Published in final edited form as:

Semin Immunol. 2009 October ; 21(5): 265–272. doi:10.1016/j.smim.2009.05.010.

The role of CD40 and CD40L in Dendritic Cells

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Keywords

CD40; CD40L; CD154; Dendritic cells

Introduction

In this review, we focus on the function of CD40-CD40L (CD154) interactions in the regulation of dendritic cell (DC) - T cell and DC-B cell cross-talk. In addition, we examine differences and similarities between the CD40 signaling pathway in DCs and other innate immune cell receptors, and how these pathways integrate DC functions. As research into DC vaccines and immunotherapies progresses, further understanding of CD40 and DC function will advance the applicability of DCs in immunotherapy for human diseases.

Background on CD40 and CD154/CD40L

CD40 is a 48 kilodalton transmembrane glycoprotein surface receptor that is a member of the Tumor Necrosis Factor Receptor superfamily (TNFRSF). The importance of CD40 in regulating functions was not appreciated for many years after CD40 was discovered. Our initial studies emphasized that CD40 was expressed on B cells and that ligating CD40 induced B cells to proliferate [1,2]. The cloning of human and mouse CD40 [3,4] facilitated the identification of CD40 ligands and the development of anti-mouse CD40 monoclonal antibodies (mAbs); analyses of mRNA and protein expression showed that CD40 is expressed on non-B cells like DCs, monocytes, epithelial cells [5–7] and even endothelial cells [8]. Nevertheless, most early studies of CD40 including our own focused on defining the role CD40 plays in B cell survival, ‘T cell help’ and isotype class switching [9–11]. The Ig deficiencies in CD40L (CD154)-impaired patients with hyper-IgM syndrome and in CD40 or CD40L knockout (KO) mice served to underscore the central role of CD40 in mature B cell functions [12–17]. However, the fact that these patients are more susceptible to certain opportunistic infections suggested CD40L-CD40 may regulate non-B functions.

More attention turned toward DCs after several groups reported that CD40 signaling induces changes in DCs, which make them more effective antigen presenting cells (APCs), such as upregulation of MHC class II and co-stimulatory molecules CD80/CD86 [18–20]. Although DCs resembled B cells in these responses to CD40 crosslinking [9], it soon became apparent that different cell types respond in distinct ways to CD40 stimulation; for instance, while CD40 ligation promotes B cells to survive, it promotes some epithelial to die [21] and others to

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produce GM-CSF [22]. Stimulation of CD40 was found to program monocytes and DCs to do things B cells do not do well, such as producing inflammatory cytokines and chemokines [23,24].

The ligand for CD40, CD154 (also known as TRAP, T-BAM, CD40 Ligand or CD40L) is a 34–39 kilodalton type II integral membrane protein. Initially CD40L was reported to be expressed principally on activated CD4⁺ T cells, which led to models that the main function of CD40L was to provide T cell help to B cells [25]. Once again, further scrutiny revealed that CD154/CD40L is also expressed on activated B cells, platelets and smooth muscle cells [25]. Furthermore, functional CD40L is induced on human blood-derived DCs after CD40 stimulation [26], and CD40L has been detected on a variety of DCs including murine Langerhans cells [27], lung DCs [28]), plasmacytoid dendritic cells (pDCs) [29] and splenic DCs [30].

B cells can also express CD40L [31–33], and CD40 has been shown to be expressed on activated CD4⁺ and CD8⁺ T cells [34]. Thus, emphasizing the CD4⁺ T cell mediated functions of CD40L may be over simplistic; any discussion of CD40 function on DCs must consider that a CD40L signal to DCs or B cells may come from not only CD4⁺ T cells but also activated DCs or B cells (Fig. 1). Studies with a related TNF-TNFR family pair, RANKL-RANK, showed that RANKL can promote DC survival [35] and that RANKL is expressed on activated DCs [36]. Since DCs interact with each other *in vivo*, forming e.g., a network in the lamina propria [37], it is quite possible that DCs interact with each other to regulate their functions through CD40L-CD40 or RANKL-RANK pathways. The C4 binding protein (C4bp) was reported to be a second ligand for CD40 [38], suggesting that CD40 can receive and bridge innate and adaptive immune signals [39]. Little is known about the role of C4bp via CD40 in DC functions.

Another important consideration regarding the function of CD40 in DCs is the change in CD40 expression from immature to mature DCs. CD40 is expressed constitutively at relatively low levels on unactivated DCs [25]. In mice levels of CD40 are used as a marker to distinguish between inactivated and activated DCs, as its expression is up-regulated on DCs after encounter with microbial products (e.g. Toll-like receptor ligands) [40] and pathogens (e.g. viruses like HIV), as well as after uptake of apoptotic cells [41].

