Raul Elgueta
Micah J. Benson
Victor C. de Vries
Anna Wasiuk
Yanxia Guo
Randolph J. Noelle

Molecular mechanism and function of CD40/CD40L engagement in the immune system

Authors' address

Raul Elgueta[1], Micah J. Benson[1], Victor C. de Vries[1], Anna Wasiuk[1],
Yanxia Guo[1], Randolph J. Noelle[1]
[1]Department of Microbiology and Immunology,
Dartmouth Medical School and The Norris Cotton Cancer
Center, Lebanon, NH, USA.

Correspondence to:

Randolph J. Noelle
Department of Microbiology and Immunology
Dartmouth Medical School and The Norris Cotton
Cancer Center
1 Medical Center Drive
Rubin Bldg, Level 7, Room 730
Lebanon, NH 03756, USA
Tel.: +1 603 653 9918
Fax: +1 603 653 9952
e-mail: Randolph.J.Noelle@Dartmouth.edu

Summary: During the generation of a successful adaptive immune response, multiple molecular signals are required. A primary signal is the binding of cognate antigen to an antigen receptor expressed by T and B lymphocytes. Multiple secondary signals involve the engagement of costimulatory molecules expressed by T and B lymphocytes with their respective ligands. Because of its essential role in immunity, one of the best characterized of the costimulatory molecules is the receptor CD40. This receptor, a member of the tumor necrosis factor receptor family, is expressed by B cells, professional antigen-presenting cells, as well as non-immune cells and tumors. CD40 binds its ligand CD40L, which is transiently expressed on T cells and other non-immune cells under inflammatory conditions. A wide spectrum of molecular and cellular processes is regulated by CD40 engagement including the initiation and progression of cellular and humoral adaptive immunity. In this review, we describe the downstream signaling pathways initiated by CD40 and overview how CD40 engagement or antagonism modulates humoral and cellular immunity. Lastly, we discuss the role of CD40 as a target in harnessing anti-tumor immunity. This review underscores the essential role CD40 plays in adaptive immunity.

Keywords: *CD40, CD40L, tumor necrosis family, TRAF proteins, humoral immunity, cellular immunity, graft tolerance, tumor immunity*

Introduction

In the initiation of an adaptive immune response, multiple signals are necessary. A primary signal is the engagement of the T-cell antigen receptor (TCR), with polypeptides derived from a protein presented in the context of major histocompatability complex II (MHC II) on the surface of antigen-presenting cells (APCs) and the binding of native antigen to a cognate B-cell receptor (BCR) complex expressed by B cells. Subsequent secondary signals involve the engagement of costimulatory molecules expressed as receptor and ligand pairs between T cells and APCs and between B cells and T cells, with CD40 ligand (CD40L) expressed by activated T cells engaging CD40 expressed by B cells and APCs (1). Other accessory signals are necessary: the secretion of cytokines functioning to further enhance, modify, and skew the responding effector cells.

Although T-cell priming and B-cell activation can occur in absence of CD40 signals, many cellular and immune functions are defective in the absence of this interaction, underscoring the importance of this ligand/receptor pair in the development of adaptive immunity (2).

Costimulatory molecules are broadly divided into two groups based on their respective homologies to founding members. The CD28/B7 family includes CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4), and binds to its ligands CD80 and CD86 (3). The tumor necrosis factor (TNF) receptor (TNFR) family, of which CD40 is a member, also includes TNFR superfamily member 4 (OX40); TNFR superfamily member 13c (BAFF-R); TNFR superfamily member 13b; (TACI), TNFR superfamily member 17 (BCMA); and TNFR superfamily member 11a (RANK) (4). Costimulatory molecules can be further subdivided based on function. For example, molecules containing positive costimulatory function can be grouped together, such as CD40L to CD40 binding, or negative costimulatory function, i.e. CTLA-4 binding CD80/CD86 (5).

The costimulatory receptor CD40 is a 48-kDa type I transmembrane protein and contains a 193 amino acid (aa) extracellular domain, 21 aa leader sequence, 22 aa transmembrane domain, and a 62 aa intracellular domain in human (90 aa in mouse) (6). In the extracellular domain of CD40, there are 22 cysteine residues that are conserved between the members of the TNFR superfamily (6). In regard to expression pattern, CD40 was initially characterized on B cells and is also expressed on dendritic cells (DCs), monocytes, platelets, and macrophages as well as by non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells (7–9).

The ligand of CD40, known as CD154 or CD40L, is a type II transmembrane protein, with a variable molecular weight between 32 and 39 kDa because of post-translation modifications (6). A soluble form of CD40L has been reported that expresses activities similar to the transmembrane form (10, 11). CD40L is a member of the TNF superfamily and is characterized by a sandwich extracellular structure that is composed of a β-sheet, α-helix loop, and a β-sheet (12). This structure allows for the trimerization of CD40L, which is also a feature of the TNF family of ligands (12). CD40L is expressed primarily by activated T cells, as well as activated B cells and platelets; and under inflammatory conditions is also induced on monocytic cells, natural killer cells, mast cells, and basophils (13). The wide expression of this costimulatory pair indicates the pivotal roles they play in different cellular immune processes.

CD40L/CD40 interactions exert profound effects on DCs, B cells, and endothelial cells, among many cells of the hematopoietic and non-hematopoietic compartments. It has been demonstrated that CD40 engagement on the surface of DCs promotes their cytokine production, the induction of costimulatory molecules on their surface, and facilitates the cross-presentation of antigen (2). Overall, the impact of CD40 signaling 'licenses' DCs to mature and achieve all of the necessary characteristics to effectively trigger T-cell activation and differentiation. CD40 signaling of B cells promotes germinal center (GC) formation, immunoglobulin (Ig) isotype switching, somatic hypermutation (SHM) of the Ig to enhance affinity for antigen, and finally the formation of long-lived plasma cells and memory B cells (14). Moreover, it has been shown that the CD40 pathway is essential for the survival of many cell types including GC B cells, DCs, and endothelial cells under normal and inflammatory conditions (15). In a pathogenic setting, the deregulation of CD40 signaling has been observed in multiple autoimmunity diseases. Together, this breadth of functions for CD40 underline the importance this receptor contains during the generation of an acquired immune response (2).

In this review, we describe CD40 signaling and the pathways implicated in this process and discuss work detailing the role of CD40 signaling in both humoral and cellular immune responses. Lastly, we will describe how CD40 targeting has led to novel anti-tumor therapies.

TRAF-dependent and -independent CD40 signaling

The engagement of CD40 by CD40L promotes the clustering of CD40 and induces the recruitment of adapter proteins known TNFR-associated factors (TRAFs) to the cytoplasmic domain of CD40 (15). The TRAF proteins activate different signaling pathways including the canonical and non-canonical nuclear factor κB (NFκB)-signaling pathways, the mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), as well as the phospholipase Cγ (PLCγ) pathway (15). Recent evidence indicates that signaling may occur independent of the TRAF proteins, as well as with Janus family kinase 3 (Jak3), which was found to be able to bind directly to the cytoplasmic domain of CD40. Binding of Jak3 has been shown to induce the phosphorylation of signal transducer and activator of transcription 5 (STAT5) (16, 17). Together, these complex pathways elicit the essential signals mediated through CD40 to impart its diverse cellular processes (Fig. 1).

Perhaps the best-characterized signaling pathways initiated during CD40 activation are the canonical and non-canonical
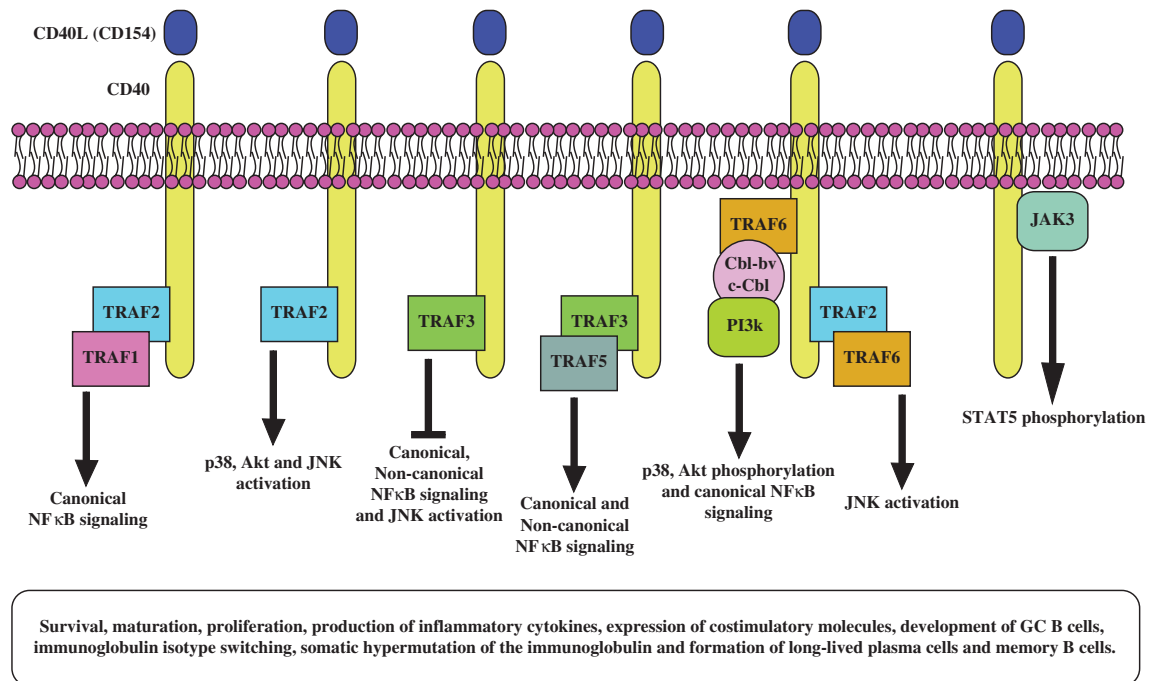
Fig. 1. CD40 signaling dependent and independent of tumor necrosis factor receptor-associated factor (TRAF) proteins. After CD40 activation, TRAFs 1, 2, 3, 5, and 6 are recruited to CD40 tail, driving different signaling pathways. Furthermore, Janus family kinase 3 can bind to the proximal cytoplasmic membrane of CD40. The signaling dependent or independent of TRAF protein regulates different cellular and immune processes.

NFκB pathways, which lie downstream of the recruitment of the TRAF proteins to the CD40 cytoplasmic domains. The canonical NFκB pathway begins with the formation of the inhibitor of κB (IκB) kinase (IKK) complex, containing the catalytic subunits IKKα, IKKβ, and the regulatory subunits IKKγ or NEMO. The activation of NFκB leads to the ubiquitin and proteasomal-dependent degradation of the IκB complex, inducing the translocation of NFκB heterodimers p50/p65 and p50/c-Rel to the nucleus. The non-canonical NFκB pathway is induced when the protein kinase NFκB-inducing kinase (NIK) activates IKKα, leading to a limited proteolysis of the precursor p100 that produces p52 protein, which associates with avian rel B and translocates into the nucleus. The result is that both signaling pathways can lead and regulate different target genes (18).

The TRAF family contains six members, designated TRAF1 through TRAF6, with all members driving cellular processes upon TNFR family engagement with their respective ligands. Characteristic of all TRAFs, members have a highly conserved carboxyl domain, which is called the TRAF domain. Upon ligand engagement, TRAF proteins are recruited to canonical aa motifs contained in the cytoplasmic domains of the TNFR family, with the TRAF domain engaging its cognate aa motif located in the TNFR cytoplasmic domains. TRAF proteins, with exception of TRAF1, contain an amino-terminal ring finger domain followed by five zinc fingers and a coiled-coil domain, with this structure called the zinc finger domain. The genetic ablation of the zinc finger domain generates a dominant negative TRAF protein that inhibits any induction of signaling mediated by the normal TRAF, demonstrating that this domain is essential for signaling (15).

Upon engagement with CD40L, CD40 directly or indirectly recruits TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 to its cytoplasmic domains. The canonical consensus binding sites for TRAF1, TRAF2, and TRAF3 are situated at the membrane distal domain of the intracellular tail of CD40 and is demarcated by the aa sequence PxQxT (19, 20). The insertion of mutations at the PxQxT domain disrupts the binding of TRAF2/3 to the CD40 cytoplasmic tail and dampens CD40 signaling by abrogating the NFκB, MAPK8 (Jnk), and p38 pathways during ligand engagement (21, 22). Our laboratory found a second, non-canonical binding site for TRAF2 at the carboxy-terminus of CD40 tail (23). Through this TRAF2 binding site, NFκB signaling is capable of being induced to control several B-cell functions (24). TRAF6 is recruited to the membrane proximal domain of CD40 to the consensus sequence QxPxEx (15). Through recruitment of TRAF proteins and their binding to different sites of CD40, TRAFs are essential in driving CD40-mediated signaling.

