Directed Delivery of CD40 Ligand from Helper T cells to Antigen-presenting B cells

By

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CERTIFICATE OF APPROVAL

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Abstract:

This dissertation presents my studies investigating the delivery of CD40 ligand (CD40L, CD154) from helper T lymphocytes to antigen-presenting B lymphocytes. CD40L is a type two transmembrane TNF superfamily cytokine made by T cells that engages CD40 on antigen-presenting cells, including B cells, initiating downstream signaling resulting in B cell proliferation, differentiation, and antibody formation. Helper T cells can produce CD40L *de novo* upon antigen-specific interactions, but they also have an intracellular secretory compartment containing a small amount of preformed CD40L that is brought to the T cell surface rapidly upon antigen recognition. In the second chapter of the dissertation, I investigate the function of preformed CD40L in the absence of *de novo* CD40L. Preformed CD40L is capable of fulfilling some functions previously assumed to require *de novo* CD40L, including upregulation of costimulatory molecules, production of cytokines by DCs, and antigen-specific proliferation of B cells. Therefore, this secretory compartment of CD40L protein may play a significant role in the adaptive immune response, especially when T cell/APC interactions are brief and not long enough for *de novo* protein production. In Chapter 3 of this dissertation, I describe my novel finding that CD40L does not remain on the T cell surface but is actually transferred to antigen-presenting B cells. Transfer is much more efficient to antigen-bearing than to bystander B cells, a finding that may explain the phenomenon of affinity maturation of the antibody response, a process that is required for generation of the high affinity antibodies that protect us from infectious agents and their toxins. In the germinal center reaction, rare somatic mutant B cells with higher affinity for antigen are selected for

survival and eventually exported as long-lived memory B cells and antibody secreting cells. Selection is known to depend on acquisition of limited survival signals from helper T cells. Transfer of CD40L proportional to the amount of antigen presented by individual B cells may allow T cells to selectively deliver a sustained signal required for survival of higher affinity mutant B cells. In the fourth Chapter of this dissertation, I investigate the functional significance of differences between the Th1 and Th2 helper T cell subsets in the segregation of molecules at the area of interaction between the helper T cells and APCs, called the immunological synapse. I found that despite distinct immunological synapse structures, preformed CD40L is delivered in an antigen-specific manner by both Th1 and Th2 helper T cells.

Chapter 1: Introduction

I. The Antibody Response

T and B cells are two of the main immune cells involved in T-dependent antibody production during an immune response. Both of these cell populations are derived from lymphoid progenitor cells during hematopoiesis, though they develop in different organs of the body prior to entry into the bloodstream. T cells develop in the thymus through a process of positive and negative selection, while B cells undergo a similar selection process in the bone marrow. Once these cells have undergone this selection, they leave these primary organs and join the circulating lymphocytes. Each T or B cell has cell surface receptors of a single specificity that are capable of binding foreign proteins. The B cell receptor includes a membrane form of the unique antibody molecule that its descendants will secrete. The T cell receptor (TCR) and B cell receptor (BCR) differ in how they detect antigens. B cell receptors recognize foreign antigens directly according to their three-dimensional structure, while TCRs recognize peptides derived from the antigen and bound to surface molecules encoded by the major histocompatibility complex (MHC) on antigen-presenting cells (APCs), including B cells, dendritic cells, and macrophages. Once T and B cells encounter antigen in secondary lymphoid structures, lymph nodes and spleen, they either participate in an extrafollicular antibody response or migrate to the B cell follicles within the secondary lymphoid structures to form specialized structures called germinal centers (1, 2). The extrafollicular antibody response produces antibody much more rapidly and without the T and B cell maturation and

differentiation processes that are involved in the germinal center reaction (3). Within germinal centers, T and B cells interact with each other, and through these cellular interactions some of the B cell descendants are selected to secrete the high affinity antibodies necessary for pathogen clearance.

Jacques Miller and Graham Mitchell discovered in 1968 that antibody formation requires collaboration of T and B cells (4, 5). A few years later, Rajewsky (6) and Mitchison (7) showed that T and B cells needed to recognize the same particle of antigen, giving rise to the concept of an antigen bridge between the two cells. It was not until the concepts of antigen-processing and presentation and MHC restriction had been discovered that it was proposed that B cells get help by acting as antigen-presenting cells (APCs) for helper T cells (8), an idea that was demonstrated experimentally some years later by Lanzavecchia (9) and Parker (10).

