by the TCR of resting T-cells. Second, co-stimulatory molecules on the membranes of APC and T-cells interact and provide an additional (antigen non-specific) stimulus, called co-stimulation. The third signal necessary for T-cell activation is stimulation by cytokines (Figure 1) [8, 9].
The CD40-CD40L dyad

This thesis is focused on the cell-type specific role of the co-stimulatory molecule dyad CD40-CD40L in chronic inflammatory diseases. The CD40-CD40L dyad is a key regulator of T-cell effector functions, DC maturation, B-cell proliferation and Ig isotype switching, and influences the expression of other costimulatory molecules on APCs [10-12]. CD40 is a protein that belongs to the tumor necrosis factor receptor (TNFR) family and can be found on APCs including B-cells, DCs and macrophages. Furthermore, CD40 is also present on non-immune cells such as endothelial cells and adipocytes [13]. The ligand for CD40, CD40L (or CD154, or gp139) is present on T-cells and platelets and is also present as a soluble form (sCD40L) in plasma after cleavage [12, 14]. Defective CD40-CD40L signaling can cause serious immune deficiency, and an example of this is the hyper IgM syndrome, a disease caused by mutations in the extracellular domain of the CD40L gene [15].

![Diagram of T cell activation by APCs](image)

**Figure 1: The three signals in T cell activation by APCs.** Activation of T-cells include the presentation of peptide fragments by MHC class II proteins on APCs to the TCR (1), signal via co-stimulatory molecules (2), and mediation by instructive cytokines (3).

Besides their role in T-cell and APC biology, CD40 and CD40L regulate the activation of other innate immune cells and non-immune cells [13]. For example, activation of CD40 on monocytes upregulates expression of adhesion molecules, chemokine receptors and chemokines, and secretion of pro-inflammatory cytokines [16, 17], whereas CD40 activation of endothelial cells induces upregulation of adhesion molecules [17].

Signal transduction after binding of CD40L to its receptor CD40 involves adaptor proteins, as the cytoplasmic domain of CD40 lacks intrinsic signaling activity [11, 18, 19]. TNFR-associated factors (TRAFs) fulfill this role, and the cytoplasmic tail of CD40 has TRAF6 and TRAF2/3/5 binding sites [17]. Binding of the TRAF proteins to
the CD40 cytoplasmic tail can activate multiple signaling cascades including NFκB, p38 mitogen-activated protein (MAP) kinase, and C-Jun N-terminal kinase (JNK) [20]. Which of these signal transduction cascades is activated is dependent on the cell-types involved and environmental conditions to which the cells are exposed. In mice with atherosclerosis and diet-induced obesity it is shown that deficiency of CD40-TRAF6, but not CD40-TRAF2,3,5 signaling ameliorates disease [21, 22]. Therefore, small molecule inhibitors (SMI) that prevent the interaction between CD40 and TRAF6 were developed [23]. Blockade of the CD40-TRAF6 signaling pathway using these SMIs ameliorates atherosclerosis and diet-induced-obesity [21, 24, 25].

Thus, activation of the CD40-CD40L dyad drives immune and non-immune cell activation resulting in pro-inflammatory responses. Research in the past two decades has shown that this dyad plays a role in the development of several chronic diseases, including multiple sclerosis, obesity, arthritis and atherosclerosis [26, 27]. So far, the mechanisms and cell types involved in these functions of CD40 and CD40L in disease are not fully unraveled.

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system (CNS) [28]. The prevalence of MS in the western world is around 1 in 1000 people and it affects women more often than men. MS is characterized by the formation of sclerotic lesions in the white matter as a result of recurrent immune reactions in the brain and spinal cord, and causes clinical symptoms such as visual problems, weakness and pain [29]. The cause of MS is still unknown, but environmental and genetic factors can probably evoke the disease [30, 31]. Before MS symptoms emerge, something (from inside or outside the CNS) triggers microglia activation. Upon activation, migration of monocytes and T-cells from the blood into the CNS is stimulated and these infiltrating cells promote destruction of the myelin cover of nerve fibers, lesion formation and axonal damage [31]. As the immune system attacks the body’s own myelin, MS is considered an autoimmune disease.