By elucidating the crystal structure of a portion of the CD40 cytoplasmic tail bound to TRAF2, McWhirter et al. (25) revealed the complexity behind the conformational changes

driven by CD40L engagement by revealing the oligomerization that occurs with CD40 in complex with TRAF2. Upon engagement with CD40L, a conformational change occurs in CD40 that exposes the docking site for TRAF2 at the canonical motif, and thus a trimeric TRAF2 is able to interact with three cytoplasmic tails of CD40 (25). In order to drive fulminant CD40 signaling, higher levels of oligomerization are required than trimerization, and it is likely that these different orders of oligomerization may result in differential recruitment of adapter and kinase proteins, particularly those that bind with low affinity to the cytoplasmic domains of CD40 such as TRAF1 or TRAF6 (26, 27). These differences in oligomerization likely account for the breadth of biologic activities induced by different monoclonal antibodies to CD40 (28).

TRAF1 protein expression is heightened during CD40 (29, 30). There is evidence demonstrating that TRAF1, as a result of the absence of a zinc finger domain, plays an important role in regulating the signaling of the other TRAF proteins (15). The TRAF1 binding site motif contained by CD40 overlaps with the binding sites for TRAF2 and TRAF3, and it appears that TRAF1 can bind weakly to the CD40 cytoplasmic domains in the absence of TRAF2 (20). The physiological implications of this observation are unclear. In APCs deficient in TRAF1, a reduction is observed in the recruitment of TRAF2 to the cytoplasmic domain of CD40, with a corresponding increase of TRAF2 degradation (31). These results were confirmed by Xie *et al.* (32) when it was demonstrated that CD40 stimulation of a B-cell line deficient in TRAF1 increases the amount of TRAF2 recruited to lipid rafts and increases the degradation of TRAF2 and TRAF3. In addition, data presented demonstrate that the recruitment of both TRAF1 and TRAF2 to CD40, during CD40L engagement, is required for activation of the canonical NFκB pathway. This was shown using B-cell line deficient for TRAF2 and TRAF1, which exhibit attenuated activation of canonical NFκB compared with single TRAF-knockout B cells (32). Together, these results suggest that TRAF1 can interact and regulate the recruitment and degradation of TRAF2. Moreover, both TRAF proteins can cooperate in the activation of the canonical NFκB pathway.

During CD40 engagement, the recruitment of TRAF2 to the tail of CD40 leads to the activation of the Jnk, p38, and thymoma viral proto-oncogene 1 (Akt) pathways. This was shown using TRAF2-deficient mouse embryonic fibroblasts (MEFs) and B cells, which exhibited attenuated activation of these pathways upon CD40 engagement (33–35). Similar results were observed in B cells transfected with a dominant negative TRAF2 (34). The engagement of CD40 induces the recruitment of TRAF2 with the kinase mitogen-activated protein kinase kinase kinase 1 (MEKK1) to CD40 tail. In experiments where CD40 was engaged on B cells lacking MEKK1, the activation of Jnk and p38 was blunted (36). MEKK1 drives the phosphorylation of Jnk and p38 and thus activates these respective pathways (36).

Despite its activating role in the aforementioned pathways, TRAF2 appears to be a negative regulator of the non-canonical NFκB pathway. The ablation of TRAF2 induces the accumulation of NIK in MEFs (37). Recent studies have elegantly demonstrated that TRAF2 cooperates with TRAF3 to constitutively degrade NIK, with engagement of another TNFR, BAFF-R, releasing this degradation and allowing for activation of the non-canonical NFκB pathway (38). It is clear that engagement of CD40 activates this pathway in a similar mechanism. Without CD40 stimulation, a complex is formed between family members of the cellular inhibitor of apoptosis (cIAP) 1 and 2, which interact with TRAF2, and which in turn interacts with TRAF3 and NIK (*Fig.* 2). Under these conditions, cIAP1/2 induces the degradation of NIK and antagonizes the non-canonical NFκB pathway (39, 40). In contrast, after CD40/CD40L engagement, the complex is destabilized, leading the recruitment of TRAF2 and TRAF3 to the cytoplasmic domains of CD40, with activation of the non-canonical NFκB pathway resulting from the subsequent accumulation of NIK as a result of TRAF2 and TRAF3 degradation following recruitment to CD40 (15). The degradation of TRAF2 is the result of self-degradation, because of its E3-ubiquitin ligase property, whereas TRAF3 degradation is dependent of cIAP1/cIAP2 (40, 41). Together, these results show that cIAP1/2 has a dual role in CD40 signaling, that in a steady state, cIAP1/2 inhibits the non-canonical NFκB signaling by degradation of NIK, whereas upon CD40 activation, cIAP1/2 promotes TRAF3 degradation, which releases NIK and promotes activation of the alternative NFκB pathway.

Initial descriptions of the role TRAF3 plays in CD40 signaling proposed that it functioned as a negative regulator of the canonical NFκB pathway (42). This hypothesis was based on experiments where a TRAF3-dominant negative protein expressed in B cells leads induction of the canonical NFκB and Jnk signaling pathways upon CD40 engagement (42, 43). These results were corroborated in experiments where CD40 signaling was studied in TRAF3-deficient B cells; it was also observed that Jnk phosphorylation and an accumulation of NIK was enhanced, as well as the recruitment of TRAF2 to lipid rafts suggesting that TRAF3 regulates TRAF2 and NIK, as was discussed above (39, 40, 43). In contrast, it was observed in epithelial cells that the enforced overexpression of TRAF3 induces canonical NFκB signaling during CD40 stimulation,
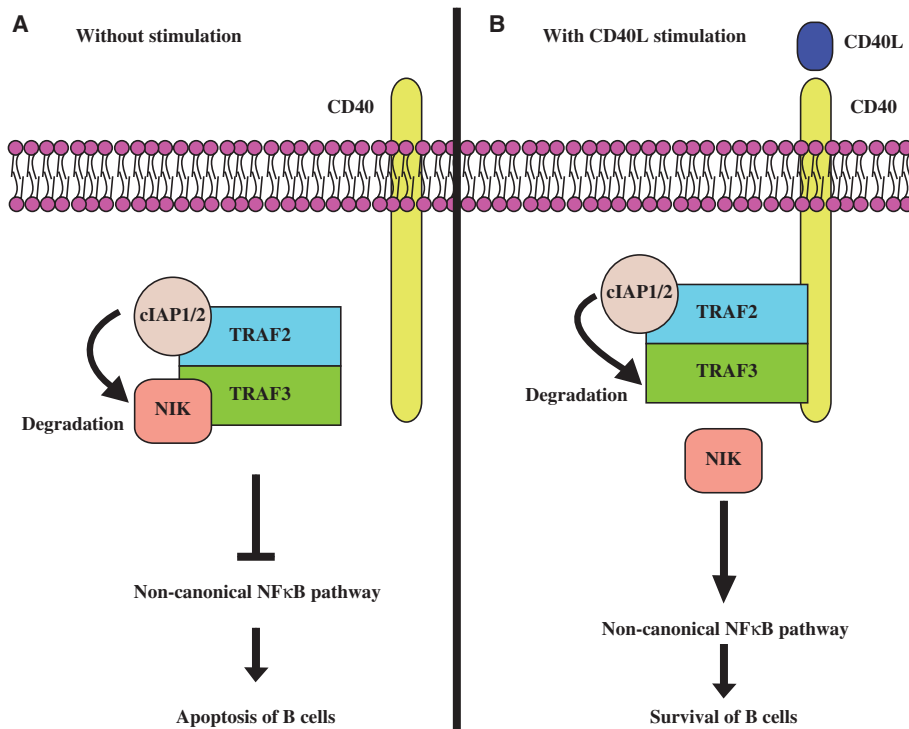
Fig. 2. Role of tumor necrosis factor receptor-associated factors (TRAFs) 2 and 3 in the inhibition of non-canonical nuclear factor κB (NFκB) pathway. Under non-stimulation, TRAF2 and TRAF3 form a complex with cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2) and NFκB-inducing kinase (NIK). cIAP1/2 degrades NIK, ablating the non-canonical NFκB pathway (39, 40). After CD40/CD40L engagement, the complex is destabilized, permitting the release of NIK from the complex inducing the non-canonical NFκB signaling. Furthermore, it induced the recruitment of TRAF2 and TRAF3 to CD40 tail and the degradation of TRAF3 by cIAP1/2 proteins.

suggesting that TRAF3 has different roles in different types of cells (44, 45). Therefore, in B cells, TRAF3 negatively regulates canonical and non-canonical NFκB pathways, as well as Jnk signaling; whereas in epithelial cells, it is able to induce canonical NFκB signaling. Additional experiments are necessary to determine the role of TRAF3 in non-canonical NFκB and Jnk signaling in epithelial cells.

Very little is known about the role of TRAF5 in CD40 signaling although TRAF5 is incapable to bind directly to CD40 cytoplasmic domains. During CD40 signaling, TRAF5 forms heterotrimers with TRAF3 (15). The role of TRAF5 signaling, during CD40 stimulation, was shown in B cells treated with small interfering RNAs (siRNAs) specific for TRAF5 or deficient for TRAF5 where canonical and non-canonical NFκB pathways were ablated. That is traduced in a reduction of the antibody production, proliferation, costimulatory molecules expression (46, 47). Further experiments are necessary to fully understand TRAF5 function in CD40 signaling.

Like other TRAF proteins, the physical interaction of TRAF6 with CD40 was demonstrated by the yeast two-hybrid assay (15). In TRAF6-deficient MEFs or epithelial cells where TRAF6 has been silenced by siRNA, these cells exhibited a reduction or abrogation in the activation of canonical NFκB, Jnk, p38,

and Akt, upon CD40 engagement. Interestingly, TRAF6 is able to interact with TRAF2 and thus regulate in a positive way CD40 signaling (21). Recently, Rowland *et al.* (48) have shown that in B cells expressing CD40 void of a TRAF6 binding site, TRAF6 was still capable of interacting with TRAF2. This suggests that TRAF6 may still have a functional role in CD40 signaling, without binding directly to CD40. Under these conditions, CD40 stimulation was capable of enhancing CD80 upregulation and inducing Jnk activation in comparison to B cells deficient in TRAF6. These data implicate TRAF6 as regulating some of its physiological functions through its interactions with TRAF2 and independent of its recruitment to the TRAF6 binding site of CD40 (48).

CD40 can induce the recruitment of TRAF6/Casitas B-lineage lymphoma b (Cbl-b)/Casitas B-lineage lymphoma (c-Cbl)/PI3K complex, which leads to Akt phosphorylation. The members of Cbl family are adapter molecules, which have E3-ubiquiting ligase properties (49). Depending on the cell type, they regulate positively or negatively a wide range of kinase and adapter proteins such as PI3K, Rous sarcoma oncogene (Src) tyrosine kinases, spleen tyrosine kinase, growth factor receptor bound protein 2, and Src homology 2 domain-containing transforming protein C1 (49). In studies using

Cbl-b-deficient DCs stimulated *in vitro* with CD40L, an ablation was observed in Akt activation without effect in the induction of the canonical NFκB pathway. These findings suggest that in DCs, the Akt activation is independent of NFκB signaling (49). As mentioned above, Cbl-b induces Akt phosphorylation through PI3K, and after the CD40 engagement, the blocking of PI3K abrogates Akt activation, demonstrating that in DCs, the Cbl-b activate PI3K, which induce the phosphorylation of Akt during CD40 signaling (50).

Physiologically, the blocking of PI3K drastically affects the survival of DCs (51). The mechanism that involves the anti-apoptotic effect of PI3K/Akt activation is dependent on the inhibition of the pro-apoptotic proteins, caspase 9, B-cell leukemia (Bcl)/lymphoma 2-associated agonist of cell death (50, 52, 53). Recently, a second mechanism was determined; PI3K can activate the mammalian target of rapamycin, which induces the expression of the anti-apoptotic protein caspase 8 and Fas-associated via death domain-like apoptosis regulator p43 (cFLIPp43) (50). Therefore, CD40, probably through TRAF6, induces the activation of PI3K signaling that protects the cell from apoptosis.

In contrast to the result described above, B cells deficient in Cbl-b have an increase of NFκB and Jnk pathways, suggesting that in B cells Cbl-b is a negative regulator of these signaling pathways (54). In this case, Cbl-b is recruited through TRAF2 to cytoplasmic tail of CD40 and the lack of Cbl-b increase TRAF2 recruitment to CD40, showing a negative role of Cbl-b in the recruitment of TRAF2 (54). Together, these results propose that in different cell types, Cbl-b can regulate different pathways dependent on which TRAF proteins are interacting.