MHC molecules are surface proteins that bind to foreign or self-peptides and are recognized by T cells through their TCR. There are two different classes of MHC molecules, MHCI and MHCII, recognized by CD8 and CD4 T cells respectively. MHCI molecules are synthesized in the endoplasmic reticulum (ER) where they are retained by the chaperone protein calnexin. The MHCI molecule then associates with β_2 microglobulin and calnexin is exchanged for calreticulin. The MHCI molecule then interacts with tapasin, ERp57, and the transporter associated with antigen processing (TAP) to form the peptide loading complex. Peptides generated in the cytosol by the proteosome are transported by TAP into the endoplasmic reticulum. These peptides bind

MHCI molecules and then are processed by ER trimming amino peptidases (ERAPs) into the optimal size. MHCI molecules bound to peptides are then packaged into COPII coated vesicles and transported via the secretory pathway to the cell surface (11, 12). CD8 T cells recognition of peptide in the context of a MHCI molecule often results in release of cytolytic granules which triggers a signaling cascade resulting in apoptosis of the cell recognized. Antigens in the MHCI pathway are usually derived from a cell endogenous source, as is the case with viral proteins synthesized within the cell. However during cross-presentation exogenous antigens are presented on class I molecules. Cross-presentation was discovered by Bevan in 1976 when he showed that CD8 T cells responding to minor histocompatibility antigens of a graft were restricted to host as well as graft MHC (13).

Antigens presented on MHC class II are usually from an exogenous source and reach the cell surface using a different pathway from antigens presented on class I molecules. Newly synthesized MHCII molecules are made in the ER where they are bound by a transmembrane protein called the invariant chain. The invariant chain associates with MHCII at the peptide-binding groove within the ER so that newly synthesized proteins and unfolded proteins cannot bind the class II molecule. The invariant chain also targets the transport of the MHCII molecules to the endosomal compartment where peptide loading occurs. In the endosomal compartment the invariant chain is cleaved by acidic proteases into a short fragment known as the class II-associated invariant-chain peptide (CLIP) which binds the class II molecule within the peptide-binding groove. B cells recognize exogenous foreign proteins through their antigen-specific receptor triggering

endocytosis of the protein. In the endocytic pathway, the foreign protein is processed into peptides by the proteolytic enzymes. In order for exogenous peptides to bind MHCII molecules in the endosomes, these molecules must bind another protein called H-2M (HLA-DM in humans) which triggers the release of CLIP. The peptide-loaded MHCII is then brought to the cell surface where it is recognized by CD4 T cells through the TCR. Antigen processing by B cells and other cells is an active process that requires a living cell. In landmark experiments, Unanue and Grey and coworkers showed that small antigenic peptides could be loaded into MHCII molecules on fixed, dead cells, short-circuiting the requirement for antigen processing, and illuminating its basic nature (14, 15).

Upon antigen recognition through cell surface receptors along with engagement of costimulatory proteins, including constitutively expressed CD28 on T cells that engages with CD80 and CD86 on APCs, T cell become activated. Downstream signaling during activation results in both B and T cell production of proteins called cytokines necessary for their mutual activation. An essential T cell cytokine necessary for antibody production is CD40 ligand (CD40L) (16). CD40L is a type II transmembrane cytokine that is synthesized once signaling downstream of TCR activation results in translocation of CD40L's transcription factors, AP-1 and NFAT, to the nucleus (17, 18). Upon TCR stimulation, CD40L is expressed at the T cell surface, where it interacts with the constitutively expressed CD40 on the surface of the B cell. This interaction leads to the production of cytokines and upregulation of CD80 and CD86 on B cells leading to the production of interleukin-2 (IL-2), IL-21, and other cytokines by T cells. These cytokines

bind to cytokine receptors on T cells and B cells resulting in their expansion via proliferation.

