CD40 in MS
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Chapter 9

General discussion
Main findings of this thesis

The overall aim of this thesis was to elucidate the cell-type specific roles of the CD40-CD40L dyad in three chronic inflammatory diseases: multiple sclerosis, diet-induced obesity, and atherosclerosis.

The main findings are:

1. Deficiency of macrophage CD40 ameliorates clinical disease in experimental autoimmune encephalomyelitis.
2. CD40-TRAF6 interactions play a dominant role in the development of neuro-inflammation and multiple sclerosis.
3. Small molecule inhibitors of CD40-TRAF6 interactions can reduce neuro-inflammation, but do not reduce disease severity in experimental autoimmune encephalomyelitis.
4. Dendritic cell CD40 plays an important role in preventing hepatosteatosis during diet-induced obesity by generating a regulatory T cell response.
5. Macrophage CD40 only plays a minor role in metabolic dysregulation during obesity.
6. Adipocyte CD40 plays a role in adipose tissue inflammation and can drive T-cell responses during diet-induced obesity.
7. Macrophage CD40 deficiency ameliorates atherosclerosis.

Role of CD40-TRAF interactions in multiple sclerosis/EAE

Blocking both CD40 and CD40L is found to be protective in experimental autoimmune encephalomyelitis (EAE) [1-4], and reviewed by [5-7]. However, inhibition of this co-stimulatory dyad is not clinically feasible as it can cause thromboembolic complications and immunosuppression [8-11]. Targeting the CD40-TRAF signaling pathways is a potential strategy for downstream blockade of CD40, with reduced risk for side effects [10, 12, 13]. We found that CD40-TRAF6 interactions play a more important role than CD40-TRAF2,3,5 interactions in EAE [Chapter 3, this thesis], but inhibition of these CD40-TRAF6 interactions with SMI6877002 was not sufficient to reduce disease severity in EAE [14]. CD40-TRAF6 signaling is the dominant TRAF pathway in macrophage CD40 signaling [15, 16], and we confirmed that macrophage CD40 is important for the development of in EAE [Chapter 3, this thesis]. Our results show that further optimization of the CD40-TRAF6 inhibitor, including improved passage over the blood-brain barrier, is necessary to increase its potential to reduce clinical signs of EAE, and macrophage targeting could be beneficial. However, it is important to keep in mind that macrophages are not the only cell type important in EAE disease development and progression, as the phenotype of the generic CD40 KO in EAE was much stronger [1].

B-cells are important producers of autoantibodies in MS [17], and CD40 signaling has a critical role in B-cell antibody production and immunoglobulin isotype switching [18].
Inhibition of CD40 in EAE reduces anti-MOG antibody production [18], but it is unclear whether B-cell CD40 also plays a relevant role in the generation of autoantibodies in MS. Interestingly, in the absence of B cells, anti-MOG antibodies can cause EAE by triggering peripheral MOG-specific T-cells after recognition of MOG by myeloid APCs [19]. Furthermore, T cells and endothelial cells also express CD40 and are found to play a role in CNS inflammation and MS [20-23]. In this thesis we investigated the role of macrophage CD40, as in the CNS of MS patients and EAE animals macrophages and activated microglia are the main CD40 expressing cell types [2, 24]. We showed that inhibition of macrophage CD40 signaling by CD40-TRAF6 SMI treatment was a safe treatment in EAE, which could reduce CNS inflammation but was not sufficient to reduce disease severity. It could be interesting to combine a macrophage CD40 blocking therapy with inhibition of the function of other cell types important in MS, for example the B or T cells. But caution is required when combining therapies, as this could increase the risk for side effects which should be avoided by developing new therapies.

Current therapies focused on reducing inflammation in MS include glucocorticoids, interferons, glatiramer acetate, and monoclonal antibodies against α4-integrin, CD52, CD25, and CD20 [25]. Although these therapies are successful for certain patient groups, they have multiple side effects including an increased risk of infection, secondary autoimmunity disease, injection side reactions, hypertension, depression, eczema and many more [26]. In addition to inhibition of the CD40-CD40L dyad, blockade of co-stimulatory molecules from the B7-CD28 superfamily in EAE is also currently being investigated. Blocking the CD28/B7/(CD80) T-cell costimulatory pathway by using CTLA4Ig has been shown to be effective in reducing EAE [27] and was found to be rather safe in a phase I clinical trial [28]. Genetic deficiency or pharmacological inhibition of the negative regulatory molecule programmed death-1 (PD-1) enhanced EAE disease severity [29, 30] and ligation of the PD-1 receptor could be a new strategy to treat MS.