Most of the studies investigating CD40 signaling demonstrate pathways dependent on TRAF proteins. However, it was observed in B cells, the membrane proximal region of the CD40 tail contains a binding domain for Jak3 (55). Revy *et al.* (56) demonstrated that in B cells, upon CD40 stimulation, Jak3 is not phosphorylated, suggesting that the recruitment of Jak3 to the cytoplasmic membrane of CD40 is not functional. However, monocytes are able to induce Jak3 phosphorylation after CD40 engagement (56). Furthermore, the inhibition of Jak3 in APCs, upon CD40 stimulation, blocks the maturation of APCs (16, 17). Additionally, Jak3 induces the transcription factor STAT5, which can dimerize and translocate to the nucleus, and leads the gene expression of the inflammatory cytokines, such as TNFα, interferon-γ (IFN-γ), and interleukin-6 (IL-6) (57). These results suggest that Jak3 is able to bind to CD40 and induce the maturation of DCs through STAT5.

In conclusion, CD40 principally mediates signaling through TRAF proteins, which can activate or inhibit different signaling pathways, dependent upon the cell type. TRAF proteins drive a wide range of cellular and immune processes and understanding these pathways will improve our ability to develop strategies to therapeutically intervene in a vast array of immune related diseases.

CD40 and humoral immunity

During initiation of a thymus-dependent (TD) humoral immune response, CD40 signaling by B cells is required for the generation of high titers of isotype-switched, high affinity antibody and for the development of humoral immune memory. The engagement of CD40 expressed on the surface of antigen-activated B cells by CD40L expressed on activated CD4^{+} T cells is essential for the initiation and progression of a TD humoral immune response. CD40 engagement triggers B-cell intercellular adhesion, sustained proliferation, expansion, differentiation, and antibody isotype switching *in vitro* (58–61). *In vivo*, CD40 engagement is required for GC formation and progression, as well as antibody isotype switching and affinity maturation, with these processes essential for the generation of memory B cells and long-lived plasma cells (62–64). The blockade of CD40/CD40L interactions through the use of blocking antibodies, genetic ablation of CD40L, or genetic ablation of CD40 completely abolishes TD humoral immunity (58).

Identification of CD40/CD40L as essential for TD humoral immunity

Historically, studies in regulation TD humoral immunity have detailed an essential role for the physical collaboration between T cells and B cells for an effective TD humoral immune response to be generated (65). This cellular collaboration is manifest as a physical interaction between antigen-activated B cells presenting antigen-derived peptides in the context of MHC class II to cognate CD4^{+} T-helper cells (Th cells) specific for the class II-restricted peptides being presented (66, 67). As a result of this immunological synapse, the Th cell provides the B cell with contact-dependent and -independent stimuli. Initially, it was thought that local release of cytokines was responsible for the ability of T cells to induce B-cell growth and differentiation. However, cytokines such as IL-4, IL-5, IL-2, IL-10, and IFN-γ alone or in combination were unable to reconstitute the abilities of T cells to stimulate sustained B-cell growth and differentiation (58, 68–71). Based on these findings, it was predicted that further cell-contact mediated mechanisms of T/B cell crosstalk existed (72). This prediction was supported by the observation that plasma membrane fractions from activated Th cells were

capable of driving B cells into cell cycle, with this induction occurring independent of cytokines (73–75). The molecule through which B cells receive this contact-dependent stimulus from T cells was identified as CD40 through experiments where agonistic monoclonal antibodies raised against CD40 were found to drive B cells into cell cycle and by using a CD40-Ig fusion protein to antagonize the ability of plasma membrane fractions to activate B cells in vitro (60, 76). By using the CD40-Ig fusion protein, CD40L expressed by activated Th cells was identified, isolated, and cloned (76, 77). Furthermore, a monoclonal antibody was raised against CD40L that antagonized the interactions of CD40 with CD40L both in vitro and in vivo (64, 76). This antibody (clone MR1) has proven to be invaluable in studies determining the role of CD40/CD40L in the regulation of humoral immunity.

The in vivo role of CD40/CD40L interactions during a humoral immune response

During infection with a pathogen, either whole pathogen or its particulates enter the lymph and the proximal draining lymphoid organs or, in the case of systemic infection, enter the spleen through the blood. Direct encounter of cognate antigen by naive follicular (NF) B cells residing in the B-cell follicles can occur. Alternatively, antigen can be acquired by tissue-resident DCs in the periphery that then home to the secondary lymphoid organs and deliver native antigen to cognate NF B cells (78–80). Lymphoid-homing DCs also present processed antigen in the context of MHC class II to Th cells in the T-cell zones of the secondary lymphoid organs, leading to the clonal activation of antigen-specific T cells. CD40/CD40L interactions between antigen-presenting DCs and Th cells are essential for the maturation and survival of DCs. In turn, the CD40-dependent maturation of DCs leads to the sustained expansion and differentiation of antigen-specific T cells.

The response of NF B cells to TD antigen and T-cell help has been well defined as a sequence of events that are temporally ordered. B-cell activation as defined by the induction of a litany of cell surface molecules can be readily observed hours after TD activation. Shortly thereafter, entry into the cell cycle can be documented. These early proliferating cells differentiate into plasmablasts secreting germline-encoded IgM and then IgG, with these plasmablasts located outside of the B-cell follicles (days 2–12 postimmunization). Activated B cells also seed secondary follicles and rapidly proliferate and interact with Th cells in a GC reaction, where isotype switching and SHM of the BCR occurs (days 9–20). GC B cells further differentiate into long-lived plasma cells and memory B cells

containing high-affinity BCRs of the switched isotypes (days 20 +) (81–83).

It is clear that CD40L plays a critical role in controlling B-cell fate during many of the aforementioned checkpoints in B-cell growth and differentiation. When Th cells are activated by DCs within the T-cell zone during a primary TD response, they transiently express CD40L (84). These activated Th cells then home toward the B-cell follicles and position themselves at the border of the B-cell follicles and T-cell zone where they encounter activated and cognate B cells, with CD40/CD40L interactions occurring between the two cell types (85). Th cells expressing CD40L are detectable at high frequencies by days 2–5 after immunization and co-localize with plasmablasts secreting antibody specific for the immunizing antigen in the T-cell zone (84). Th cells expressing CD40L are essential in activating NF B cells to differentiate into plasmablasts as blockade of CD40/CD40L interactions between responding Th cells and cognate B cells completely ablates the plasmablast response (64). In contrast to the TD immune response, abrogation of the CD40/CD40L pathway has no impact on the thymus-independent (TI) antibody response. When mice are immunized against the TI-type II antigen TNP-Ficoll, no difference in the strength of the humoral immune response is seen between mice treated either with anti-CD40L antibody or with control antibody (64). Similar results were observed in studies comparing the ability of CD40L knockout mice to mount an immune response to TNP-Ficoll in comparison to wildtype mice (86). In summation, CD40 signaling in antigen-responding B cells is essential for their differentiation into plasmablasts in response to a TD antigen.

CD40/CD40L interactions in the initiation and progression of the GC

In parallel to the plasmablast response, a subset of oligoclonal antigen-responding NF B cells which have been similarly activated in the T-cell zones colonize the B-cell follicles where they form GC structures (81, 83). The fate of whether a responding B cell differentiates into a plasmablast or whether it seeds a GC is dictated by a number of B-cell intrinsic or extrinsic factors. The intrinsic factors include the germline-encoded affinity of the B cell for antigen, with this affinity exerting a tremendous impact on B-cell fate. That is, high initial affinity BCR tends to drive the B cells into the plasmablast lineage, whereas moderate affinity imprints the B cells to seed the GC (87–90). Extensive triggering via CD40/CD40L is an example of a cell extrinsic factor that modifies B-cell fate. There clearly is a range of CD40 signaling that is 'acceptable'

in inducing a GC response. In the absence of CD40 stimulation, no GC response is observed. As CD40L signaling is provided, a GC response initiates and matures. However, in the presence of enhanced CD40 stimuli during a primary immune response, either with agonistic anti-CD40 antibodies or elevated T-cell help, B cells are selectively triggered to differentiate down the plasmablast lineage and divert from a GC response (91). In this situation of agonistic anti-CD40, the antigen-responding B cells uniformly differentiate into plasmablasts localized outside of the B-cell follicles, and no GC is formed. A similar scenario, albeit to a less severe degree, occurs when the numbers of activated, cognate T cells are enhanced by an order of magnitude. After priming with the carrier protein keyhole limpet hemocyanin (KLH) 7 days prior to immunization with the hapten/carrier (4-hydroxy-3-nitrophenyl) acetyl conjugated to KLH to simulate enhanced T-cell help, responding B cells are again shuttled from seeding a GC and instead differentiate into plasmablasts; although in this case GCs do appear (91). It is clear that the strength and duration of a CD40 signal, while required for the onset of humoral immunity, also has a substantial impact on the fate of antigen-responding B cells (91).

Once committed to the GC lineage, B cells encounter a specialized subset of activated Th cells called T follicular helper cells (T_{FH} cells), that provide the necessary cytokine and cell-contact signals, including CD40L, required to sustain the GC reaction (92–94). By means yet unclear, after T/B-cell encounter at the edges of the periarteriolar lymphoid sheath and B-cell follicles, a subset of activated Th cells further differentiate into T_{FH} cells and are licensed to enter the B-cell follicles by the heightened expression of the chemokine receptor chemokine (C-X-C motif) receptor 5 (CXCR5) (93, 95). The chemokine ligand for CXCR5 is chemokine (C-X-C motif) ligand 13 (CXCL13), and this chemokine coordinates the positioning and structure of the B-cell follicles as mature B cells, which express CXCR5 (96–100). T_{FH} cells are characterized by their expression of CD40L, ICOS, CXCR5 and the secretion of the cytokine IL-21, which stimulates B-cell proliferation, isotype switching, and differentiation into plasma cells (93). CD40L has been shown to be critical for T_{FH} cells function.

As the GC reaction matures, the structure becomes polarized into light and dark zones, with each zone situated to play a role in GC B-cell competition for antigen and T-cell help (83). The GC dark zone is so named because of the presence of a high density of rapidly dividing B cells, called GC centroblasts, whose large nuclei project a dark appearance during histological analysis. It is at the GC dark zone that SHM occurs (101).

The GC dark zone is maintained by the chemokine, chemokine (C-X-C motif) ligand 12 (CXCL12), with this chemokine secreted by the resident stromal cells (102). Accordingly, the receptor for this chemokine, chemokine (C-X-C motif) receptor 4 (CXCR4), is expressed on GC centroblasts (102, 103). The GC light zone is situated at the pole of antigen entry, and within this zone resides follicular dendritic cells (FDCs) on which antigen complexes are tethered by binding complement receptors CD21 and CD35 and the FcγRIIb (104). At the GC light zone, FDCs and light zone stromal cells secrete CXCL13 and it is here that T_{FH} cells are found (102, 103). GC B cells residing in the light zone are called centrocytes and are characterized by the expression of CXCR5 (102). Within the GC light zone, the interactions between centrocytes, T_{FH} cells, and complexed antigen occur in a critical competition and result in the selection of centrocytes exhibiting the highest affinities for antigen, as they receive survival and differentiation signals through their BCR and through CD40 engagement. It should be noted that the role of immune complexes on FDC in affinity maturation has been brought into question by Shlomchik and coworkers (105).

GC B cells expressing BCRs exhibiting inferior affinities for antigen are deleted by Fas-dependent apoptosis as they do not receive sufficient BCR and CD40 survival signals. The engagement of CD40 by a GC centrocyte during a productive immune synapse with a T_{FH} cell induces cellular survival. This was initially shown in studies observing that GC B cells could be rescued *in vitro* with the administration of agonistic anti-CD40 antibody or CD40L (106, 107). The engagement of CD40 by centrocytes protects the cell from undergoing Fas-mediated apoptosis by inducing the anti-apoptotic proteins Bcl/B-cell lymphoma x (Bcl-XL) and c-FLIP (108–110). It is clear that T_{FH} cells provide centrocytes with signals in addition to CD40L in order to propagate survival and differentiation to memory and plasma cells, with IL-21 occupying such a role in PC differentiation (93). Classic *in vitro* studies by MacLennan and coworkers (106) observed that centrocytes are induced to differentiate into memory B cells upon culture with activated memory Th cells but not activated naive Th cells, indicating that further signals may exist (106). How these other signals, which are derived from T_{FH} impact centrocyte differentiation into memory B cells and PCs are in the process of being elucidated.