Signaling downstream of CD40 results in activation of multiple pathways, including the mitogen-activated protein (MAP) kinase pathways as well as both the canonical and non-canonical NF-κB pathways. These pathways result in the production of cytokines, proteins necessary for APC survival, and prolonged pMHC presentation. Engagement of CD40 by CD154 leads to the clustering of CD40 and recruitment of the TRAF (tumor necrosis factor receptor-associated factor) molecules to the cytoplasmic domain of CD40. Canonical NF-κB signaling is rapid and results from degradation of IKBα, which is part of the catalytic subunit of the IKK complex (inhibitor of NF-κB kinase). Activation of IKK results in the phosphorylation of the subunit IκBα which triggers its ubiquitination and proteosomal degradation. This leads to translocation of the NF-κB complexes, p50/RelA or p50/c-Rel to the nucleus leading to transcription of inflammatory mediators. Non-canonial NF-κB pathway activation is slower than canonical as it requires new protein synthesis. In this pathway, binding of TRAFs to engaged CD40 interferes with constitutive degradation of NIK (NF-κB inducing kinase) by TRAF2/3/cIAP (cellular inhibitors of apoptosis) complex, such that NIK accumulates in the cytoplasm. NIK phosphorylates the IKKα complex and IKKα then phosphorylates p100. This targets p100 for processing by the proteasome resulting in the production of p52 dimers (p52/Rel-B, p52/p65, and p52/c-Rel) that then translocate to the nucleus resulting in the production of inflammatory genes products. (19, 20)

The T cell, through activation of signaling downstream of CD40L and CD40, is capable of inducing all the costimulatory signals it needs from the APC to become fully activated and proliferate during an immune response. Since this T cell/APC inflammatory pathway is self-amplifying, it is necessary to have checks in place to make sure the immune response does not respond in a manner that would be harmful towards a host. These checkpoints exist in many forms. For example, recognition of pMHC by a TCR without costimulation results in an anergic or unresponsive T cell state. Additionally there are inhibitory receptors on T cells, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) that can engage with receptors on APCs and inhibit the activation of an immune response (21). Specifically CTLA-4 has a higher affinity for CD80 and CD86 on APCs than CD28, and therefore CTLA-4 outcompetes CD28 for interaction with CD80/86 and blocks productive interactions between these two cell types. PD-1 is another inhibitory molecule that suppresses the activation of T cells when engaged by PD-L1 on APC (22). There is also subset of T cells called regulatory T cells (Tregs) whose main role during an active immune response is to dampen the inflammatory response. Tregs have many mechanisms to suppress the activation of other T cells and APCs (23, 24). They interact with APCs and downregulate costimulatory molecules on their surface (25) via transendocytosis dependent on CTLA-4 (26). Tregs also produces cytokines, such as IL-10 and TGFβ, which are known to dampen immune responses (27). These inhibitory molecules may be the reason why T cells may be unresponsive to tumor antigens, and recently they have proven to be very effective therapeutic targets for neutralizing antibodies in some patients with some kinds of cancer (28, 29).

Despite the numerous checkpoints in place to prevent deleterious immune responses towards self-proteins, productive immune responses against foreign pathogens usually develop, and increased knowledge about how these responses occur may lead to improved vaccines. During an immune response to infection or vaccination, a small group of activated T cells and B cells that have received appropriate signals from other cells differentiate into memory cells (30, 31). These memory cells can more rapidly proliferate and respond to a secondary challenge with the same foreign antigen. Each person has a unique pool of memory cells based on the pathogens and vaccines that they have been exposed to during their lifetime. Immune responses also result in the differentiation of long-lived plasma cells. They secrete large quantities of protective antibodies that have undergone the processes of affinity maturation and class switching. Affinity maturation is the process by which antibodies acquire mutations that have an increased ability to bind antigen. Class switching is the process by which an antibody changes its class allowing it to interact with different effector molecules while retaining specificity for antigen. A better understanding of the molecules involved in immune responses will allow immunologists to design more effective vaccines, as well as gain a more thorough understanding of the basic immunology of antibody development.

II. T cell help for B cells

A. *In vivo*

In vivo studies of T cell help for B cells have mainly focused on reactions occurring within germinal centers in mice. The germinal center is divided into light and dark zones due to the cellular composition and density in these areas. The light zone of the germinal center is where T cells interact with B cells to deliver CD40L (32). B cells with the highest affinity receptors are able to outcompete other B cells for survival signals, migrate into the dark zone, and proliferate. The dark zone of the germinal center has the highest density of proliferating B cells (33). The result of the selection processes in the germinal center is the production of high affinity antibodies that can effectively and rapidly resolve a pathogenic challenge upon future exposure. Selection of high affinity antibodies depends upon somatic hypermutation, or the acquisition of random mutations by B cell antibody due to the expression of activation-induced cytidine deaminase (AID). AID has been detected mostly in the dark zone, so somatic hypermuation has been thought to occur at this location (34-36). However these studies are inconclusive as far as the location of the process of affinity maturation, because AID is also detected in the light zone and differences in cell densities and the movement of cells between zones make such calculations difficult (37-41).