The co-stimulatory CD40-CD40L dyad plays an important role in multiple sclerosis. The number of CD40L+ T-cells is increased in the CNS from patients with MS and macrophages and microglia present in MS lesions express CD40 [32]. In order to investigate the role of CD40 in MS, the experimental autoimmune encephalomyelitis (EAE) animal model is often used. We know that genetic deficiency of CD40 and its ligand, and treatment with anti-CD40L antibodies protect mice and marmoset monkeys against EAE development [32-37], and reviewed by [38]. In EAE, blockade of CD40 signaling reduces CNS immune cell infiltration and can inhibit clinical relapses [32, 35, 39]. CD40 is expressed on macrophages and microglia in the CNS
of patients suffering from MS [32, 40]. The importance of CD40 on monocyte-derived macrophages is not yet investigated. As CD40 needs the TRAF adaptor molecules for signaling it is interesting to explore which of the signaling pathways is most important in MS and whether blocking this pathway could be of therapeutic interest.

**Obesity and the metabolic syndrome**

Obesity, defined as a body mass index >30 kg/m$^2$, is a medical condition in which excess body fat has accumulated to the extent that it exerts a negative effect on health. Obesity is usually caused by a chronic imbalance between energy intake and physical activity. The number of people with obesity is growing worldwide and includes both adults and children. Obesity causes a substantial burden in human health problems and their associated financial costs [41]. Obesity is a risk factor for the metabolic syndrome, which is characterized by insulin resistance, glucose intolerance, development of hepatosteatosis, and dyslipidemia [42]. Metabolic dysregulation is linked to a variety of cardiovascular diseases such as atherosclerosis, myocardial infarction, and stroke, as well as diabetes mellitus type II [43].

Both innate and adaptive immune cells are involved in the development of the systemic low-grade inflammation linked to obesity [21]. Elevated levels of cytokines and adipokines derived from the adipocytes and leukocytes in the adipose tissue, such as TNF, IL-6, MCP-1 and leptin are significantly correlated with dysfunctional insulin signaling and insulin resistance [44, 45]. Interactions between immune cells and adipocytes increase the expression of pro-inflammatory chemokines and cytokines in the adipose tissue, further enhancing the accumulation of leukocytes. This pro-inflammatory milieu also results in systemic inflammation, including immune cell infiltration in plasma and organs outside the adipose tissue, for example in liver, brain and pancreas [42].

CD40-CD40L signaling plays a central role in obesity-associated inflammation [46]. The leading hypothesis is that suppression of the CD40-CD40L signaling cascade can reduce inflammation and metabolic effects associated with obesity. Genetic ablation of CD40L resulted in amelioration of adipose tissue inflammation [47, 48]. Surprisingly, CD40$^{-/-}$ mice displayed worsened insulin resistance and excessive inflammation of adipose tissue compared with wild-type mice [21, 49-51]. In these four papers reporting the same phenotype, different theories are described to explain the variances in phenotype between the CD40L deficient and the CD40 deficient mice. One theory describes the opposing roles of the CD40-TRAF signaling pathways in obesity as loss of CD40-TRAF2,3,5 signaling aggravates diet-induced-obesity (DIO) while loss of CD40-TRAF6 signaling is protective in DIO [21]. The other theories suggest a tissue specific effect of CD40 signaling. The adipose tissue of
CD40−/− mice has increased mRNA expression of the endothelial cells markers CD31 and von Willebrand factor [49], and deficiency of CD40 on T-cells increases adipose tissue inflammation [50] and aggravates insulin resistance [51]. Although there are some theories, it is not completely clear what causes the phenotype of the CD40 deficient mice in obesity and it is therefore of interest to continue the search how CD40 deficiency aggravates diet induced obesity.

Atherosclerosis

Atherosclerosis is a lipid driven chronic inflammatory disease of the arterial wall [52, 53]. It is the major cause of cardiovascular diseases (CVD) such as myocardial infarction and ischemic stroke, which is responsible for 31% of all deaths worldwide [54]. Development of atherosclerosis is characterized by the formation of plaques in the arterial wall. Low density lipoprotein (LDL) can be modified within the arterial wall and this initiates an inflammatory response, causing continuous recruitment of inflammatory cells into the plaque and degradation of its extracellular matrix (ECM) [52]. Growth of the atherosclerotic plaque results in narrowing of the lumen and can cause occlusion and ischemia. When plaques have a large necrotic core and thin fibrous cap they are classified as vulnerable plaques. These plaques can rupture and initiate thrombosis, and depending on the arterial bed affected, can induce myocardial infarction or stroke [55].