CD40 signaling from activated T cells at the inductive phase of the GC response as well as from T_{FH} cells throughout the duration of the GC response is essential for the completion of the GC response. Blockade of CD40/CD40L interactions or genetic ablation of the CD40L or CD40 gene prevents the

clonal proliferation of antigen-responding B cells as well as blocks the generation of the GC structure by preventing these cells from receiving signals from activated Th cells through CD40 (63, 64, 86). As such, if anti-CD40L antibody is injected at the peak of the GC response, the GC is immediately shut down (111). After anti-CD40L antibody administration, the Ig affinity of the plasma cells in the bone marrow was decreased in comparison to control antibody-treated mice, supporting the hypotheses that (i) affinity maturation occurs in the GC and progresses over time, and (ii) CD40/CD40L interactions are essential for this progression. Together, these data indicate that CD40 signaling initiates and propagates the GC, and that abrogating this reaction through CD40 block prevents the full benefits of the GC, as measured by affinity maturation.

The contributions of TRAF recruitment during CD40 signaling on B-cell fate

Early events in the CD40 signaling cascade control the fate of antigen-specific B cells. Upon CD40 multimerization by CD40L, TRAFs are recruited to the CD40 cytoplasmic tail as discussed in detail earlier in this review. The respective contributions of TRAFs 1, 2, 3, 5, and 6 recruitment to CD40 in regulating humoral immunity was studied in vivo by generating transgenic mice containing CD40 receptors with mutations in defined CD40 binding sites for TRAFs (24, 112–114). Upon CD40L binding to CD40, TRAF2 and TRAF3 directly bind to the cytoplasmic tail of CD40. TRAF1 and TRAF 5 are also recruited to CD40 through indirect associations with CD40-bound TRAFs (20, 115, 116). Through disruption of TRAF6-binding site within the CD40 cytoplasmic domain, we selectively ablated affinity maturation and the generation of plasma cells after immunization. Mutagenesis of both the TRAF6 and TRAF2–TRAF3 sites was essential for arresting GC formation in response to immunization. CD40-induced B-cell proliferation and early Ig production occurred even when all TRAF sites were ablated. These studies demonstrated that specific CD40–TRAF associations control well-defined aspects of humoral immunity (112). The fact that the B-cell proliferative response was intact in mice where both the TRAF2–TRAF3 and TRAF6 sites were ablated suggested another functional site in the CD40 cytoplasmic tail. Indeed, our laboratory identified a second non-canonical TRAF2 binding motif in the C-terminus of CD40 (23, 24). All early B-cell activation events, including plasmablast generation are intact in the transgenic mice that express only the c-terminal TRAF2 binding motif in the CD40 tail. When immunized with T-dependent antigen,

the mice are still impaired to develop GC demonstrating that either or both, TRAF3 and TRAF6 are necessary to form GC structures (23, 24). In summary, TRAFs are essential intermediaries in CD40-induced humoral immunity.

Perhaps the most striking example of how essential CD40/CD40L interacts are in generating humoral immunity is the clinical manifestations that arise in humans carrying mutations in the CD40L gene which generate non-functional CD40L. This syndrome is X-linked hyper-IgM syndrome and is characterized by reduced levels of serum IgG, IgA, and IgE and increased levels of IgM (117). Because of inactivation of functional CD40L, TD immune responses in these patients are severely impaired, including the generation of GCs, memory B cells, and isotype-switched plasma cells secreting protective antibody. Patients with CD40L deficiency are thus susceptible to life-threatening bacterial infections. Thus, the importance of CD40/CD40L interactions in controlling humoral immunity has been displayed in an unfortunate clinical presentation.

The role of CD40/CD40L in cell-mediated immunity

There is little doubt as to the essential role of CD40/CD40L in the development of TD humoral immunity. While initially thought to be only involved in regulating TD immunity, CD40/CD40L is also critical in the development of cell-mediated immunity (CMI). There is clear and unquestionable evidence that the development of some CMI responses require CD40/CD40L (118). This is most evident in the role of this ligand–receptor pair in the development of cytotoxic T cells to tumors, virus, and alloantigens (119, 120) and its role in the development of a litany of T-cell-dependent autoimmune diseases (121). In this review, we discuss its role in regulating allograft tolerance and rejection, as a model that clearly delineates the role of this ligand–receptor pair in controlling critical checkpoints in the development of CMI.

One of the first pathways to be targeted in an attempt to modify allograft rejection was the CD28/B7 pathway. It was demonstrated that by using a CTLA-4-Ig (a soluble form of the high-affinity receptor to B7 molecules), long-term allograft survival of islet cells (122, 123) and cardiac allografts (124) could be achieved. By competitively binding to B7 molecules, CTLA-4-Ig blocked CD28 signaling to the T cells, therefore limiting activation of the alloreactive T-cell compartment. Other means by which to disrupt T-cell activation have been through the blockade of the CD40/CD40L pathway. Originally, it was thought that anti-CD40L antibody monotherapy would be effective because it limited APC

maturation and downmodulated the B7–CD28 interaction, resulting in the lack of a signal 2 and T-cell anergy (125, 126). However, anti-CD40L antibody as a monotherapy has not been successful in inducing allograft tolerance (127, 128). Studies by Waldmann and coworkers (129) indicate that failure to induce tolerance using antibody anti-CD40L therapy is insufficient because of its inability to block rejection elicited by CD8^{+} T cells. Highly immunogenic allografts, such as those of heart and skin, require stronger tolerogenic therapies. Among these, the combination of costimulatory blockade with immunosuppressive drugs (rapamycin with antibody anti-CD40L and CTLA-4-Ig) has been shown to result in long-term graft acceptance (130). Interestingly, the effect of cyclosporine A impedes the tolerogenic effects of anti-CD40L antibody and CTLA-4-Ig. The explanation for this resides in the ability of cyclosporine to block IL-2 production and likely the expansion of regulatory T cells (131).

Given the failures of anti-CD40L antibody monotherapy in many graft systems, we asked if we could pretolerize a host to allow that host to accept an allograft. The specific system that we and others (132–137) have exploited to understand the role of CD40L in the regulation of graft rejection involves the pretolerization of the host with alloantigen (donor-specific transfusion, DST) and anti-CD40L antibody, followed by grafting. In this system, donor alloantigen is provided by an intravenous infusion of T-depleted donor splenocytes or heparinized whole blood. When used in combination with anti-CD40L antibody, this has been shown to induce allospecific T cell tolerance, as measured by suppression of both CD8^{+} and CD4^{+} alloreactive T cells (127–129, 138) and enhanced persistence of allografts of skin, kidney, pancreatic islets, heart, and lung (132–137).

The underlying cellular and molecular mechanisms that account for the DST and anti-CD40L antibody T-cell tolerance have been studied (135). Studies into this phenomenon have provided insights into the process of allospecific tolerance, peripheral tolerance, and the role of regulatory T cells in the survival of allografts. Blockade of CD40L by anti-CD40L antibody impairs the maturation of the host DCs (upregulation of CD80/86) and incapacitates the abilities of these DCs from inducing productive T-cell activation. As such, the alloantigen-presenting, non-matured DCs induce abortive activation and anergy of the host alloreactive T-cell pool. Among the most relevant findings were that indirect presentation of alloantigen was essential for tolerance induction. Thus, the infused DST was rapidly processed by host APCs and presented via indirect presentation to the host alloreactive T cells in a tolerogenic manner because of the CD40L blockade. The tolerogenic impact of DST and anti-CD40L antibody in this system was to induce a rapid (days 3–4), systemic, abortive expansion of the alloreactive T cells that resulted in profound anergy. One additional unique feature of this system was that we could quantify the magnitude of the T-cell unresponsiveness by purifying anergic cells. In these cases, the unresponsiveness was determined to be >95% on a per-cell basis. Hence, infusion of DST and anti-CD40L antibody resulted in a pre-emptive induction of tolerance within the alloreactive T-cell compartment, thereby silencing the alloreactive response days prior to the time when the allograft had the opportunity to elicit an immune response (135).

We now envision that the infused DST rapidly undergoes apoptosis and is presented by host APCs. It is of interest to note that anti-CD40L antibody may facilitate the apoptosis of the DST by depriving it of a CD40 signal. At the same time, this blocking antibody impairs the maturation of host APCs, committing them to the tolerogenic presentation of DST-derived allopeptides. Delivery of peptides via apoptotic cells appears to be an extremely efficient means to induce peripheral tolerance. In analogous studies, Steinman and coworkers have shown that B cells deficient in transporter associated with antigen processing 1 protein and hyperosmotically loaded with OVA can induce abortive expansion and anergy of OVA-specific cytolytic T lymphocytes (CTLs) in vivo via indirect presentation (139). Similarly, antigens expressed on dying pancreatic cells (140) induce tolerance via indirect presentation. More recently, antigens targeted directly to defined DC surface molecules (such as DEC-205) and antigens delivered to immature DCs have been shown to induce profound antigen-specific tolerance, as predicted by these earlier studies.

CD40-dependent maturation of DCs is far more complex than simply the upregulation of costimulatory molecules. As a consequence of CD40 signaling of DCs, there is heightened expression and increased stability of the MHC/alloantigen complex (141, 142). Furthermore, increased life span of the DC is considered a major factor in the success of CD40-triggered DCs in driving CMI. CD40-mediated survival is based on the upregulation of Bcl-XL, an anti-apoptotic factor and is NFκB dependent (143, 144). Passive apoptosis of T cells induced by the absence of growth/survival factors, such as IL-2, IL-7, and IL-15 imposed by CD40L blockade also can impact on T cell immunity (145–147).

Central to the induction of alloreactive T-cell tolerance by many interventions involving costimulatory blockade is the induction of regulatory T cells (148). The functional importance of this population has been repeatedly demonstrated by

graft loss resulting from anti-CD25 antibody treatment and the observations of overt infiltration of regulatory T cells into the graft observed by immunohistochemical analysis (135, 149). By using forkhead box p3 (Foxp3)-expressing T cells (150) or Foxp3-green fluorescent protein positive T cells (Victor C. de Vries, Randolph J. Noelle, unpublished observation), it was confirmed that the tolerance can be transferred by just the regulatory T cells. However, one cannot discount the massive reduction of alloreactive effector T cells by anti-CD40L antibody and DST in facilitating graft survival. As anticipated, it has been shown that the maturation status of DCs regulates the generation of antigen-specific regulatory T cells. Likewise, strong costimulation by DCs after treatment with agonistic CD40 and/or lipopolysaccharide diminished regulatory T cells expansion and, CD40-deficient DCs or blocking with anti-CD40L antibody greatly enhanced regulatory T cells development from naive TCR-transgenic T cells (151, 152) to regulatory T cells.

Combination therapies using CD40L blockade

From studies using anti-CD40L antibody, it is clear that although graft survival is significantly prolonged, indefinite tolerance is not achieved. In murine models, permanent allograft survival using anti-CD40L antibody and DST requires thymectomy (153). Over the years, additional strategies have been explored to prolong graft survival mediated by anti-CD40L antibody. Most approaches make use of blocking antibodies to other costimulatory molecules, such as CD28 and OX40, or agonistic antibodies for coinhibitory molecules, such as CTLA-4 (154). More recently, the roles of newer members in the costimulation family have been targeted in combination with anti-CD40L, such as PD1/PD-L1 and the ICOS/B7RP-1 (155–157). In addition, antibodies interfering with adhesion directly or indirectly, such as lymphocyte function-associated antigen-1 (LFA-1) and/or CD45RB have shown to be beneficial (158–163). Additionally, promising results come from studies targeting IL-2, IL-7, and IL-15 in combination with CD40L antibody (164–166) because it was shown that signaling through the common γ-chain was the reason why diabetic mice were resistant to CD40/CD28-induced tolerance (167).

Additive suppression by blocking other costimulatory pathways

The clonal deletion of alloreactive T cells can be accelerated in the absence of CD80/CD86 expression and in the absence of CD40L costimulation (147). Blocking of either pathway will lead to prolonged graft survival in some models. Anti-CD28

or anti-CD80/86 antibody is based on blocking costimulation. Although both approaches target the same pathway, the anti-CD28 antibody is mainly based on passive apoptosis of T cells, whereas the anti-CD80/86 antibody is based on actively inducing cell death. Therefore, CD40 resembles anti-CD28 blockade with combination therapies, showing variable results on graft survival (127, 150, 168–170). In addition to blocking costimulation, the engagement of CD80/CD86 by CTLA-4-Ig, has been shown to upregulate the expression of indole amine 2,3-dioxygenase (IDO), a process believed to normally occur by the interaction of regulatory T cells and DCs (171). IDO degrades tryptophan leading to local removal of this essential aa needed for T-cell proliferation (172). However, regulatory T cells may also mediate suppression by eliciting IDO from DCs (171).

Using double knockouts for CD28 and CD40L revealed that these mice could still reject allografts and it was shown that the OX40/OX40L pathway was critical in this process. The 4-1BB/4-1BBL, ICOS/B7RP-1 or CD27/CD70 pathways were not involved in facilitating graft rejection in the double-knockout mice (173). Later, it was shown that OX40 played an important role in rejection mediated by memory T cells (157). OX40 signaling, in addition to activating effector T cells also turns off the suppressive function of regulatory T cells (174). It must be noted that multiple publications have shown that blocking the OX40/OX40L pathway in the absence of CD40L leads to long-lasting pancreatic islet graft survival (175). The effect of OX40L blockade is mainly because of impairment of rejection, which is mediated by CD4$^+$ T cells and to a lesser extent to CD8$^+$ T cell (173, 176).