Prior to the development of knock-out mice, many of the studies investigating the role of T and B cells *in vivo* during immune responses were performed using irradiation and antibodies to deplete specific lymphocyte populations. Lymphocytes are sensitive to radiation, therefore irradiation of mice depletes all lymphocyte populations. Either B or T cells were then transferred back into these mice and T cell help was assessed by the ability to make antibody responses to the antigen presented during the response. As

mentioned earlier, these experiments determined that both T and B cells were required for a productive antibody response. These early experiments also led to the discovery of the importance of MHC in the development of antibodies (42-44). CD4 helper T cells recognize peptide displayed on MHC class II on APCs. When blocking antibodies are added against MHC II molecules, productive interaction cannot occur between T and B cells and antigen-specific antibodies are not produced. This was shown *in vivo* with the injection of antibodies against MHC Class II molecules (45). Although these early experiments were essential to discovering the nature of T cell/B cell collaboration in antibody development *in vivo,* due to the early timing of the response investigated, they often did not involve germinal center formation, but instead the extrafollicular responses mentioned in the beginning of this chapter.

Recent technological advances in fluorescent microscopy and mouse models have allowed for in depth study of cellular interactions within germinal centers. Two photon microscopy techniques allow cells to be imaged in real time during different time points of an immune response. Differential fluorescent labeling of T and B cells undergoing antigenic challenge has allowed immunologists to determine that interaction times within germinal centers are actually much shorter than previously thought (46-49). Using antibodies for different markers of the light and dark zone and differences in B cell densities in the light and dark zone of the germinal centers, it has become possible to track individual cells as they move between different zones of the germinal center (37, 46). Knock-out mouse models have also provided essential information as to which molecules are required for the development and maintenance of T and B cell interactions

during germinal center formation. These models have determined that the adhesion molecules SLAM **(**Signaling lymphocyte activation molecule) and SAP (SLAM associated protein) are necessary for T/B collaboration and germinal center formation (50-53).

B. *In vitro*

I*n vitro* studies advanced the basic understanding of the way in which T cell recognized B cells for the production of antibodies. The capability of B cells to efficiently process and present antigen bound to their antigen receptors was first demonstrated *in vitro* using a polyclonal model for antigen. In this experiment, rabbit anti-mouse immunoglobulin (Ig) was used in place of antigen because it binds to all mouse B cell receptors and is then processed and presented to helper T cells. The T cells had been primed against the rabbit Ig, so they were able to recognize and respond to B cells presenting this antigen (54).

Early experiments also showed that there were differences in the requirements for T cell help *in vivo* and *in vitro*. In contrast to *in vivo* situations, under non-antigen limiting concentrations, both bystander (cells not displaying antigen) and antigen-specific cells are helped *in vitro*. This was shown to be due to cytokines produced by helper T cells and the high concentration of antigen available to cells in *in vitro* culture conditions (55, 56). Activation of B cells has also been hypothesized to be due to non-specific uptake of antigen by B cells and their ability to present this antigen to T cells as the ability to interact with T cells was necessary for antigen-specific antibody production (57).

Although under certain circumstanced bystander B cells are helped *in vitro*, this response is usually less than antigen-specific interactions within the same culture.

Activation of bystander B cells continues to be an active area of research as this type of activation may be the cause of certain inflammatory diseases like autoimmune diseases. The immune system must maintain the ability to respond to foreign antigens, while preventing inflammatory responses to self-proteins. Recent studies suggest that the activation and expansion of memory B cells that are solely antigen-specific may be due to the limited amount of antigen that is available *in vivo* (58). It has also been suggested that there may be transfer of antigen from the antigen-specific to bystander B cells which could potentially trigger bystander activation (59). Further understanding of the signals involved in activation and selection of memory B cells is needed to understand development of antibodies.