CD40 Signaling pathways in DCs

The role the CD40-CD40L pair plays in regulating B cell functions is well documented [9, 11,42]. The signaling motifs within CD40's cytoplasmic tail required for extrafollicular B cell differentiation versus germinal center formation *in vivo* are different [43]. CD40 ligation clearly inhibits the apoptosis of immature B cells including transitional B cells [44,45]. A number of CD40-responsive genes have been identified including Bcl-2 family members cIAPs, c-myc, signaling elements like TRAF1 and Pim-1 kinase and cell surface molecules like CD40 itself, CD23 and CD21. A number of these genes are dependent on the NF-κB signaling pathway [42,46–49]. These data suggest CD40 can program both survival and differentiation in B cells.

The signaling pathways induced downstream of CD40 engagement on DCs also have been studied and activate multiple genes important for DC function. The cytoplasmic tail of CD40 contains sites for the recruitment of TNF Receptor Associated Factor family of proteins (TRAFs). CD40 ligation results in the trimeric clustering and the signaling pathways activated downstream CD40 depends on the specific TRAF being recruited. The recruitment of TRAFs after CD40 ligation initiates signaling cascades that activate genes involved in cytokine production, as well as upregulation of co-stimulatory molecules such as CD80 and CD86, and other maturation markers [50]. CD40 signaling can recruit TRAFs 2, 3, 5 and 6, although

TRAF6 appears to be the predominant player utilized in DCs [34]. The details of CD40 signaling have been reviewed elsewhere, and thus will not be discussed in great detail here [25,34]. The recruitment of TRAF2 and TRAF3 occurs downstream of CD40 signaling in human tonsillar B cells but not in monocyte-derived immature DCs (iDCs) [46]. Vidalain et al. reported TRAF3 (and to a lesser extent TRAF2) could be co-immunoprecipitated from CD40-containing lipid rafts stimulated human iDCs after CD40 ligation, and suggested that TRAF2 and TRAF3 may also be recruited downstream of CD40 signaling in DCs [51]. However, the authors did not show direct binding of TRAF2 or TRAF3 to the cytoplasmic tail of CD40, and in addition acknowledged that the use of detergent insolubility as the only criterion for whether a protein associated with the lipid raft was flawed. Since then no additional studies have been published to support this finding. At this time, TRAF6 appears to be the only member of the TRAF family that clearly is recruited downstream of CD40 ligation in DCs.

These signaling pathways downstream of CD40 ligation in DCs are similar to those activated by other receptors such as Toll-like receptors (TLRs) and RANK-RANKL (TRANCE R-TRANCE). However, CD40 ligation also induces functions distinct from signaling through TLRs or RANK. Thus CD40 induces important DC functions via signaling pathways that are both common with and unique from other receptor interactions.

CD40 and TLR signaling pathways

One of the major DC functions regulated by CD40 ligation is the production of cytokines. In DCs, TRAF6 is recruited downstream of CD40 ligation [52], resulting in activation of the p38 MAP Kinase (MAPK) and JNK (Jun Kinase) [53] leading to production of cytokines such as IL-12p40 [23] and IL-6 [54]. TRAF6 is also involved in the signaling pathways downstream of TLR ligation via MyD88 and TRIF signaling [40]. DCs from TRAF6 KO mice are unable to upregulate maturation markers MHC II and CD86, and cannot produce IL-6 or IL-12p40 in response to LPS or CD40L [55].

Both TLR and CD40 signaling can induce the activation of NF- κ B, which turns on a number of genes important to DC function. A recent study showed that CD40 ligation-induced DC antigen presentation required the activation of NF- κ B-inducing kinase (NIK) to activate non-canonical NF- κ B2 (p52/p100) [56]. DCs from alymphoplasia (aly) mice expressing a point mutation of NIK impairing its interaction with the IKK complex were unable to cross-present OVA antigen to CD8⁺ T cells. These aly DCs were also unable to cross-present OVA antigen to CD8⁺ T cells after LPS stimulation, which signals through both the MyD88 and TRIF pathways [40]. While it remains controversial whether TLR signaling truly induces NF- κ B activation through the non-canonical pathway physiologically [40]. It appears that TLR and CD40 signaling overlap in the activation of NIK to turn on NF- κ B2 through the non-canonical signaling pathway.