In conclusion, the blocking of CD40L leads to prolonged graft survival and seems to be synergistic when combined with most of the other members of both the TNF-family and B7-family of costimulatory molecules. Although it seems that CD40 and CD28 are the most prominent members in the regulation of peripheral tolerance, there are additive, but redundant roles of the other family members impacting specific subset of T cells involved in maintaining tolerance.

CD40-based immunotherapy in cancer

CD40/CD40L is critical for the development of protective anti-tumor immunity and CD40 can be a cancer-associated target in antibody-based therapies. In fact, CD40 was first discovered as an antigen expressed in bladder carcinoma and paralleled its identification as a receptor on B cells (13, 177–181).

CD40 expression in tumors

The broad range of expression of CD40 on normal healthy cells translates to its extensive expression on a variety of tumors. Reports have shown that CD40 is widely expressed on both murine and human melanoma, prostate, and lung cancers (182, 183), as well as in carcinomas of the nasopharynx, bladder, cervix, and ovary (184–187). CD40 expression has been reported on both non-Hodgkin's lymphomas (NHLs) and Hodgkin's lymphomas and on other hematologic malignancies, such as lymphocytic leukemia, lymphoma, multiple myeloma, and acute myeloid leukemia (188–191).

Direct effect of CD40/CD40L interaction on tumor cells

As in normal B cells, CD40 ligation in certain B-cell malignancies causes an increase in the expression of anti-apoptotic factors such as Bcl-XL, TNF α-induced protein 3 (A20), Bcl/B-cell lymphoma 2 related protein A1a (Bfl-1), survivin, and cFLIP. These factors protect the cell from apoptosis induced by apoptotic agent, such as, serum withdrawal, IgM, anti-Fas, TNF-related apoptosis-induced ligand (TRAIL) or DNA-damaging agents (109, 192–195). It has been suggested that low-level constitutive engagement of CD40 may facilitate malignant cell growth. Studies with non-Hodgkin's lymphoma, Burkitt's lymphoma, and chronic lymphocytic leukemia cells have shown that these cells express low levels of CD40L, and through and autocrine pathway sustain cell proliferation. In addition, the low-levels of CD40L expression protect these cells from apoptosis (196–198). Recently, human CD40-positive breast tumor biopsies were shown to co-express CD40L, and that co-expression confers oncogenic effects in vitro (177). Furthermore, the co-expression of CD40 and its ligand, in immortalized human epithelial cells induce an increase in their proliferation, motility, and invasion (177). These results suggest that neoplastic growth utilizes the CD40/CD40L pathway independent of the immune system to sustain proliferative capacity and survival. Furthermore, by expression of the receptor/ligand pair, tumors are able to manipulate both T-cell and APC compartments most likely contributing to the establishment of the immunosuppressive tumor microenvironment.

Conversely, transient activation of CD40 on carcinomas and some B-cell malignancies results in direct anti-proliferative effects and apoptosis. Both in vitro and in vivo treatment of Burkitt's lymphoma, primary high-grade B-cell lymphoma, and multiple myeloma cells with CD40L results in a reduction in the proliferation of these tumor cells (189, 199). Under CD40 stimulation, carcinoma cells can be induced to undergo apoptosis by the increased expression of Bcl2-associated X protein and the upregulation of membrane-bound cytotoxic ligands of the TNF family, such as FasL, TNF, and TRAIL (200, 201). Cell death induction seen by CD40L occurs even when protein synthesis is blocked, suggesting that the production of anti-apoptotic factors may override the cell death induction signal provided by CD40L (202, 203). CD40L triggering of bladder and ovarian carcinomas in vitro results in growth inhibition and enhanced susceptibility to apoptosis, induced by anti-neoplastic drugs, TNF-α, Fas, and ceramide (203). These observations were confirmed in an in vivo system, where CD40L alone or in combination with chemotherapy could significantly inhibit the growth of transplantable breast or ovarian tumors and confer overall survival (204, 205). Hence, CD40 activation can have a direct anti-tumor effect on several tumors, even in the absence of any additional immune responses and cells. The direct cytotoxic effect of CD40L on CD40-expressing tumors has been studied using immuno-compromised mice, where it was observed that inhibition of breast carcinoma and B-cell lymphoma growth (199, 200) can be observed in the absence of an intact immune system.

In summary, CD40 activation can induce the function of various downstream signaling pathways including both, pro-apoptotic and anti-apoptotic proteins. It is clear that the function of direct CD40 stimulation on malignancies is dependent on the state of differentiation of the stimulated cells and the type of malignancy, as reviewed extensively elsewhere (206).

CD40/CD40L is central to the development of protective anti-tumor immunity

CD40 is a potent stimulator of the immune system and, as such, CD40/CD40L interaction on immune cells has been extensively studied for its involvement in the development of protective anti-tumor immunity. The first report implicating CD40/CD40L interaction as a critical factor in the generation of protective immunity by means of tumor vaccines came from Mackey et al. (207). It was shown that anti-CD40L monoclonal antibody treatment inhibited the generation of protective immune responses and prevented the therapeutic value of potent tumor vaccines. These results were confirmed using CD40-deficient mice. These mice were unable to generate a protective anti-tumor immune response following a protective vaccination regime (207).

Cognizant of the fact that CD40L was critical for inducing protective tumor immunity, it was anticipated that agonistic

anti-CD40 antibodies would be powerful adjuvants for induce tumor immunity. Indeed, early studies showed that CD40 agonistic antibody generated CTL responses that were able to eradicate tumor in a lymphoma system. It was also seen that CD40 ligation could overcome peptide-induced peripheral CTL tolerance and increase anti-tumor vaccine efficacy (208–210). However, use of CD40 as a solitary agonist to induce immunity came into question.

Several reports have confirmed that CD40 stimulation can enhance anti-tumor immune responses by means of DC maturation (211). Activation of DCs with agonist of CD40 results in their increased survival, secretion of IL-1, IL-6, IL-8, IL-12, TNF-α, and macrophage inflammatory protein-1α. Additionally, CD40 activation induces the upregulation of costimulatory molecules such as MHC class II, LFA-3, CD80, and CD86 and promotes antigen presentation, priming and cross-priming of Th and CTL, respectively (206). However, the use of agonist anti-CD40 antibody alone accelerates the deletion of tumor-specific CTL in the absence of antigen vaccination in a mouse-melanoma model system (212). While immunotherapeutic regimes utilizing CD40-dependent IFN-responses lead to successful early anti-tumor efficacy, the treatment with CD40 agonist alone impairs the development of tumor-specific T cells (180). It is clear that robust development of adaptive immunity relies on the interplay of both innate and adaptive immunity, and therefore the contribution of Toll-like receptor (TLR) agonists to CD40-dependent stimulation of adaptive immunity was evaluated. Recently, our group showed that the combinatorial use of CD40 and TLR agonists induces profound frequencies of anti-tumor-specific T cells and provides therapeutic efficacy in a number of tumor models. An increase in the frequencies of tumor-reactive CD8^{+} T cells that efficiently infiltrate the tumor-burdened organ resulted in the generation of potent tumor-specific memory CD8^{+} T cells. These results suggest that future CD40-dependent anti-tumor approaches should encompass activators of both innate and adaptive immune systems (213).

Studies seeking to utilize CD40 ligation for tumor immunotherapy have been approached using gene delivery of the CD40L gene to DCs and tumor cells. Expression of CD40L in a small proportion of tumor cells was enough to generate a long-lasting systemic anti-tumor immune response in mice that was shown to be dependent in CTLs (214). Further, it was demonstrated that CD40 ligation can bypass the absolute requirement of CD4^{+} T cells during immunization with melanoma antigen gene-modified DCs (215). Other gene therapies have been successful in murine tumor models, e.g. *ex vivo* virus delivery to either DC or tumors by use of recom-

binant adenovirus expressing CD40L. This approach has proven effective in syngeneic tumor models of colorectal carcinoma, lung carcinoma and melanoma (216, 217). It is likely that CD40 monotherapy is effective in these models because the adenoviral delivery system provides the other necessary TLR signal to drive the development of therapeutic immunity.

The experience of targeting CD40 in human cancer clinical trials

To date, three main rationales have been used in the development of clinical phase I studies which target the CD40 pathway. First, that CD40 can act as a tumor-associated antigen to which a cytotoxic antibody can be targeted. Second, CD40 engagement by an antibody can transduce a pro-apoptotic signal to a tumor cell to facilitate its death. Third, an agonistic anti-CD40 antibody can enhance immunity to the tumor and facilitate the development of therapeutic immunity.

A summary of some of the CD40-directed clinical studies can be found in *Table* 1 where we list the three main approaches to CD40 targeting. These three are CD40L gene therapy, recombinant protein therapy, and anti-CD40 therapy (179, 181, 218–226). Most of the reported therapies are relatively well tolerated, although each treatment exhibits some adverse reactions.

The use of a human trimeric recombinant CD40L in patients with solid tumors was development based on the pro-apoptotic activities of this molecule on human tumors in recombination gene knockout mice (227). Patients with advanced solid tumors or intermediate- or high-grade NHL received CD40L subcutaneously daily for 5 days in a phase I dose-escalation study. Doses continued until disease progression was noted. CD40L induce liver toxicity noted by the increased levels of liver transaminases in the serum. Six percent of patients had a partial response with one patient having a complete response. Since the time of the study in 2001, no further studies have been reported (219).

Humanized or fully human monoclonal IgG antibodies have since been developed, such as, CP-870,893 (Pfizer Inc., New York, NY, USA), SGN-40 (Seattle Genetics Inc., Bothell, WA, USA), and HCD 122 (Novartis/XOMA Basel, Switzerland). For the Pfizer studies, patients in this phase I trial with advanced solid tumors received single doses of the CD40 agonistic antibody, CP-870,893 intravenously. Dose-limiting toxicity was observed with the most common adverse event being cytokine release syndrome including chills, rigors, and fever. 14% of all patients and 27% of melanoma patients had objective partial responses at restaging. CP-870,893 was well

Table 1. Human clinical trail therapy in cancer patients

Tumor	Treatment	Clinical observations	Mechanism	References
CD40L gene therapy B-CLL (n = 11)	CD40L-transfected leukemia cells, I.V. transfer	No sever adverse reaction Flu-like symptoms Reduced leukemic cell count (>50% at 2–4 weeks postinfusion and lymph node size (>90% reduction)	T-cell sensitization Agonist	Wierda et al. (221) Kipps et al. (222)
CD40L-expressing leukemia cells with IL-2-expressing fibroblasts (n = 10)	CD40L and IL-2 transduced leukemic blasts and skin fibroblasts, S.C. transfer	No severe adverse reaction 90% 5-year survival rate	Cytotoxic effector-cell activity Generation of IgG Ab Agonist	Rousseau et al. (226)
Recombinant protein therapy Solid carcinomas and NHL (n = 32)	Recombinant CD40L, S.C. injections, daily ×5	Some adverse reaction Injection site reaction Increased AST/ALT 38% Responders with stable disease 2 Partial responses	Undetermined Agonist	Vonderheide et al. (219) Younes et al. (220)
Anti-CD40 monoclonal antibody therapy Advanced solid tumors (n = 29)	CP-870,893 (human IgG2, Pfizer, New York, NY, USA), I.V. infusion	Some adverse reaction Cytokine release syndrome (chills, rigors, fever) 14% Partial responses of all patients and 27% in melanoma patients	Transient depletion of CD19+ B cells in blood Agonist	Vonderheide et al. (219)
NHL (n = 29)	SGN-40 (humanized IgGI, Seattle Genetics, Bothell, WA, USA), I.V. infusion	Some adverse reactions Cytokine release syndrome 14% Partial response and 3.5% complete response	Weak agonist	Forero-Torres et al. (218)
Multiple myeloma (n = 23)		Some adverse reaction Cytokine release syndrome 17% of patients had decreases in M-protein	CD19+ B cell decrease Weak agonist	Hussein et al. (225)
CLL and multiple myeloma (n = 24)	HCD 122 (human IgGI, Novartis/XOMA, Basel, Switzerland)	Some adverse reactions Transient infusion reactions 8% Partial response	Blocks CD40L-induced cell survival Mediates ADCC Antagonist	Byrd et al. (181) Bensinger et al. (179)

B-CLL, B and chronic lymphocytic leukemias; I.V., intravenous; S.C., subcutaneous; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NHL, non-Hodgkin's lymphoma, Ig G, immunoglobulin G; IL-2, interleukin-2.

tolerated and was shown to be biologically active, and was associated with anti-tumor activity. The authors concluded that further studies were warranted. While the presumed mechanism of active are still unresolved, the intent is that CP-870,893 will induce tumor immunity.