III. CD40L

Although antigen processing and presentation by B cells explained how T cells and B cells could recognize the same antigen leading to changes in both lymphocyte populations, CD40L, the main helper molecule involved in this response, was not discovered until much later. Early investigations in T cell help for B cells lead to the discovery that something present in activated plasma membranes of effector T cells could trigger proliferation of B cells (60). Finally in 1992, the main cytokine necessary for this B cell proliferative response was determined to be CD40L (61). CD40L was identified

using CD40-Ig to probe the activated T cell membranes. CD40-Ig is a fusion protein created from the extracellular domain of CD40 bound to the Fc region of human IgG During the same time, an antibody against CD40L was developed by creating antibodies against antigens differentially expressed on the activated T cell membranes compared to resting T cells (61). This antibody was capable of blocking the B cell proliferation induced by interactions with helper T cells. During the same year, Armitage et al discovered murine CD40L by using CD40-Ig to probe the murine thymoma cell line EL-4 (62). mRNA was extracted from EL-4 cells that had been sorted for their ability to bind CD40 and used to create an expression library that CD40L was cloned from using the CD40-Ig probe. Cells were then transfected with CD40L and they were shown to have the ability to induce B cell proliferation and IgE secretion in the presence of exogenous IL-4.

CD40L also plays a vital role in the development of germinal centers. Without CD40L, somatic hypermutation, the process by which B cell antibodies acquire mutations that allow for selection of higher binding affinity for an antigen, does not occur. In both CD40 (63) and CD40L knock-out mice, germinal centers do not develop (64-66). Additionally, if a mouse is allowed to develop germinal centers and then is injected intravenously at day 6, 8, and 10 post-immunization with a blocking antibody against CD40L, by day 12 post-immunization the germinal centers completely dissolved (67). This is suggested to be due to the fact that CD40L is a necessary survival signal for the germinal center B cells. CD40L is also known to be important in human germinal center development, because people who cannot produce this protein have to be treated with immunoglobulin replacement therapy to reverse their susceptibility to a wide range of respiratory, fungal,

and bacterial infections (68-71). The gene encoding CD40L is found on the X-chromosome so these deficiencies often appear in young boys (71).

Due to crucial role of CD40L in the development of an adaptive immune response, regulation of CD40L mRNA has been an active area of study. Regulation of CD40L mRNA is often described as biphasic (72). During the initiation of CD40L transcription, there is rapid production as well as degradation of CD40L mRNA. The peak production of CD40L mRNA is at 6 hours following TCR stimulation. During this peak mRNA production, mRNA stability is the lowest due to high rates of degradation. After 12 hours, the RNA binding protein, polypyrimidine tract-binding protein (PTB), stabilizes CD40L mRNA expression in both the nucleus and cytoplasm (73) by binding the CU-rich elements in the 3' untranslated region and blocking adjacent signals that promote RNA degradation (74).

IV. Preformed CD40L

Although it is generally thought that CD40L has to be made *de novo* during each T cell interaction with an APC, some studies have revealed an intracellular compartment of preformed CD40L in memory and effector helper T cells that is rapidly mobilized to the cell surface by TCR engagement (75, 76). This would allow for rapid delivery of CD40L during the short interactions between T and B cells recently determined to occur in germinal centers, and previously shown to be necessary for the survival of antigen-specific B cells at these sites. This is similar to how CD107a (LAMP-1) is rapidly expressed at the surface of CD8 cytotoxic T lymphocytes during degranulation following

TCR stimulation. During recognition of antigen on a target cell, degranulation also results in the release of cytotoxic granules necessary to kill target cells. Using antibodies against different subcellular markers, the intracellular compartment containing CD40L was further characterized as a secretory lysosome within both effector and memory CD4 T cells (76). Intracellular CD40L within this compartment colocalized with the proteins lysosomal-associated membrane protein-2 (LAMP-2), Cathepsin D, and Fas Ligand (FasL), but not with CTLA-4 (76). Both FasL and CTLA-4 can be delivered to the T cell surface following TCR stimulation and calcium flux, much like CD40L (77-83). Due to similarities in how these effector molecules are delivered to the T cell surface, they might follow similar paths to the cell surface, using some of the same molecular motors and trafficking proteins. Trafficking of CD40L to the T cell surface upon stimulation through the TCR as well as the functional significance of preformed CD40L is further investigated in Chapter 2 of this dissertation.