Although the cytoplasmic tail of CD40 can potentially recruit other TRAF family members, only TRAF6 has been clearly shown to be active in CD40 signaling pathways in DCs. While further research is required to elucidate if other TRAF members are involved in CD40 signaling in DCs, the finding that CD40 signaling in DCs depends on TRAF6 suggest that CD40 signaling of DCs normally only induces non-canonical activation of NF- κ B. In contrast, signaling through MyD88 and TRIF can activate NF- κ B through the canonical pathway (formation of Rel-A/p50 heterodimers) [40]. In addition, MyD88 and TRIF, activates unique pathways not triggered via CD40 signaling, such as the activation of interferon (IFN) response factors (IRFs).

CD40 signaling, however, can synergize with TLR9 signaling induce production of Type I IFN by human plasmacytoid DCs (pDCs): Kerkmann *et al.* detected enhanced production of IFN α by human pDCs stimulated with CpG-B and CD40L [57]. Since the addition of IFN-receptor (IFNR) blocking antibodies did not abrogate production of IFN α in response to CpG-

B and CD40L, the authors concluded that CD40L enhancement of IFN α production was independent of an IFNR positive feedback loop. Neither IRF7 nor IRF3 transcription was induced by CD40L stimulation, and thus CD40L appears to promote IFN production through a pathway distinct from that of TLR9 signaling.

CD40 and RANK-RANKL Signaling

Another member of the TNFRSF important in regulating DC function is RANK (Receptor Activator of NF- κ B, also known as TRANCE-Receptor or TRANCE-R). Although a number of studies have examined the function of RANK in DCs, it is important to keep in mind that RANK expressed on cells other than DCs including osteoclasts, B cells and fibroblasts [58, 59]. Signaling through RANK is regulated through competitive binding of RANK to its ligand (RANKL, or TRANCE) by a soluble receptor called osteoprotegerin (OPG) [60]. CD40L stimulation of DCs upregulates the expression in DCs of both RANK [58] and OPG [61], underscoring the interconnectiveness of these two receptor-ligand pairs.

Similar to CD40, signaling through RANK-RANKL induces the production of pro-inflammatory cytokines. The production of cytokines by murine bone marrow-derived DCs (BMDCs) after either RANKL or CD40L stimulation induces the production of IL-1 β , IL-1R α , IL-6 as well as the T cell/natural killer (NK) cell-activating cytokine IL-15 [62]. However, CD40L stimulation also induces production of IL-12, which is important for inducing Th1 responses [23]. The RANK signaling pathway in DCs, like CD40 mainly utilizes TRAF6, but other TRAFs have also been implicated in RANK signaling of DCs, [59,63]. Evidence that RANKL and CD40L trigger distinct signaling pathways in DCs comes from studies showing that RANKL signaling can enhance the survival of CD40L-stimulated DCs [64] and that DCs transduced with a CD40L adenoviral vector produce significantly more IL-12 and express more co-stimulatory molecules than DCs overexpressing RANKL [65]. Conversely, RANKL can enhance the expansion of CD8 $^{+}$ T cells in vivo under conditions where CD40L is not effective [66].

RANKL (TRANCE) -RANK signaling promotes the survival of DCs, most likely by the upregulation of Bcl-x $_L$ [59], although it upregulates Bcl-x $_L$ to a lesser extent than CD40L signaling [67]. The expression of Bcl-2 is also induced in RANKL-treated BMDCs [59]. Similar to RANK, ligation of CD40 induces signals that promote cell survival, thus making CD40 an important molecular player involved in the regulation of DC apoptosis and survival. In murine DCs, CD40 ligation on DCs can induce survival by protection against Fas-induced apoptosis [67] as well as through upregulation of anti-apoptotic protein Bcl-x $_L$ [68]. Both CD40L and RANKL-induced DC survival requires NF- κ B p50 and cRel [69]. In human iDCs, CD40 ligation induces upregulation of anti-apoptotic protein Bcl-2, which has been correlated to protection against Fas-induced apoptosis [70].

CD40 signaling of DCs induces expression of OPG [61]. Constitutive RANKL-RANK interactions between DCs sustain DC viability and Bcl-2 expression [36,71]. However, OPG blocks both RANK-dependent DC survival and cytokine production [36]. Thus, CD40 signaling of DCs and OPG production may induce feedback regulation that limits the duration of DC-mediated activation.