Dacetuzumab (SGN-40) is a weak agonist and is currently in multiple phase II trials in multiple myeloma and diffuse large-cell lymphoma. Seattle Genetics reported in December of 2008 that data from a phase II trial demonstrated objective responses at well-tolerated doses in heavily pretreated patients with diffuse large B-cell lymphoma. SGN-40 is a weak agonist and is believed to trigger apoptosis.

A phase I trial with HCD122, a CD40 antagonist, was well tolerated in patients with relapsed and refractory multiple myeloma. The majority of adverse events were mild to moderate in severity. HCD122 monotherapy achieved a partial response in one out of the six patients treated. HCD122 is believed to facilitate tumor apoptosis by depriving B-cell tumors of growth promotion via CD40 and mediating lysis by antibody-dependent cellular cytotoxicity (228). The most likely direction toward which CD40-directed tumor therapy should be moving is in combination with alternative or more established therapies, such as radiation, cancer vaccine, chemotherapy, anti-CTLA-4 antibody blocking, TLR agonists, and cytokines (205, 209, 210, 213, 229, 230).

In conclusion, the overall effect of CD40 therapy in the activation of the immune system and direct tumor cytotoxicity is most likely to act synergistically in the purpose of establishing an anti-tumor effect. Additionally, it is currently generally agreed upon that optimal DC activation depends on synergistic triggering of several 'danger' receptors by the administration of exogenous TLR ligands and CD40 agonists to elicit maximum anti-tumor T-cell response or therapeutic effect. Taking lessons from the last few decades of anti-cancer drug development, it is currently pretty well accepted that the cure for or treatment of tumors will have to be a combinatorial approach that acts both on the tumor itself, as well as on the level of immune response induction.

Conclusion

The wide-range expression of CD40 in normal as well as tumor cells shows the pivotal role that CD40 is playing in diverse cellular and immune processes. The understanding of CD40 signaling in a humoral and cellular context will address to improve therapies against pathogens and tumors. In contrast, the blocking of CD40/CD40L engagement will drive to generation of tolerance during transplant or in autoimmunity diseases.

References

1. Bretscher PA. A two-step, two-signal model for the primary activation of precursor helper T cells. Proc Natl Acad Sci USA 1999;**96**:185–190.
2. Quezada SA, et al. CD40/CD154 interactions at the interface of tolerance and immunity. Annu Rev Immunol 2004;**22**:307–328.
3. Bromley SK, et al. The immunological synapse and CD28-CD80 interactions. Nat Immunol 2001;**2**:1159–1166.
4. Au PY, Yeh WC. Physiological roles and mechanisms of signaling by TRAF2 and TRAF5. Adv Exp Med Biol 2007;**597**:32–47.
5. Linsley PS, et al. Immunosuppression *in vivo* by a soluble form of the CTLA-4 T-cell activation molecule. Science 1992;**257**:792–795.
6. van Kooten C, et al. CD40-CD40 ligand. J Leukoc Biol 2000;**67**:2–17.
7. Bancherau J, et al. Functional CD40 antigen on B cells, dendritic cells and fibroblasts. Adv Exp Med Biol 1995;**378**:79–83.
8. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8^{+} T cells in the generation of CD8^{+} T cell memory. Science 2002;**297**:2060–2063.
9. van Kooten C, Banchereau J. Functions of CD40 on B cells, dendritic cells and other cells. Curr Opin Immunol 1997;**9**:330–337.
10. Graf D, Muller S, Korthauer U, van Kooten C, Weise C, Kroczek RA. A soluble form of TRAP (CD40 ligand) is rapidly released after T-cell activation. Eur J Immunol 1995;**25**:1749–1754.
11. Mazzei GJ, et al. Recombinant soluble trimeric CD40 ligand is biologically active. J Biol Chem 1995;**270**:7025–7028.
12. Karpusas M, et al. 2 A crystal structure of an extracellular fragment of human CD40 ligand. Structure 1995;**3**:1031–1039.
13. Carbone E, et al. A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. J Environ Monit 1997;**185**:2053–2060.
14. Danese S, Sans M, Fiocchi C. The CD40/CD40L costimulatory pathway in inflammatory bowel disease. Gut 2004;**53**:1035–1043.
15. Bishop GA, Moore CR, Xie P, Stunz LL, Kraus ZJ. TRAF proteins in CD40 signaling. Adv Exp Med Biol 2007;**597**:131–151.
16. Saemann MD, et al. Prevention of CD40-triggered dendritic cell maturation and induction of T-cell hyporeactivity by targeting of Janus kinase 3. Am J Transplant 2003;**3**:1341–1349.
17. Saemann MD, Kelemen P, Zeyda M, Bohmig G, Staffler G, Zlabinger GJ. CD40 triggered human monocyte-derived dendritic cells convert to tolerogenic dendritic cells when Jak3 activity is inhibited. Transplant Proc 2002;**34**:1407–1408.
18. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004;**25**:280–288.
19. Pullen SS, et al. High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. Biochemistry 1999;**38**:10168–10177.
20. Pullen SS, Miller HG, Everdeen DS, Dang TT, Crute JJ, Kehry MR. CD40-tumor

necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. Biochemistry 1998;**37**:11836–11845.

21. Davies CC, Mak TW, Young LS, Eliopoulos AG. TRAF6 is required for TRAF2-dependent CD40 signal transduction in nonhemopoietic cells. Mol Cell Biol 2005;**25**:9806–9819.

22. Manning E, Pullen SS, Souza DJ, Kehry M, Noelle RJ. Cellular responses to murine CD40 in a mouse B cell line may be TRAF dependent or independent. Eur J Immunol 2002;**32**:39–49.

23. Lu LF, Cook WJ, Lin LL, Noelle RJ. CD40 signaling through a newly identified tumor necrosis factor receptor-associated factor 2 (TRAF2) binding site. J Biol Chem 2003;**278**:45414–45418.

24. Lu LF, et al. The in vivo function of a noncanonical TRAF2-binding domain in the C-terminus of CD40 in driving B-cell growth and differentiation. Blood 2007;**110**:193–200.

25. McWhirter SM, Pullen SS, Holton JM, Crute JJ, Kehry MR, Alber T. Crystallographic analysis of CD40 recognition and signaling by human TRAF2. Proc Natl Acad Sci USA 1999;**96**:8408–8413.

26. Haswell LE, Glennie MJ, Al-Shamkhani A. Analysis of the oligomeric requirement for signaling by CD40 using soluble multimeric forms of its ligand, CD154. Eur J Immunol 2001;**31**:3094–3100.

27. Pullen SS, Dang TT, Crute JJ, Kehry MR. CD40 signaling through tumor necrosis factor receptor-associated factors (TRAFs). Binding site specificity and activation of downstream pathways by distinct TRAFs. J Biol Chem 1999;**274**:14246–14254.

28. Barr TA, Heath AW. Functional activity of CD40 antibodies correlates to the position of binding relative to CD154. Immunology 2001;**102**:39–43.

29. Schwenzer R, et al. The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-kappaB and c-Jun N-terminal kinase. J Biol Chem 1999;**274**:19368–19374.

30. Zapata JM, et al. TNFR-associated factor family protein expression in normal tissues and lymphoid malignancies. J Immunol 2000;**165**:5084–5096.

31. Arron JR, Pewzner-Jung Y, Walsh MC, Kobayashi T, Choi Y. Regulation of the subcellular localization of tumor necrosis factor receptor-associated factor (TRAF)2 by TRAF1 reveals mechanisms of TRAF2 signaling. J Exp Med 2002;**196**:923–934.

32. Xie P, Hostager BS, Munroe ME, Moore CR, Bishop GA. Cooperation between TNF receptor-associated factors 1 and 2 in

CD40 signaling. J Immunol 2006;**176**: 5388–5400.

33. Hostager BS, Haxhinasto SA, Rowland SL, Bishop GA. Tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient B lymphocytes reveal novel roles for TRAF2 in CD40 signaling. J Biol Chem 2003;**278**:45382–45390.

34. Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y. TRAF2 is essential for Jnk but not NF-kappaB activation and regulates lymphocyte proliferation and survival. Immunity 1997;**7**:703–713.

35. Yeh WC, et al. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 1997;**7**:715–725.

36. Gallagher E, et al. Kinase MEKK1 is required for CD40-dependent activation of the kinases Jnk and p38, germinal center formation, B cell proliferation and antibody production. Nat Immunol 2007;**8**:57–63.

37. Vince JE, et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007;**131**:682–693.

38. Gardam S, Sierro F, Basten A, Mackay F, Brink R. TRAF2 and TRAF3 signal adapters act cooperatively to control the maturation and survival signals delivered to B cells by the BAFF receptor. Immunity 2008;**28**:391–401.

39. Vallabhapurapu S, et al. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat Immunol 2008;**9**: 1364–1370.

40. Zarnegar BJ, et al. Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol 2008;**9**:1371–1378.

41. Brown KD, Hostager BS, Bishop GA. Regulation of TRAF2 signaling by self-induced degradation. J Biol Chem 2002;**277**:19433–19438.

42. He JQ, Oganesyan G, Saha SK, Zarnegar B, Cheng G. TRAF3 and its biological function. Adv Exp Med Biol 2007;**597**:48–59.

43. Xie P, Hostager BS, Bishop GA. Requirement for TRAF3 in signaling by LMP1 but not CD40 in B lymphocytes. J Exp Med 2004;**199**:661–671.

44. Propst SM, Estell K, Schwiebert LM. CD40-mediated activation of NF-kappa B in airway epithelial cells. J Biol Chem 2002;**277**:37054–37063.

45. Urbich C, Mallat Z, Tedgui A, Clauss M, Zeiher AM, Dimmeler S. Upregulation of TRAF-3 by shear stress blocks CD40-mediated endothelial activation. J Clin Invest 2001;**108**:1451–1458.

46. Hauer J, et al. TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor

of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs. Proc Natl Acad Sci USA 2005;**102**:2874–2879.

47. Nakano H, et al. Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. Proc Natl Acad Sci USA 1999;**96**:9803–9808.

48. Rowland SL, Tremblay MM, Ellison JM, Stunz LL, Bishop GA, Hostager BS. A novel mechanism for TNFR-associated factor 6-dependent CD40 signaling. J Immunol 2007;**179**:4645–4653.

49. Arron JR, et al. A positive regulatory role for Cbl family proteins in tumor necrosis factor-related activation-induced cytokine (trance) and CD40L-mediated Akt activation. J Biol Chem 2001;**276**: 30011–30017.

50. Davies CC, Mason J, Wakelam MJ, Young LS, Eliopoulos AG. Inhibition of phosphatidylinositol 3-kinase- and ERK MAPK-regulated protein synthesis reveals the proapoptotic properties of CD40 ligation in carcinoma cells. J Biol Chem 2004;**279**: 1010–1019.

51. Yu Q, Kovacs C, Yue FY, Ostrowski MA. The role of the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphoinositide-3-OH kinase signal transduction pathways in CD40 ligand-induced dendritic cell activation and expansion of virus-specific CD8$^+$ T cell memory responses. J Immunol 2004;**172**: 6047–6056.

52. Cardone MH, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;**282**:1318–1321.

53. Datta SR, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997; **91**:231–241.

54. Qiao G, et al. Negative regulation of CD40-mediated B cell responses by E3 ubiquitin ligase Casitas-B-lineage lymphoma protein-B. J Immunol 2007;**179**:4473–4479.

55. Hanissian SH, Geha RS. Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. Immunity 1997;**6**:379–387.

56. Revy P, et al. Activation of the Janus kinase 3-STAT5a pathway after CD40 triggering of human monocytes but not of resting B cells. J Immunol 1999;**163**:787–793.

57. Pine R, Canova A, Schindler C. Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. EMBO J 1994;**13**:158–167.

58. Banchereau J, et al. The CD40 antigen and its ligand. Annu Rev Immunol 1994;**12**:881–922.

59. Barrett TB, Shu G, Clark EA. CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. J Immunol 1991; **146**:1722–1729.

60. Clark EA, Ledbetter JA. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50. Proc Natl Acad Sci USA 1986;**83**: 4494–4498.

61. Jabara HH, Fu SM, Geha RS, Vercelli D. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. J Exp Med 1990;**172**:1861–1864.

62. Foy TM, Durie FH, Noelle RJ. The expansive role of CD40 and its ligand, gp39, in immunity. Semin Immunol 1994;**6**:259–266.

63. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, Noelle RJ. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J Exp Med 1994;**180**:157–163.

64. Foy TM, Shepherd DM, Durie FH, Aruffo A, Ledbetter JA, Noelle RJ. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. J Exp Med 1993;**178**:1567–1575.