V. Immunological Synapse

The immunological synapse is the area of interaction between a T cell and an APC. These cellular interactions are the basis for many of the effects of the adaptive immune response, thereby giving much value to the study of formation of this specialized structure. The study of the immunological synapse has made been more accessible with the development of the planar lipid bilayer system with fluorescently labeled proteins (84, 85). The lipid bilayer mimics an APC in that once a T cell recognizes foreign peptides in

the context of MHC on the bilayer, an immunological synapse is formed. The lipid bilayer consists of lipid and proteins tethered to the lipid membrane via glycosyl-phosphatidylinositol (GPI) linkage. This allows for the proteins to move laterally within the lipid bilayer. The minimal membrane proteins necessary for T cell synapse formation were determined to be the adhesion molecule, ICAM-1, and peptide-loaded MHC (pMHC). T cells added to the lipid bilayer recognize peptide engaged MHC molecules and then adhere to the bilayer through the adhesion molecules, LFA-1 (present on the T cells) and ICAM (the adhesion molecule present on the lipid bilayer). After this interaction occurs, an immunological synapse with distinct zones of interacting proteins forms. The immunological synapse consists of cSMAC (central supramolecular activation cluster), pSMAC (peripheral), and dSMAC (distal) (86). The cSMAC is the area of TCR and pMHC interactions; pSMAC is the area of interaction of adhesion molecules and where larger molecules that are excluded from the cSMAC accumulate; and the dSMAC is most external ring of the immunological synapse (87, 88). TCR signaling precedes synapse formation and actively signaling TCR microclusters initiate peripherally but migrate towards the cSMAC, where signaling stops and TCR is thought to be degraded (89).

Since the discovery of the immunological synapse, much research has been devoted to determining its function. It has been suggested that such a structure could function in asymmetrical division of effector and memory T cells, delivery of effector molecules, or signaling (90-93). Different signaling proteins are known to accumulate in the different areas of the immunological synapse, therefore one could imagine that the immunological synapse functions as a scaffold to recruit appropriate proteins necessary for its function

(93). For example, CD45, which is known to have an inhibitory function in regards to T cell signaling, is excluded from the cSMAC while TCR and activating kinases and associated downstream signaling molecules accumulate there (94, 95). Furthermore, since TCR is degraded at the cSMAC, it has also been suggested to be the site responsible for the modulation of TCR signaling activity during immune responses. It was shown that at high antigen concentrations of antigen TCR is degraded within the synapse, but it also amplified TCR signaling at lower antigen concentrations (96). It has also been suggested that immunological synapse could function in directional secretion of soluble cytokines and cytolytic molecules toward the APC (97). The example often provided is the restricted delivery of cytolytic granules by cytotoxic T lymphocytes to infected targets through the cSMAC in the center of the adhesion ring (98-100). The synapse has also been investigated in terms of asymmetrical cell division (101). The microtubule organizing center and many other proteins including fate-determining transcription factors are recruited to the proximal or distal side of the T cell associated with APC contact. When the cell divides, this segregation allows for the daughter cells to assume different fates: the proximal cell ends up with more effector proteins (effector T cells), while the distal cell may become a precursor of the memory cells.

Recently the immunological synapse has also been compared to intraflagellar transport machinery (102, 103). Although T lymphocytes lack cilia, they have all the major signaling components necessary for this transport system and a few intraflagellar transport proteins and associated motor proteins. Knock-down of these proteins results in impaired T cell activation and accumulation of phosphorylated tyrosine (104). The intraflagellar proteins in the T cell associate with the TCR, and formation of this protein

complex has been shown to be necessary for exocytosis that occurs at the synapse (102). Both cilia and the immunological synapse involve the recruitment of trafficking proteins, motors, microtubules, and signaling molecules necessary for exocytic events that occur at these specialized structures. Therefore the immunological synapse may be a functional homolog to cilia used by non-hematopoietic cell types (102, 103).

Although none of these proposed theories regarding the function of the immunological synapse excludes the others and synapses may have multiple functions as well as others not yet proposed, I describe my investigation of the function of the immunological synapse in the delivery of effector cytokines and induction of signaling in the target APCs in Chapter 4 of this dissertation. Griffiths has shown that the cytotoxic T lymphocytes form an immunological synapse that functions to deliver molecules involved in the processes of killing target cells. Limiting the delivery of cytotoxic effector molecules to the contact area between the cytotoxic T cell and the APC allows the T cell to selectively target individual APCs, and allows bystander, uninfected cells, to escape cell death. We and others propose that CD40L may be delivered in a similar restricted manner to limit help to solely antigen-specific B cells, thereby limiting activation of bystander B cells. As mentioned above, it was thought that cytokines had to be made *de novo* following APC interaction, but Koguchi et al. showed that CD40L is stored in an intracellular compartment and is brought to the T cell surface rapidly following TCR engagement (76). The ability to deliver CD40L rapidly may be necessary during immunological synapse formation between T and B cells in germinal centers, where interaction times are relatively short. The delivery of cytokines within the immunological synapse has been proposed and studied for a few other cytokines, but the