CD40 and DC Subsets

There are multiple DC subsets in mice and humans [72,73]; however, the growing expanse of DC subset definitions has limited the analysis of CD40 expression and function being conducted for each subset. Human peripheral blood pDCs (CD4 $^{+}$,CD123 $^{+}$, BDCA1 $^{+}$, BDCA4 $^{+}$) and myeloid DCs (mDCs: CD4 $^{+}$ CD11 $^{+}$ CD33 $^{+}$) upregulate CD40 in response to TLR9 (CpG) or TLR4 (LPS) agonists [74]. The pDC subset stimulated with CpG upregulated

CD40 to a greater extent compared to mDCs stimulated with LPS *in vitro*, while conversely, CD40 upregulation was not induced in LPS-stimulated pDCs [74,75]. pDCs and mDCs both upregulated CD40 to similar levels in response to the TLR7 ligand Imiquimod [75,76]. Thus, the level of CD40 expression in peripheral blood DCs is not subset specific but rather dictated by the signaling pathway and receptor utilized when the DC becomes activated.

Indeed, a recent study examining the expression of maturation markers including CD40 on human pDCs versus mDCs in response to Hepatitis C virus (HCV) showed that HCV inhibits TLR7-mediated upregulation of CD40 in pDCs, but not TLR3-mediated upregulation of CD40 in mDCs [77]. Also, Veckman and Julkenen showed that human pDCs increased more CD40 after exposure to *Streptococcus pyogenes* and Influenza A compared to mDCs [78]. Influenza A induced greater fold CD40 induction in pDCs compared to *S. pyogenes*, whereas in mDCs, Influenza A failed to induce CD40 upregulation. Again differences in the upregulation of CD40 expression appear to be more dependent on the type of stimulation rather than the DC subset.

However, this may not always be the case. In human lungs, basal expression of CD40 and other co-stimulatory markers CD80 and CD86 appear to be higher in mDCs compared to pDCs [28]. In addition, mature DC subsets in the human thymus differentiated by the expression of CD11b show slightly different expression patterns of CD40 [79]. Additional work is required to compare expression of CD40 in subsets localized to other tissues, particularly in mucosal tissues, spleen and lymph nodes.

The broad classification of human DCs has been used in defining mouse DC subsets [73]. In the mouse spleen, there appears to be no difference in levels of basal CD40 expression between splenic CD8⁻CD4⁺ and CD8⁻CD4⁻ myeloid DCs [80]. In the liver, CD11c^{hi}B220⁻ DC subsets express higher basal levels of CD40 expression compared to that of CD11c^{int}B220⁻ subsets, and there appears to be little or no expression of CD40 on the B220⁺CD11c^{int} population [81]. Activation of DC subsets using murine cytomegalovirus (MCMV) induces high upregulation of CD40 on the CD11c^{hi}B220⁻ population, but not CD11c^{int}B220⁺ DCs [81]. In addition, CD8 expression on hepatic DC subsets is correlated with higher basal expression of CD40 compared to CD8⁻ DC populations [82].

Expression Levels of CD40 and DC Function

The term ‘regulatory DCs’ has been associated with subsets of DCs that are distinct from ‘immature’ or ‘tolerogenic’ DCs that present signal 1 (e.g. antigen peptide-MHC complex) but not a co-stimulator signal 2, and thus can induce anergy in cognate lymphocytes [83,84]. It describes subsets of DCs found in both mice and humans that induce T cells with poor proliferative capacity, and produce IL-10 and TGF- β rather than Th1 or Th2 cytokines. Notably, these DCs also induce the generation of regulatory T cells (T_{regs}) [85–88]. DCs possessing these regulatory effector functions encompass a heterogeneous and broad range of DCs that are induced under various types of stimuli and infection [89], so we will narrow this discussion to a specific subset identified in mice [86–88].

Wakkach *et al.* identified one particular population of regulatory CD11c^{int}CD45RB^{hi} DCs [88]. This population is found in the spleen and the lymph nodes and has the capacity to induce tolerance by promoting differentiation of T_{reg} cells through secreting IL-10. Stromal cells may be required for the differentiation and support of this DC subset. One characteristic of the CD45RB⁺ regulatory DCs is that they do not upregulate CD40 even after infection or TLR stimulation; indeed, they do not upregulate surface CD40 even in response to LPS [86,88] or *Plasmodium falciparum* infection [90].