**CD40 as a suppressor of inflammation in diet-induced obesity**

While blockade of the CD40-CD40L dyad is protective in EAE, the role for CD40 and its ligand are not that well understood in diet-induced obesity (DIO). Deficiency of CD40L was found to ameliorate the metabolic complications of obesity [31, 32], while CD40 deficient mice [33-36] display worsened symptoms of DIO. As CD40 is expressed on different cell-types involved in DIO, we hypothesized that a cell-type specific role for CD40 could explain the surprising phenotype of the CD40 deficient mice. The findings in this thesis indicate an important protective role for dendritic cell (DC) CD40 in DIO [Chapter 5, this thesis], while macrophage and adipocyte CD40 have a minor contribution to DIO development [Chapter 6 and 7, this thesis]. The preliminary results of the adipocyte CD40 deficient mice in DIO did show local effects on adipose tissue inflammation. We observed that loss of adipocyte CD40 resulted in an increase in effector T cells, showing that adipocyte CD40 does play an
unexpected role in the functioning of the immune system. However, the potential of the adipocyte to function as an immune cell has already been suggested as they express MHCII and co-stimulatory molecules [37]. In future experiments we will further explore this function of the adipocyte and how CD40 contribute to this.

Dendritic cell CD40 deficient mice had increased hepatosteatosis which we could explain by a reduction in the number of circulating and tissue specific regulatory T cells (Tregs) [Chapter 5, this thesis]. Tregs are protective in obesity as they suppress the immune response and influence the behavior of other T cell populations [38]. Adipose tissue Tregs express high levels of the anti-inflammatory cytokine IL-10 [39]. IL-10 can suppress expression of pro-inflammatory markers and is found to restore the expression of GLUT4, an important glucose transporter [39]. Depletion of Tregs worsens insulin sensitivity in mice [38].

Despite the decreased Treg numbers in dendritic cell CD40 deficient mice, we did not observe signs of altered inflammation in these obese mice. Interestingly, we did observe strongly increased circulating lipid levels and lipid accumulation in the metabolic organs. Tregs mediate hepatic lipid metabolism via control over the expression of VLDL binding protein sortilin-1 in the liver, stimulating clearance of VLDL and CM lipoproteins [40]. DC depletion in mice results in increased serum cholesterol levels [41], suggesting that DC-T cell interactions affect lipid homeostasis in the liver.

In the DIO study in this thesis the mice developed hepatosteatosis, the first sign of non-alcoholic fatty liver disease, which is defined by the accumulation of lipids without inflammation [42]. The decrease in Tregs could be the start of an inflammatory process resulting in development of non-alcoholic steatohepatitis (NASH). It has been described that depletion of DCs results in increased NASH development [43]. We will further investigate the role of DC-CD40 on liver metabolic pathways, liver inflammation and fibrosis by inducing NASH in CD40fl/flCD11ccre mice.

In addition to the role for dendritic cell CD40 in obesity described in this thesis, other studies have also tried to explain the particular phenotype of obese CD40 deficient mice. It is shown that the CD40-TRAF pathways have opposite functions in DIO, as the loss of CD40-TRAF6 interactions is found to be protective, but loss of CD40-TRAF2,3,5 interactions worsens symptoms of DIO [33, 44]. Furthermore, CD40 expressing (CD8+) T cells have a protective function in DIO, as loss of this cell subset causes worsened glucose tolerance and insulin resistance [35, 36]. Additionally, the involvement of non-classical receptors for CD40L, and other ligands for CD40 could be considered to contribute to the opposing phenotype of the CD40 and CD40L deficient mice. For example, CD40L can bind to Mac-1 and in atherogenesis this interaction promotes leukocyte recruitment to the arterial wall [45, 46]. However, a prominent role for CD40L-Mac1 interactions in DIO is unlikely, as it is known that
Mac1 signaling does not play a role in recruitment of adipose tissue macrophages during DIO, but does enhance macrophage inflammatory gene expression [47].

Together, the results in this thesis provide novel insights in the cell-type specific function of CD40 in obesity and identify stimulation of DC-CD40 as possible therapeutic strategy in DIO [Chapter 5, this thesis]. However, the new insights are not sufficient to fully explain the worsened phenotype observed in the generic CD40 KO mice in DIO. DC-CD40 plays an important role in the development of hepatosteatosis, but the worsened adipose tissue inflammation and insulin resistance are not yet explained. The role of the remaining CD40 expressing cell types, including the B cells, T cells and endothelial cells, should also be investigated to obtain a complete overview of CD40 cell type specific function in DIO. Moreover, most likely the phenotype is not caused by CD40 function of one cell type, but by a combined contribution of different CD40 cell type functions specific for the disease conditions.