delivery of the transmembrane cytokine CD40L to the APC has not been investigated in detail. Huse et al. showed that IL-2 and IFNγ are delivered in a restricted manner towards antigen-specific cells, while other cytokines including IL-4 and TNFα are delivered multidirectionally, away from the synapse (105). However the precise molecular details involved in this delivery were not investigated in this study, and CD40L was not investigated, so much remains to be revealed about how effector proteins are delivered to APCs. Huse et al. showed that the cytokines that were delivered multidirectionally used different trafficking molecules compared to those delivered in an antigen-specific manner. Rab3d and Rab19 were shown to colocalize with IL-2 and IFNγ, while syntaxin 6 colocalized with TNFα. Other trafficking molecules colocalized with both groups of cytokines. Rho GTPases have also been shown to play an important role in immune synapse formation and delivery of cytokines at the immunological synapse (106, 107). Cdc42 was necessary for actin remodeling and clearance at the immunological synapse, without which IFNγ release at the synapse is inhibited (108, 109).

Inhibiting both T cell polarity and actin clearance at the immunological synapse has been shown to affect the delivery of effector molecules to APCs. A few groups have investigated how actin clearance impacts the release of lytic granules and killing of targets cells by cytotoxic T and NK cell at synapses (110, 111). Similarly, delivery of cytokines by T helper cells is also affected. CD40L-mediated production of IL-12 by the APC was inhibited at the immunological synapse when T cell polarity was blocked (112) It has also been shown that there are different exocytic compartments for delivery of different signaling proteins to the synapse. Lymphocyte-specific protein tyrosine kinase (Lck), a signaling molecule downstream of TCR activation, and linker for activation of T

cells (LAT) are distributed in distinct secretory compartments prior to delivery to the immunological synapse (113). Each depends on different trafficking proteins to be expressed at the immunological synapse. Lck depends on myelin and lymphocyte protein (MAL), while LAT depends on synaptotagmin-7 and calcium. Therefore, it will be important to determine each protein's distinct exocytic compartment and the proteins involved in regulation of different compartments prior to delivery at the immunological synapse. The previously mentioned colocalization study shows that CD40L is contained in a secretory vesicle distinct from CTLA-4 (76).

VI. Immunological Synapse Structure of different Helper T cell Subsets

The immunological synapse structure depends on the subset of CD4 helper T cell engaging with the APC (25, 94, 114). Th1 cells have a bull's eye synapse structure, with the adhesion molecules forming a ring around the central interaction of TCR and pMHC, while Th2 cells have a multifocal synapse structure with multiple TCR-pMHC interaction sites surrounded by zones of interactions of adhesion molecules (Figure 1, (94)). Regulatory T cells also have a distinct immunological synapse structure. Usually they form a motile synapse, which has been termed kinapse in studies of other T cell subsets (85). In this immunological synapse structure the ICAM molecules form a horseshoe structure in the leading edge of the T cell while the TCR molecules trail behind in the uropod (25). The reason for the difference in immunological synapse structure between the Th1 and Th2 cell subsets is not known, and could potentially have to do with the different functional roles that Th1 and Th2 cells play in adaptive immune responses and

affect the delivery of effector molecules, like CD40L. This hypothesis is investigated in Chapter 4 of this dissertation.