Another recent study reported the induction of DCs possessing similar effector functions to the CD11c^{int}CD45RB^{hi} DCs, and which fail to upregulate CD40 in response to *Trypanosome*

cruzi exposure *in vitro* [91]. However this study employed the use of bone-marrow derived DCs (BMDCs) and moreover, did not use CD45RB as a marker. Thus it is difficult to compare whether these cells are the same subset as identified by Wakkach *et al.*

Whether CD40 signaling is associated with the function of these DC subsets has yet to be determined. Given the low expression of CD40 on the regulatory DCs, it would be of interest to know if TNFR family members other than CD40 such as RANK regulate this DC subset. Furthermore, one could speculate that the decreased expression of co-stimulatory molecules on these DCs may contribute to their decreased capacity to induce effector T helper cells and preferential induction of T_{regs}. Indeed, it was shown that steady-state DCs without anti-CD40 activation were the most efficient at the induction of CD4⁺CD25⁺Foxp3⁺ T_{regs} from naive CD4⁺CD25⁻ T cells in the periphery. [92]. The authors targeted splenic CD8α⁺ DC subsets with a fusion protein of an anti-DEC205 antibody conjugated to the hemagglutinin antigen from the Influenza virus (anti-DEC-HA), which by itself does not alter maturation status of steady-state DCs [93]. DCs targeted with low doses of anti-DEC205-HA were most efficient at inducing *de novo* CD25⁺Foxp3⁺ T_{regs} from naive CD4⁺CD25⁻Foxp3⁻ progenitors when compared to DCs targeted with high dose antigen or in the presence of anti-CD40 stimulus.

A more recent study examined murine DCs that displayed a 'semi-mature' phenotype and its role in regulating T cell responses in collagen-induced rheumatoid arthritis (CIA) [94]. The authors stimulated murine BMDCs with naked DNA plasmid versus LPS and showed that treatment with plasmid induced levels of cytokines and expression of co-stimulatory molecules (including CD40) that were intermediate between LPS and unstimulated BMDCs, and hence termed these DCs as 'semi-mature'. Functionally, semi-mature DCs were similar to LPS-stimulated mature DCs in ability to induce T_{regs} and provided protection against CIA. Although there was no enhanced efficacy of 'semi-mature' DCs to induce T_{regs} compared to LPS-stimulated DCs expressing higher levels of CD40, the levels of IL-10 and TGF-β transcription were the highest in animals that had received transfer of 'semi-mature' DCs in the peripheral lymph nodes early after transfer. Thus 'semi-mature' DCs may induce alternate T cell programming that result in protection from CIA.

Cytokine and pharmacological treatments also affect the expression of CD40 and other co-stimulatory molecules associated with maturation status of DCs. The use of pharmacological treatments such as Vitamin D can modulate the expression of surface CD40 and overall maturation of DCs. These DCs have been shown to induce T_{regs} and increase IL-10 production resulting in increased tolerance to transplants and decreased diabetes in mouse models [85].

Sato *et al.* initially showed that human or murine DCs cultured with immunomodulatory cytokines could result in suppression of effector T cell responses [87]. They showed that human DCs cultured in the presence of IL-10 and TGF-β could induce expansion of CD4⁺CD25⁺ and IL-10 secreting CD8⁺CD28⁻ T_{reg} cells that could suppress CD4⁺ T cell functions. In a model of xenogeneic graft-versus-host disease (XGVHD), murine BMDCs cultured with IL-10 and TGF-β (which also expressed low levels of co-stimulatory molecules and had decreased capacity to induce expansion of primed human T cells) were transferred into a SCID mouse grafted with human peripheral blood leukocytes (hu-PBL-SCID). The hu-PBL-SCID mice receiving murine BMDCs cultured with IL-10 and TGF-β had significantly higher rates of survival from XGVHD compared to hu-PBL-SCID mice that received murine BMDCs cultured in GM-CSF alone.

In another study, Lan *et al.* showed that Balb/c murine BMDCs generated in GM-CSF with IL-10 and TGF-β are more resistant to LPS-induced upregulation of CD40 and preferentially induced higher percentages of CD4⁺CD25⁺Foxp3⁺ T_{regs} *in vitro* compared to BMDCs generated with GM-CSF alone [95]. In an MHC-mismatch rejection model, BMDCs grafts

from Balb/c mice were tolerated by C57BL/10J recipient mice if the BMDCs were generated in the presence of IL-10/TGF- β . In addition, tolerized grafts were shown to have significant numbers of CD4⁺Foxp3⁺ infiltrates compared to rejected grafts, thus supporting the notion that IL-10/TGF- β alters BMDC function in the regulation of T cell responses.