The co-stimulatory CD40-CD40L dyad has a critical role in the development of atherosclerosis. Genetic deficiency or pharmacological inhibition of CD40L reduces the extent of atherosclerosis and induces a stable plaque phenotype that is high in fibrosis and low in inflammation [47, 49, 56-59]. Targeting of CD40L seems to be a promising strategy to reduce atherosclerosis and to stabilize atherosclerotic plaques. Unfortunately, clinical trials using an anti-CD40L antibody were halted as a result of thromboembolic complications, particularly because CD40L is present on platelets and can interact with the integrin αIibβ3 in platelets, thereby stabilizing thrombi [60, 61]. Antagonizing CD40, the receptor for CD40L, or its signaling intermediates would be an alternative approach for treating human atherosclerosis. Similar to CD40L deficient mice, CD40−/−Apoe−/− mice also have a reduction in atherosclerosis and develop a stable plaque phenotype [22]. Blockade of the complete CD40-CD40L dyad can result in immunosuppression and is therefore not feasible as a therapeutic target. In order to avoid these side effects, it could be interesting to target cell type specific CD40 functions.

Both CD40 and CD40L are expressed on almost all cell types present in human and experimental atherosclerotic lesions [62-64]. Monocytes play a key role in atherosclerosis progression as they are recruited to the inflamed arterial wall where they accumulate lipids and transform into macrophages or foam cells. Foam cells are
characteristic for atherosclerosis development, they ingest oxidized LDL and secrete various elements involved in plaque growth. Excessive lipid uptake by the foam cells results in cytotoxicity, cell death and necrotic core formation [53, 65]. CD40 expressed on macrophages can bind CD40L resulting in the induction of multiple pro-inflammatory, proteolytic, and pro-thrombotic chemokines and cytokines. Moreover, different matrix metalloproteinases (MMPs) are produced that degrade the ECM components of the fibrous cap [66]. All these factors induced by macrophage activation via CD40 can contribute to atherosclerosis progression.

**Aim and outline of the thesis**

This thesis focuses on the cell-type specific role of the CD40-CD40L dyad in inflammatory diseases including multiple sclerosis, the metabolic syndrome, and atherosclerosis.

Our hypothesis is:

Inhibition of CD40 in a cell type specific manner and/or inhibition of the CD40 downstream signaling adaptor proteins (TRAFs) can ameliorate chronic inflammatory diseases and avoid the side effects observed with complete blockade of the CD40-CD40L dyad.

Sub-hypotheses focused on in this thesis:

1. Inhibition of macrophage CD40 and/or the CD40 downstream signaling adaptor proteins (TRAFs), reduces the initiation and progression of multiple sclerosis.

2. Inhibition of CD40 on macrophages, dendritic cells and adipocytes differentially affects the metabolic and inflammatory complications of diet-induced obesity.

3. Inhibition of macrophage CD40 diminishes the initiation and progression of atherosclerosis.

In Chapter 2 we review the current knowledge on the role of the CD40-CD40L dyad in multiple sclerosis and EAE. Chapter 3 focuses on the role of macrophage CD40 and the CD40-TRAF2/3/5 and CD40-TRAF6 signaling pathways in MS. In chapter 4 the therapeutic potential of small molecule inhibitors of the CD40-TRAF6 interactions in neuroinflammation is investigated.

As described in the introduction, CD40L−/− mice and CD40−/− mice show opposing phenotypes during DIO, where CD40L deficiency protects against obesity and CD40 deficiency worsens obesity. CD40 is expressed by a variety of cell types including macrophages, DCs, endothelial cells and adipocytes and we therefore hypothesize that CD40 exerts cell-type specific functions in obesity and its metabolic dysregulation. In this thesis we investigate the cell-type specific role of CD40 in a
mouse model of diet-induced obesity. In **chapter 5** we describe the role of dendritic cell- CD40, in **chapter 6** the role of CD40 on macrophages and in **chapter 7** we discuss the role of adipocyte-CD40 in diet induced obesity.

As a role for macrophage CD40 in atherogenesis has been postulated we studied the role of macrophage CD40, using mice deficient in macrophage CD40 signaling in **Chapter 8**.

Finally, **Chapter 9** is a general discussion of the thesis, which summarizes the findings and discusses future perspectives.
References