65. Mitchison NA. T-cell-B-cell cooperation. Nat Rev Immunol 2004;**4**:308–312.

66. Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature 1985;**314**:537–539.

67. Rock KL, Benacerraf B, Abbas AK. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. J Exp Med 1984;**160**:1102–1113.

68. Loughnan MS, Nossal GJ. Interleukins 4 and 5 control expression of IL-2 receptor on murine B cells through independent induction of its two chains. Nature 1989;**340**: 76–79.

69. Loughnan MS, Takatsu K, Harada N, Nossal GJ. T-cell-replacing factor (interleukin 5) induces expression of interleukin 2 receptors on murine splenic B cells. Proc Natl Acad Sci USA 1987;**84**:5399–5403.

70. Noelle R, Krammer PH, Ohara J, Uhr JW, Vitetta ES. Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. Proc Natl Acad Sci USA 1984;**81**:6149–6153.

71. Oliver K, Noelle RJ, Uhr JW, Krammer PH, Vitetta ES. B-cell growth factor (B-cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proc Natl Acad Sci USA 1985;**82**:2465–2467.

72. Bartlett WC, Michael A, McCann J, Yuan D, Claassen E, Noelle RJ. Cognate interactions between helper T cells and B cells. II. Dissection of cognate help by using a class II-restricted, antigen-specific, IL-2-dependent helper T cell clone. J Immunol 1989;**143**:1745–1754.

73. Bartlett WC, McCann J, Shepherd DM, Roy M, Noelle RJ. Cognate interactions between helper T cells and B cells. IV. Requirements for the expression of effector phase activity by helper T cells. J Immunol 1990;**145**:3956–3962.

74. Brian AA. Stimulation of B-cell proliferation by membrane-associated molecules from activated T cells. Proc Natl Acad Sci USA 1988;**85**:564–568.

75. Hodgkin PD, Yamashita LC, Coffman RL, Kehry MR. Separation of events mediating B cell proliferation and Ig production by using T cell membranes and lymphokines. J Immunol 1990;**145**:2025–2034.

76. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc Natl Acad Sci USA 1992;**89**:6550–6554.

77. Armitage RJ, et al. Molecular and biological characterization of a murine ligand for CD40. Nature 1992;**357**:80–82.

78. Bergtold A, Desai DD, Gavhane A, Clynes R. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity 2005;**23**:503–514.

79. Pape KA, Catron DM, Itano AA, Jenkins MK. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. Immunity 2007;**26**:491–502.

80. Wykes M, Pombo A, Jenkins C, MacPherson GG. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. J Immunol 1998; **161**:1313–1319.

81. Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. J Exp Med 1991;**173**: 1165–1175.

82. Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. J Exp Med 1992;**176**:679–687.

83. MacLennan IC. Germinal centers. Annu Rev Immunol 1994;**12**:117–139.

84. Van den Eertwegh AJ, et al. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T–B cell interactions. J Exp Med 1993;**178**:1555–1565.

85. Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. Science 1998;**281**:96–99.

86. Xu J, et al. Mice deficient for the CD40 ligand. Immunity 1994;**1**:423–431.

87. Benson MJ, Erickson LD, Gleeson MW, Noelle RJ. Affinity of antigen encounter and other early B-cell signals determine B-cell fate. Curr Opin Immunol 2007;**19**: 275–280.

88. O'Connor BP, et al. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. J Immunol 2006;**177**:7723–7732.

89. Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. J Exp Med 2006;**203**: 1081–1091.

90. Phan TG, et al. High affinity germinal center B cells are actively selected into the plasma cell compartment. J Exp Med 2006;**203**: 2419–2424.

91. Erickson LD, et al. Short-circuiting long-lived humoral immunity by the heightened engagement of CD40. J Clin Invest 2002;**109**:613–620.

92. Breitfeld D, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B-cell follicles, and support immunoglobulin production. J Exp Med 2000;**192**:1545–1552.

93. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol 2008;**26**:741–766.

94. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med 2000;**192**:1553–1562.

95. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J Exp Med 1999;**190**:1123–1134.

96. Ansel KM, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature 2000;**406**:309–314.

97. Bowman EP, et al. Developmental switches in chemokine response profiles during B cell differentiation and maturation. J Exp Med 2000;**191**:1303–1318.

98. Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific

anatomic compartments of the spleen. Cell 1996;**87**:1037–1047.

99. Gunn MD, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature 1998;**391**:799–803.

100. Legler DF, Loetscher M, Roos RS, Clark-Lewis I, Baggiolini M, Moser B. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. J Exp Med 1998;**187**:655–660.

101. Rogerson B, Hackett J Jr, Peters A, Haasch D, Storb U. Mutation pattern of immunoglobulin transgenes is compatible with a model of somatic hypermutation in which targeting of the mutator is linked to the direction of DNA replication. EMBO J 1991;**10**:4331–4341.

102. Allen CD, et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol 2004;**5**:943–952.

103. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. Science 2007;**315**:528–531.

104. Liu YJ, et al. Follicular dendritic cells specifically express the long CR2/CD21 isoform. J Exp Med 1997;**185**:165–170.

105. Hannum LG, Haberman AM, Anderson SM, Shlomchik MJ. Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. J Exp Med 2000;**192**:931–942.

106. Casamayor-Palleja M, Feuillard J, Ball J, Drew M, MacLennan IC. Centrocytes rapidly adopt a memory B cell phenotype on co-culture with autologous germinal centre T cell-enriched preparations. Int Immunol 1996;**8**:737–744.

107. Holder MJ, et al. Suppression of apoptosis in normal and neoplastic human B lymphocytes by CD40 ligand is independent of Bcl-2 induction. Eur J Immunol 1993;**23**:2368–2371.

108. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC. Mechanism of antigen-driven selection in germinal centres. Nature 1989;**342**:929–931.

109. Hennino A, Berard M, Krammer PH, Defrance T. FLICE-inhibitory protein is a key regulator of germinal center B cell apoptosis. J Exp Med 2001;**193**:447–458.

110. Tuscano JM, Druey KM, Riva A, Pena J, Thompson CB, Kehrl JH. Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. Blood 1996;**88**:1359–1364.

111. Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. J Exp Med 1998;**187**:885–895.

112. Ahonen C, et al. The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. Nat Immunol 2002;**3**:451–456.

113. Jabara H, et al. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. Immunity 2002;**17**:265–276.

114. Yasui T, et al. Dissection of B cell differentiation during primary immune responses in mice with altered CD40 signals. Int Immunol 2002;**14**:319–329.

115. Cheng G, Cleary AM, Ye ZS, Hong DI, Lederman S, Baltimore D. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science 1995;**267**:1494–1498.

116. Lee HH, Dempsey PW, Parks TP, Zhu X, Baltimore D, Cheng G. Specificities of CD40 signaling: involvement of TRAF2 in CD40-induced NF-kappaB activation and intercellular adhesion molecule-1 up-regulation. Proc Natl Acad Sci USA 1999;**96**:1421–1426.

117. Ochs HD, Hollenbaugh D, Aruffo A. The role of CD40L (gp39)/CD40 in T/B cell interaction and primary immunodeficiency. Semin Immunol 1994;**6**:337–341.

118. D'Orlando O, et al. Outside inside signalling in CD40-mediated B-cell activation. J Biol Regul Homeost Agents 2007;**21**:49–62.

119. Agarwal A, Newell KA. The role of positive costimulatory molecules in transplantation and tolerance. Curr Opin Organ Transplant 2008;**13**:366–372.

120. Vonderheide RH. Prospect of targeting the CD40 pathway for cancer therapy. Clin Cancer Res 2007;**13**:1083–1088.

121. Toubi E, Shoenfeld Y. The role of CD40-CD154 interactions in autoimmunity and the benefit of disrupting this pathway. Autoimmunity 2004;**37**:457–464.

122. Lenschow DJ, et al. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA-4lg. Science 1992;**257**:789–792.

123. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity 1994;**1**:793–801.

124. Baliga P, et al. CTLA-4Ig prolongs allograft survival while suppressing cell-mediated immunity. Transplantation 1994;**58**:1082–1090.

125. Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J Exp Med 1993;**177**:925–935.

126. Ranheim EA, Kipps TJ. Tumor necrosis factor-alpha facilitates induction of CD80 (B7-1) and CD54 on human B cells by activated T cells: complex regulation by IL-4, IL-10, and CD40L. Cell Immunol 1995;**161**:226–235.

127. Jones ND, et al. CD40-CD40 ligand-independent activation of CD8^{+} T cells can trigger allograft rejection. J Immunol 2000;**165**:1111–1118.

128. Lehnert AM, et al. Blockade of the CD28 and CD40 pathways result in the acceptance of pig and rat islet xenografts but not rat cardiac grafts in mice. Transpl Immunol 2001;**9**:51–56.

129. Honey K, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4^{+} T cell tolerance and linked suppression. J Immunol 1999;**163**:4805–4810.

130. Li Y, Zheng XX, Li XC, Zand MS, Strom TB. Combined costimulation blockade plus rapamycin but not cyclosporine produces permanent engraftment. Transplantation 1998;**66**:1387–1388.

131. Kang HG, Zhang D, Degauque N, Mariat C, Alexopoulos S, Zheng XX. Effects of cyclosporine on transplant tolerance: the role of IL-2. Am J Transplant 2007;**7**:1907–1916.

132. Chalermskulrat W, et al. Combined donor specific transfusion and anti-CD154 therapy achieves airway allograft tolerance. Thorax 2006;**61**:61–67.

133. Larsen CP, et al. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. Transplantation 1996;**61**:4–9.

134. Parker DC, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. Proc Natl Acad Sci USA 1995;**92**:9560–9564.

135. Quezada SA, et al. Mechanisms of donor-specific transfusion tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation. Blood 2003;**102**:1920–1926.

136. Wood ML, Gottschalk R, Monaco AP. Comparison of immune responsiveness in mice after single or multiple donor-specific transfusions. J Immunol 1984;**132**:651–655.

137. Zheng XX, et al. Blockade of CD40L/CD40 costimulatory pathway in a DST presensitization model of islet allograft leads to a state of Allo-Ag specific tolerance and permits subsequent engraftment of donor strain islet or heart allografts. Transplant Proc 1999;**31**:627–628.

138. Jarvinen LZ, Blazar BR, Adeyi OA, Strom TB, Noelle RJ. CD154 on the surface of CD4^{+} CD25^{+} regulatory T cells contributes to skin

transplant tolerance. Transplantation 2003;**76**:1375–1379.

139. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. J Exp Med 2002;**196**: 1091–1097.

140. Coulombe M, Yang H, Wolf LA, Gill RG. Tolerance to antigen-presenting cell-depleted islet allografts is CD4 T cell dependent. J Immunol 1999;**162**:2503–2510.

141. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;**392**:245–252.

142. Frleta D, et al. Distinctive maturation of in vitro versus in vivo anti-CD40 mAb-matured dendritic cells in mice. J Immunother 2003;**26**:72–84.

143. Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. Immunity 2002;**16**:257–270.

144. Wong BR, et al. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 1997;**186**:2075–2080.

145. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. Nat Med 1999;**5**:1298–1302.

146. Wekerle T, et al. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. J Immunol 2001;**166**:2311–2316.

147. Wells AD, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. Nat Med 1999;**5**:1303–1307.

148. Graca L, Le Moine A, Cobbold SP, Waldmann H. Antibody-induced transplantation tolerance: the role of dominant regulation. Immunol Res 2003;**28**:181–191.

149. Banuelos SJ, et al. Regulation of skin and islet allograft survival in mice treated with costimulation blockade is mediated by different CD4^{+} cell subsets and different mechanisms. Transplantation 2004;**78**:660–667.

150. Guillonneau C, et al. Anti-CD28 antibodies modify regulatory mechanisms and reinforce tolerance in CD40Ig-treated heart allograft recipients. J Immunol 2007;**179**:8164–8171.

151. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 2007;**204**: 1765–1774.

152. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3^{+} CD4^{+} regulatory T cells. Proc Natl Acad Sci USA 2008;**105**: 9331–9336.

153. Markees TG, et al. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA-4. J Clin Invest 1998;**101**:2446–2455.

154. Vincenti F. Costimulation blockade in autoimmunity and transplantation. J Allergy Clin Immunol 2008;**121**:299–306;quiz 7–8.

155. Mai G, del Rio ML, Tian J, Ramirez P, Buhler L, Rodriguez-Barbosa JI. Blockade of the PD-1/PD-1L pathway reverses the protective effect of anti-CD40L therapy in a rat to mouse concordant islet xenotransplantation model. Xenotransplantation 2007;**14**:243–248.

156. Schilbach K, et al. PD-1-PD-L1 pathway is involved in suppressing alloreactivity of heart infiltrating T cells during murine GVHD across minor histocompatibility antigen barriers. Transplantation 2007;**84**: 214–222.