While the role of CD40 signaling and expression on DCs has not been directly connected to the induction or maintenance of T_{reg}s, the levels of CD40 expression on DCs may influence CD40-CD40L interactions between DCs and T cells and thus lymphocyte programming by DCs. In this regard, it is relevant to consider how CD40 and/or CD40L signaling of DCs and how that affects regulation of B and T lymphocytes. Thus we next discuss the differential expression of CD40 and CD40L on DCs and lymphocytes and how crosstalk between these molecules may affect DC-lymphocyte interactions.

Regulation of Lymphocytes by CD40⁺ and CD40L⁺ DCs

CD40 not only is expressed on APCs such as B cells and DCs, but also has been detected on activated T cells (Fig. 1) [34]. Similarly, CD40L not only is expressed on activated T cells, but also on activated human and murine DCs (Fig. 1) [26,30]. Thus, it is important to consider the role of possible CD40L-CD40-dependent bi-directional crosstalk that occurs between DCs and lymphocytes. Indeed it was shown early on that CD40-CD40L interactions between T cell and DCs provides reciprocal effects that both regulate T cells and DCs [96,97]. While it is clear that CD40L crosslinking can signal T cells e.g., to produce IFN- γ [98,99], it is not clear how ligation of CD40L on B cells or DCs affects their functions.

CD40 expression on DCs is clearly important for the effector functions regulating lymphocytes. As noted above ligation of CD40 via anti-CD40 mAbs or CD40L induces upregulation of co-stimulatory molecules, adhesion molecules, and the Th1-polarizing cytokine IL-12 in both mouse and human DCs [23,100,101]. The similarities between human and mouse CD40 enabled mouse genetics to be used for further understanding the role of CD40 in DC functions. CD40 KO (CD40^{-/-}) mice were generated by two different groups, one with the CD40 defect targeted to hematopoietic cells [102], the other, with the defect restricted to lymphocytes [103]. Initially, both groups showed that CD40 deficient mice were impaired in the generation of T-dependent antibody responses. Later the CD40^{-/-} mouse model was used to study DC-lymphocyte interactions initially in the context of cardiac allograft rejection [104] and subsequently by Mackey *et al.* [96].

Mackey *et al.* showed that transfer of CD40^{+/+} DCs into CD40^{-/-} mice could rescue recipients from death by tumor challenge [96]. Exogenous IL-12 could partially rescue CD40L^{-/-} mice from succumbing to tumor challenge. The authors concluded that the absence of CD40-induced IL-12 production following cognate T cell-DC interactions results in a defect in mounting effective Th1 responses and susceptibility to tumor load. However, a later study showed that CD40L^{-/-} splenic APCs produced IL-12 to similar levels as that of CD40^{+/+} mice following a tumor allograft challenge, but this did not protect CD40L^{-/-} mice from succumbing to tumor load [105]. The authors reported a defect in priming tumor-specific CTLs in CD40L^{-/-} mice which could not be rescued with the addition of exogenous CD40L *in vivo*. Thus the requirement for CD40-CD40L interactions may not solely be due to the lack of IL-12 production in defective CD40 signaling on DCs, and suggests that CD40-CD40L interactions between APCs and T cells involves other mechanisms (e.g. production of other cytokines, direct signaling via CD40) in the priming of effector T cells.

CD40 may also be involved in differentiation of T cell subsets other than Th1 effectors. As mentioned, “regulatory DCs” expressing low levels of CD40 preferentially induces T_{reg} generation. A recent study by Iezzi *et al.* illustrated a requirement for the expression of CD40

and interaction with CD40L on CD4⁺ T cells to drive to Th17, but not Th1 differentiation [106]. CD40^{-/-} mice had lower numbers of IL-17 and IFN- γ -producing CD4⁺ T cells compared to wildtype mice after immunization with a lymphocytic choriomeningitis virus peptide compared to CD40^{+/+} mice, but the numbers of IFN- γ -producing cells could be rescued in CD40^{-/-} using a higher dose of antigen. However, both CD40^{-/-} and CD40^{+/+} mice were able to induce comparable numbers of IFN- γ -producing cells after immunization with *Listeria monocytogenes*. In addition, CD40^{-/-} mice also are protected from MOG peptide-induced experimental autoimmune encephalomyelitis (EAE), a disease whose immunopathology is associated with IL-17-producing CD4⁺ T cells [107,108]. The protection in CD40^{-/-} mice from EAE was correlated with decreased Foxp3⁺, IL-17⁺ or IFN- γ ⁺ T cell infiltrates into the central nervous system.