**Macrophage CD40 in atherosclerosis**

CD40 and CD40L both play an important role in atherosclerosis [15, 48-53] and modulation of the pathway offers therapeutic potential. Deficiency of the CD40-TRAF6 interactions, and not CD40-TRAF2,3,5 interaction is protective in atherosclerosis [15]. CD40-TRAF6 interactions are mainly important for macrophage CD40 signaling, and inhibition of these CD40-TRAF6 interactions by small molecule inhibitors (SMI) reduced atherosclerotic plaque development in mice [16]. Improved efficacy of treatment by nanoparticle encapsulated CD40-TRAF6 SMIs confirmed the important role for macrophage CD40 in atherosclerosis development [16, 54]. In this thesis we investigated the role of macrophage CD40 by using CD40fl/flLysMcre mice and show that macrophage CD40 is important for plaque development and leukocyte infiltration, but surprisingly does not affect plaque stability [Chapter 8, this thesis]. Stable plaques found in the generic CD40 knock out mice were composed of smooth muscle cells in a collagen matrix [15]. Vascular smooth muscle cells (vSMC) are the main collagen producing cells in atherosclerotic plaques [55], and also express CD40 [56]. Stimulation of vSMC with CD40L increases vSMC proliferation and migration [57], IL-6 production [56] and suppresses the expression of the enzyme prolyl-4-hydroxylase α1 (P4Ha1) involved in collagen synthesis [58, 59]. Preliminary results from a study were CD40 was depleted on SMCs show reduced plaque sizes, but have not evaluated the plaque stability yet [60].

Although blocking macrophage CD40 could reduce atherosclerosis in mice, blocking of the CD40-TRAF6 signaling pathway had stronger effects and is therefore a more interesting and promising therapeutic target in atherosclerosis. In addition to the role in macrophage signaling [15], CD40-TRAF6 signaling is found to be important in vascular SMCs in a model for neointima formation [61, 62]. This might contribute to the beneficial function of the CD40-TRAF6 SMI in reducing atherosclerosis, although
no effects on vSMC proliferation or collagen production were observed when the CD40-TRAF6 SMI was given to vSMC in culture.

**Future perspective: cell type specific drug targeting to increase therapeutic potential of CD40 blockade**

In this thesis we have shown that the role of CD40 is cell type specific and dependent on disease conditions. We have shown that macrophage CD40 is important in the development of MS and atherosclerosis, but hardly affects diet-induced obesity. While blockade of the CD40-CD40L dyad is generally thought to be anti-inflammatory, blocking CD40 in DIO results in increased inflammation [33-36]. An important contributor to the cell type specific function of CD40 are the different CD40-TRAF downstream signaling pathways involved [10, 63]. Deficiency of the CD40-TRAF2,3,5 signaling pathway worsens DIO [33] and atherosclerosis [15], but ameliorates EAE (this thesis). Genetic deficiency of CD40-TRAF6 is protective in multiple diseases [13, 15, 33], and CD40-TRAF6 inhibition by our small molecule inhibitor is effective in reducing atherosclerosis [16, 54], DIO [33, 44], sepsis and lupus [13], but was not efficient in reducing EAE disease severity [64]. CD40 can perform multiple functions dependent on signaling pathways involved, therefore a disease specific approach should be considered when targeting CD40-CD40L signaling as a therapeutic strategy.

Targeting the CD40-CD40L dyad has therapeutic potential for different diseases [5, 12, 13, 15, 31, 33], but complete CD40-CD40L blockade can lead to immune suppression and thrombo-embolic complications [8-11]. Cell type specific targeting of CD40 or CD40L could be an interesting approach to avoid these side effects. We showed that macrophage CD40 signaling plays an important role in EAE and atherosclerosis. Drug targeting of monocytes and macrophages for CD40 blockade can be achieved via multiple ways [65]. An example is the degradation of CD40 mRNA by using CD40 short interfering RNA (siRNA) [66] targeted to a macrophage-based receptor, for example transmembrane (tm)TNFα [67], although this receptor is not only present on macrophages. Another approach for drug delivery to macrophages is via surface engineered nanoparticles, liposomes and dendrimers [65]. In atherosclerosis, drug targeting of macrophages in the plaques can be achieved via nanomedicine-based delivery [68]. Reconstituted high-density lipoprotein (HDL) particles loaded with the CD40-TRAF6 SMI have already shown to be effective in reducing atherosclerosis [16, 54]. Macrophage targeting via nanoparticle delivery of drugs is also investigated and found to be effective in animal models of multiple sclerosis [69].

As we found a protective role for dendritic cell CD40 in obesity it would be interesting to target these cells with CD40 stimulating agents. Specific delivery of therapeutic agents to DCs can be achieved via incorporation in DC derived exosomes [70], via liposomes [71], via molecular probes, for example ‘aptamers’ [72], and via
nanoparticles [73] targeted to DC receptors, for example the Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN; CD209) receptor [73]. However, although our results suggest that upregulation of DC-CD40 could be beneficial in treatment of DIO, further research is necessary as constitutive expression of CD11c-CD40 during atherosclerosis caused increased T cell numbers, neutrophilia and development of inflammatory bowel disease [74].

Overall conclusions
This thesis provides further evidence that CD40 has different functions per cell type and per disease. Cell-type specific targeting of CD40 modulating compounds could increase treatment efficacy and reduce the risk of side effects such as immunosuppression.
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