157. Vu MD, Clarkson MR, Yagita H, Turka LA, Sayegh MH, Li XC. Critical, but conditional, role of OX40 in memory T cell-mediated rejection. J Immunol 2006;**176**: 1394–1401.

158. Berney T, et al. The effect of simultaneous CD154 and LFA-1 blockade on the survival of allogeneic islet grafts in nonobese diabetic mice. Transplantation 2003;**76**: 1669–1674.

159. Kobayashi T, Harb G, Rayat GR. Prolonged survival of microencapsulated neonatal porcine islets in mice treated with a combination of anti-CD154 and anti-LFA-1 monoclonal antibodies. Transplantation 2005;**80**:821–827.

160. Lunsford KE, Koester MA, Eiring AM, Horne PH, Gao D, Bumgardner GL. Targeting LFA-1 and cd154 suppresses the in vivo activation and development of cytolytic (cd4-Independent) CD8^{+} T cells. J Immunol 2005;**175**:7855–7866.

161. Nicolls MR, Coulombe M, Beilke J, Gelhaus HC, Gill RG. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. J Immunol 2002;**169**:4831–4839.

162. Rayat GR, Gill RG. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. Diabetes 2005; **54**:443–451.

163. Wang Y, Gao D, Lunsford KE, Frankel WL, Bumgardner GL. Targeting LFA-1 synergizes with CD40/CD40L blockade for suppression of both CD4-dependent and CD8-dependent rejection. Am J Transplant 2003;**3**:1251–1258.

164. Lee JH, Ha J, Kim SH, Kim SJ. IL-2 pathway blocking in combination with anti-CD154 synergistically establishes mixed macrochimerism with limited dose of bone marrow cells and prolongs skin graft survival in mice. J Korean Med Sci 2006;**21**: 1005–1011.

165. Pan H, et al. Anti-CD25 mAb, anti-IL2 mAb, and IL2 block tolerance induction through anti-CD154 mAb and rapamycin in xenogeneic islet transplantation. Transplant Proc 2007;**39**:3452–3454.

166. Wang Y, Dai H, Liu Z, Cheng X, Tellides G, Dai Z. Neutralizing IL-7 promotes long-term allograft survival induced by CD40/CD40L costimulatory blockade. Am J Transplant 2006;**6**:2851–2860.

167. Demirci G, Strom TB, Li XC. Islet allograft rejection in nonobese diabetic mice involves the common gamma-chain and CD28/CD154-dependent and -independent mechanisms. J Immunol 2003;**171**: 3878–3885.

168. Li XC, Strom TB. A rare find – cells that improve bone marrow transplantation. Nat Med 2000;**6**:866–867.

169. Trambley J, et al. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. J Clin Invest 1999;**104**:1715–1722.

170. Williams MA, et al. Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. J Immunol 2000;**165**: 6849–6857.

171. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol 2005;**6**:338–344.

172. Grohmann U, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol 2002;**3**:1097–1101.

173. Demirci G, et al. Critical role of OX40 in CD28 and CD154-independent rejection. J Immunol 2004;**172**:1691–1698.

174. Vu MD, et al. OX40 costimulation turns off Foxp3^{+} Tregs. Blood 2007;**110**: 2501–2510.

175. Chen M, Xiao X, Demirci G, Li XC. OX40 controls islet allograft tolerance in CD154 deficient mice by regulating FOXP3^{+} Tregs. Transplantation 2008;**85**:1659–1662.

176. Habicht A, Najafian N, Yagita H, Sayegh MH, Clarkson MR. New insights in CD28-independent allograft rejection. Am J Transplant 2007;**7**:1917–1926.

177. Baxendale AJ, et al. Constitutive activation of the CD40 pathway promotes cell transformation and neoplastic growth. Oncogene 2005;**24**:7913–7923.

178. Bedian V, et al. *In vitro* characterization and pre-clinical pharmacokinetics of CP-870,893, a human anti-CD40 agonist antibody. J Clin Oncol 2006;**24**:2539. (Abstract).

179. Bensinger W, et al. A phase 1 dose escalation study of a fully human, antagonist anti-CD40 antibody, HCD 122 (formerly CHIR-12.12), in patients with relapsed and refractory multiple myeloma. Blood 2006;**108**:3590. (Abstract).

180. Berner V, et al. IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. Nat Med 2007;**13**:354–360.

181. Byrd JC, et al. Pharmacokinetics and pharmacodynamics from a first-in-human phase 1 dose escalation study with antagonist anti-CD40 antibody, HCD 122 (formerly CHIR-12.12) in patients with relapsed and refractory chronic lymphocytic leukemia. Blood 2006;**108**:2837. (Abstract).

182. Tan J, et al. CD40 is expressed and functional on neuronal cells. EMBO J 2002;**21**:643–652.

183. Young LS, Dawson CW, Brown KW, Rickinson AB. Identification of a human epithelial cell surface protein sharing an epitope with the C3d/Epstein-Barr virus receptor molecule of B lymphocytes. Int J Cancer 1989;**43**:786–794.

184. Cooke PW, James ND, Ganesan R, Wallace M, Burton A, Young LS. CD40 expression in bladder cancer. J Pathol 1999;**188**:38–43.

185. Altenburg A, Baldus SE, Smola H, Pfister H, Hess S. CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. J Immunol 1999;**162**:4140–4147.

186. Gallagher NJ, Eliopoulos AG, Agathangelo A, Oates J, Crocker J, Young LS. CD40 activation in epithelial ovarian carcinoma cells modulates growth, apoptosis, and cytokine secretion. Mol Pathol 2002;**55**:110–120.

187. Agathanggelou A, Niedobitek G, Chen R, Nicholls J, Yin W, Young LS. Expression of immune regulatory molecules in Epstein-Barr virus-associated nasopharyngeal carcinomas with prominent lymphoid stroma. Evidence for a functional interaction between epithelial tumor cells and infiltrating lymphoid cells. Am J Pathol 1995;**147**:1152–1160.

188. Kato K, Cantwell MJ, Sharma S, Kipps TJ. Gene transfer of CD40-ligand induces autologous immune recognition of chronic lymphocytic leukemia B cells. J Clin Invest 1998;**101**:1133–1141.

189. Pellat-Deceunynck C, Amiot M, Robillard N, Wijdenes J, Bataille R. CD11a-CD18 and CD102 interactions mediate human myeloma cell growth arrest induced by CD40 stimulation. Cancer Res 1996;**56**:1909–1916.

190. Teoh G, et al. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. Blood 2000;**95**:1039–1046.

191. Aldinucci D, et al. CD40L induces proliferation, self-renewal, rescue from apoptosis, and production of cytokines by CD40-expressing AML blasts. Exp Hematol 2002;**30**:1283–1292.

192. Choi MS, Boise LH, Gottschalk AR, Quintans J, Thompson CB, Klaus GG. The role of Bcl-XL in CD40-mediated rescue from anti-mu-induced apoptosis in WEHI-231 B lymphoma cells. Eur J Immunol 1995;**25**:1352–1357.

193. Sarma V, et al. Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. J Biol Chem 1995;**270**:12343–12346.

194. Zhang X, et al. Up-regulation of Bcl-xL expression protects CD40-activated human B cells from Fas-mediated apoptosis. Cell Immunol 1996;**173**:149–154.

195. Lee HH, Dadgostar H, Cheng Q, Shu J, Cheng G. NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc Natl Acad Sci USA 1999;**96**:9136–9141.

196. Pham LV, et al. A CD40 Signalosome anchored in lipid rafts leads to constitutive activation of NF-kappaB and autonomous cell growth in B cell lymphomas. Immunity 2002;**16**:37–50.

197. Challa A, et al. Population depletion activates autonomous CD154-dependent survival in biopsylike Burkitt lymphoma cells. Blood 2002;**99**:3411–3418.

198. Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol 2000;**164**:2200–2206.

199. Funakoshi S, et al. Inhibition of human B-cell lymphoma growth by CD40 stimulation. Blood 1994;**83**:2787–2794.

200. Wingett DG, Vestal RE, Forcier K, Hadjokas N, Nielson CP. CD40 is functionally expressed on human breast carcinomas: variable inducibility by cytokines and enhancement of Fas-mediated apoptosis. Breast Cancer Res Treat 1998;**50**:27–36.

201. Eliopoulos AG, et al. CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily. Mol Cell Biol 2000;**20**:5503–5515.

202. Hess S, Engelmann H. A novel function of CD40: induction of cell death in transformed cells. J Environ Monit 1996;**183**:159–167.

203. Eliopoulos AG, et al. CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene 1996;**13**:2243–2254.

204. Hirano A, et al. Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. Blood 1991;**93**:2999–3007.

205. Tong AW, et al. Growth-inhibitory effects of CD40 ligand (CD154) and its endogenous expression in human breast cancer. Clin Cancer Res 2001;**7**:691–703.

206. Tong AW, Stone MJ. Prospects for CD40-directed experimental therapy of human cancer. Cancer Gene Ther 2003;**10**:1–13.

207. Mackey MF, et al. Protective immunity induced by tumor vaccines requires interaction between CD40 and its ligand, CD154. Cancer Res 1997;**57**:2569–2574.

208. French RR, Chan HT, Tutt AL, Glennie MJ. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nat Med 1999;**5**:548–553.

209. Diehl L, et al. CD40 activation *in vivo* overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat Med 1999;**5**:774–779.

210. Sotomayor EM, et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through *in vivo* ligation of CD40. Nat Med 1999;**5**:780–787.

211. van Mierlo GJ, et al. CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. Proc Natl Acad Sci USA 2002;**99**:5561–5566.

212. Kedl RM, Jordan M, Potter T, Kappler J, Marrack P, Dow S. CD40 stimulation accelerates deletion of tumor-specific CD8(+) T cells in the absence of tumor-antigen vaccination. Proc Natl Acad Sci USA 2001;**98**:10811–10816.

213. Ahonen CL, et al. Enhanced efficacy and reduced toxicity of multifactorial adjuvants compared with unitary adjuvants as cancer vaccines. Blood 2008;**111**:3116–3125.

214. Grossmann ME, Brown MP, Brenner MK. Antitumor responses induced by transgenic expression of CD40 ligand. Hum Gene Ther 1997;**8**:1935–1943.

215. Ribas A, et al. CD40 cross-linking bypasses the absolute requirement for CD4 T cells during immunization with melanoma antigen gene-modified dendritic cells. Cancer Res 2001;**61**:8787–8793.

216. Kikuchi T, Crystal RG. Anti-tumor immunity induced by *in vivo* adenovirus vector-mediated expression of CD40 ligand in

tumor cells. Hum Gene Ther 1999;**10**:1375–1387.

217. Kikuchi T, Moore MA, Crystal RG. Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood 2000;**96**:91–99.

218. Forero-Torres A, et al. A humanized antibody against CD40 (SGN-CD40) is well tolerated and active in non-Hodgkin's lymphoma (NHL): results of a phase 1 study. J Clin Oncol 2006;**24**:7534. (Abstract).

219. Vonderheide RH, et al. Phase I study of recombinant human CD40 ligand in cancer patients. J Clin Oncol 2001;**19**:3280–3287.

220. Younes A. CD40 ligand therapy of lymphoma patients. J Clin Oncol 2001;**19**:4351–4353.

221. Wierda WG, Cantwell MJ, Woods SJ, Rassenti LZ, Prussak CE, Kipps TJ. CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. Blood 2000;**96**: 2917–2924.

222. Kipps TJ, Chu P, Wierda WG. Immunogenetic therapy for B-cell malignancies. Semin Oncol 2000;**27**:104–109.

223. Schultze JL, Anderson KC, Gilleece MH, Gribben JG, Nadler LM. A pilot study of combined immunotherapy with autologous adoptive tumour-specific T-cell transfer, vaccination with CD40-activated malignant B cells and interleukin 2. Br J Haematol 2001;**113**: 455–460.

224. Vonderheide RH, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. J Clin Oncol 2007;**25**:876–883.

225. Hussein MA, et al. A phase I humanized anti-CD40 monoclonal antibody (SGN-40) in patients with multiple myeloma. Blood 2005;**106**:3576 (Abstract).

226. Rousseau RF, et al. Immunotherapy of high-risk acute leukemia with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation. Blood 2006;**107**:1332–1341.

227. Alexandroff AB, et al. Role for CD40-CD40 ligand interactions in the immune response to solid tumours. Mol Immunol 2000;**37**:515–526.

228. Luqman M, et al. The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. Blood 2008; **112**:711–720.

229. Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. Cancer Res 2003;**63**:4490–4496.

230. Honeychurch J, Glennie MJ, Johnson PW, Illidge TM. Anti-CD40 monoclonal antibody therapy in combination with irradiation results in a CD8 T-cell-dependent immunity to B-cell lymphoma. Blood 2003;**102**: 1449–1457.