Although Iezzi *et al.* did not test if transfer of CD40^{+/+} DCs or T_{regs} could induce Th17 effectors or IL-17 production resulting in exacerbation of EAE in CD40^{-/-} mice, their data emphasize the importance of CD40 signaling in enabling DCs to induce effector populations such as T_{regs} and Th17 in certain antigen environments *in vivo*. Thus CD40 plays a role in DC-mediated priming of multiple effector T cell responses in addition to the Th1 subset. As more novel effector T cell subsets continue to be defined, further work will be required to understand the direct role of CD40 signaling in DCs in regulation of these subsets.

CD40 Expression on DCs and the Regulation of B cells

Both DCs and B cells express CD40 constitutively, and both cell types interact with and regulate T cells by ligating CD40L. As such, CD40-CD40L interactions have not been heavily studied in the context of DC-B cell interactions. However, CD40L is expressed on activated B cells and DCs (Fig. 1). Thus it is possible that B cells and DCs directly interact via CD40-CD40L. This seems all the more likely given the growing literature showing that DCs regulate B cell responses through cell contact-dependent mechanisms independent of CD4⁺ T cell help [109–111].

Wykes and MacPherson showed that the expression of CD40 on DCs and B cells is required for optimal proliferation of B cells in absence of any exogenous stimuli [111]. The loss of CD40 on B cells also diminishes long term survival of B cells in the presence of DCs. While the mechanism of this interaction has not been established, it suggested that CD40 expressed on DCs played a direct role in regulating B cell proliferation, and perhaps more in regulation of B cell homeostasis.

Bergtold *et al.* showed that BMDCs directly enhanced B cell proliferation in response to anti-CD40 compared to B cells cultured with anti-CD40 alone [109]. DCs lacking activating receptors Fc γ RI and Fc γ RIII utilize the inhibitory Fc γ RIIB to internalize immune complexes, and recycles antigens to the surface for presentation to B cells in B cell-DC clusters. The loss of Fc γ RI and Fc γ RIII on DCs also increased B cell proliferation and CD86 expression in response to anti-CD40 stimulation, especially if the DCs were pulsed with anti-IgM containing immune complexes. Thus the recycling of immune complexes taken up by DCs via Fc γ RIIB endocytosis induced optimal B cell activation. It remains unclear if CD40 stimulation of B cells, DCs or both cell types was necessary for optimal B cell proliferation. Since B cells and DCs can express both CD40 and CD40L (Fig. 1), it is possible that CD40-driven proliferation could occur in the absence of T cells.

Cytokines produced by DCs activated by CD40 signaling can regulate T cell-independent B cell antibody responses. T-cell dependent regulation of B cell antibody responses is thought to require CD40L on T cells interacting with CD40 on B cells [112]. Litinskiy *et al.* showed that DCs activated by CD40L induces the expression of TNF family member cytokines B lymphocyte stimulator protein (BLyS, also known as BAFF, TALL-1, THANK and zTNF4)

and a proliferation-inducing ligand (APRIL) [113]. BAFF binds to BAFF Receptor (BAFF-R) and B cell maturation antigen (BCMA) on B cells, while APRIL binds to transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BCMA on B cells [114]. BAFF and APRIL promoted B cell survival, class switch recombination and secretion of IgG and IgA antibodies. The addition of anti-BAFF, BCMA-Ig or TACI-Ig to neutralize DC-derived BAFF or APRIL abrogated antibody production and class switching, but was not observed with the addition of CD40-Ig. The authors concluded that B cell antibody responses are regulated by cytokines produced during CD40 signaling on DCs, and not through DC-induced CD40 signaling on B cells.

This model is supported by subsequent studies illustrating the importance of DC-derived BAFF and APRIL in regulating B cell antibody responses. Konrad *et al.* showed that BMDCs pulsed with intestinal bacteria induced IgA production from B cells in the Peyer's patches and that IgA production could be abrogated with blockade of BAFF or APRIL by TACI-Ig fusion protein [115]. Later Tezuka *et al.* showed that mice deficient in nitric oxide production (iNOS KO mice) have DCs that secrete decreased amounts of APRIL [116]; as a result T-independent IgA class switching and secretion is impaired unless the mice are adoptively transferred with wildtype DCs which can produce APRIL. Finally, we found that BAFF produced by human monocyte-derived DCs enhances anti-IgM but not CD40L-activated B cells [117]. Collectively these studies illustrate that cytokines produced by DCs via CD40 signaling can regulate B cell antibody responses.

CD40L Expression on DCs and the Regulation of Adaptive Immunity

As mentioned, CD40L is expressed on activated T cells, and is important both in providing co-stimulation for effector T cell functions [118–121] as well as inducing proper 'licensing' of DCs and APCs to prime CD8⁺ T cells to become CTLs [83,122–124]. Most of these studies were conducted using mice with CD40L deficiency in all cells of the hematopoietic compartment. Again it is important to remember that CD40L is expressed not only on T cells but also on human and murine DCs [26,28–30].

We found that human peripheral blood DCs as well as tonsillar B cells bound to an anti-CD40L monoclonal antibody [26]. Expression of CD40L was upregulated via CD40 ligation or PMA/Ionomycin treatment of isolated DCs. DCs pre-activated through CD40 induced B cells to produce IgG and IgA antibodies by a CD40L-CD40 pathway since exogenous anti-CD40L blocked antibody production. These data support the idea that DCs also regulate B cells via CD40-CD40L interactions (Fig. 1).

Johnson *et al.* focused on the role of CD40L in CD4-independent CD8⁺ T cell responses [30]. As with human DCs [26], murine splenic DCs expressed CD40L after CD40 ligation and also after TLR stimulation. In an *in vitro* mixed lymphocyte reaction, CD40L^{-/-} DCs induced less proliferation of CD40^{+/+} CD8⁺ T cells. Conversely, CD40^{-/-} CD8⁺ T cells had decreased proliferative response to CD40L^{+/+} DCs. These findings strongly suggest that CD40L on DCs and CD40 on CD8⁺ T cells are required for efficient CD8⁺ T cell priming.

Other *in vivo* studies by Johnson *et al.* implicated CD40L and CD40 in the priming of CTLs by DCs [30]. Mixed bone marrow chimeric mice reconstituted with CD40L^{-/-} and CD40L^{+/+}CD11c⁺-DTR bone marrow were generated; depletion of CD40L^{+/+} CD11c-DTR DCs decreased specific lysis of OVA-coated splenocytes that had been transferred into the chimeric mice. CD4⁺ T cells were depleted with anti-CD4 antibody prior to transfer of OVA-coated splenocytes, showing that CD40L expression on DCs could induce CTL priming in the absence of CD4⁺ T cell help. Thus the authors concluded that CD40L expression on DCs primes CD4-independent CTL responses.

The mechanism by which CD40L expression on DCs induces CTL priming has not been elucidated. It is possible that CD40L signaling on DCs induces the production of cytokines that regulate lymphocytes. Another possibility is that DCs expressing CD40L induces CD40 signaling on activated lymphocytes. While the mechanism remains unknown, these studies reveal that DCs and lymphocytes can express CD40 and CD40L, and can participate in reciprocal crosstalk interactions (Fig. 1).

Concluding Remarks

CD40 was initially characterized as a co-stimulatory molecule expressed on APCs that played a central role in B and T cell activation. However, this molecular pair functions in the regulation of both APCs and effector lymphocytes (Fig. 1). As we understand more about the number of different DC and T cell subsets, we are likely to find that CD40-CD40L interactions play important and distinct roles in regulating these novel subsets. In addition, as we continue to understand how innate immunity cells directly regulate B cells and antibody responses, the influence of CD40 and CD40L in these interactions should be further clarified. Further insights into the functions of CD40-CD40L interactions will advance our understanding of immune cell crosstalk and interdependent regulation of the immune system.

Acknowledgments

Grant Information: Supported by NIH grants AI52203 and DE16381.

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Figure 1. Schematic showing CD40 and CD40L expression on DCs and Lymphocytes. (Left) Initial model of CD40-CD40L interactions between T cells and DCs

CD40 is upregulated on activated DCs and CD40L is expressed on activated T cells.

Engagement of CD40 on DCs induces positive signaling that leads to expression of CD80/86 and the production of IL-12 that skews towards Th1 differentiation in CD4⁺ T cells. In addition to IL-12, CD40L signaling in T cells induces IFN- γ production. **(Right) Model of CD40-CD40L crosstalk interactions between T cells, B cells and DCs.**

CD40 and CD40L are both reciprocally expressed on activated DCs and lymphocytes, and may engage in multi-directional crosstalk between cell types. CD40 signaling on DCs induces secretion of IL-12 which promotes Th1 differentiation, IL-10 that induces T_{regs} or other cytokines that induces Th17 differentiation. CD40 signaling also induces BAFF and APRIL, which along with T cell-derived IL-2 and IL-4 [125], induces B cell class switching and secretion of IgG and IgA antibodies.