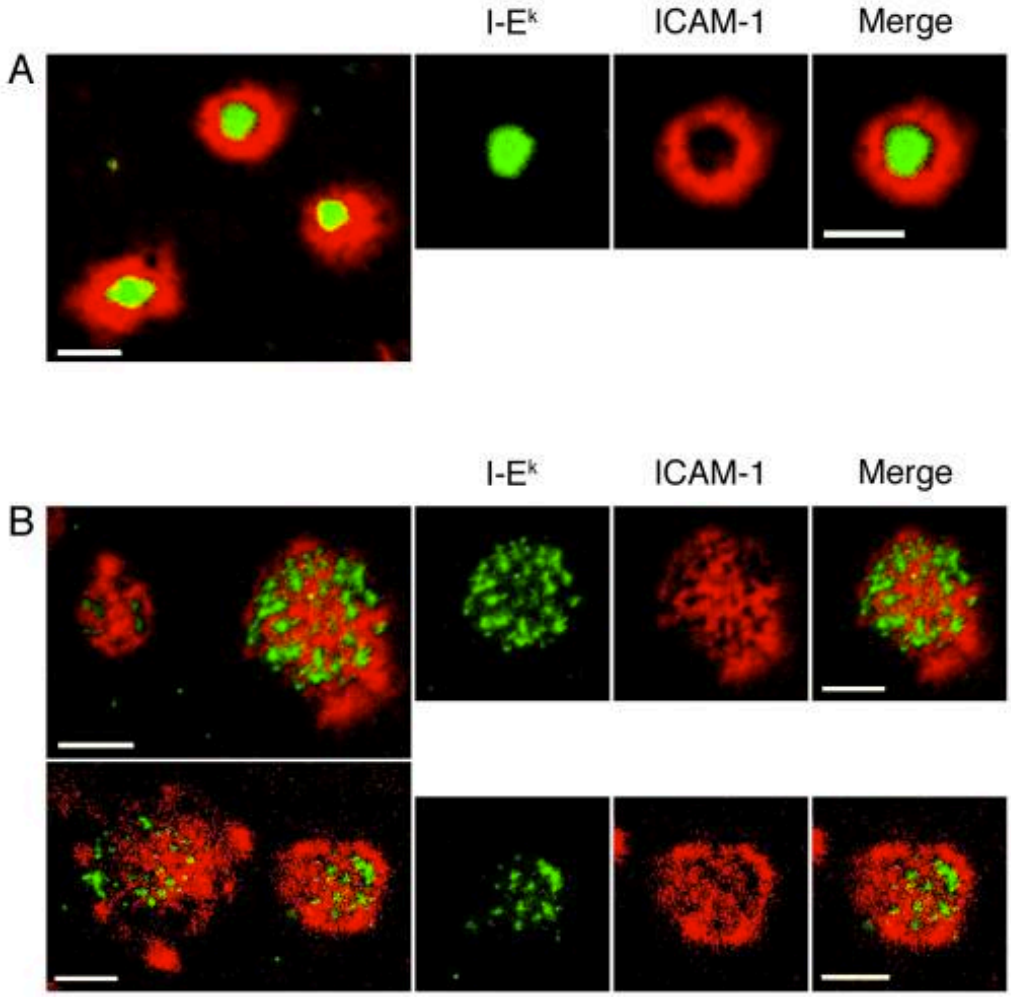
Many new subsets of T helper cells have been described recently, the most relevant for antibody development and T/B cell interaction being T follicular helper cells (Tfh). These cells reside in germinal centers owing to expression of the surface receptor CXCR5 (115). Prior to the discovery of Tfh, Th2 were traditionally thought to be the T cell subset involved in activation of B cells due to fact that these cells secreted IL-4, IL-5, IL-10, and IL-13, all of which promote B cell growth. Therefore it was assumed that Th2 cells were most likely the T cell subset involved in antibody production, while Th1 cells were necessary for clearance of intracellular pathogens like viruses due to the cytokines they produced, including IFNγ and the T cell growth factor, IL-2, and help for cytolytic CD8 T cells. However, this theory was quickly modified with the discovery of T follicular helper cells (Tfhs) in 2005 (116). These effector T cells express the master transcription factor Bcl-6 (117, 118) and are the T cell subset that exists in germinal centers. The development of an IL-21 reporter mouse made further investigation of Tfhs *in vivo* possible because IL-21 is the main cytokine expressed by this T cell population shown to be vital for both Tfh and antibody development (118-121). Interestingly, this subset seems to be heterogeneous in nature in that there are both Th1-like and Th2-like Tfh cells (122). T regulatory follicular helper cells have also been detected in germinal centers and have been shown to be involved in the downregulation of costimulatory molecules on B cells in germinal centers (123). However it still remains to be determined which immunological synapse structure Tfh cells form upon interaction with APCs, which may

provide clues as to the delivery of CD40L within germinal centers, the primary site of T cell dependent antibody development.

VII. Transfer of CD40L upon T cell/APC interaction

Recently the idea of transfer of molecules leading to active signaling in APCs has become an active area of investigation in studies involving the immunological synapse. The center of the immunological synapse was thought to be a region of TCR degradation and limited active TCR signaling. However Michael Dustin's group has shown that TCR microvesicles exocytosed in the synapse and transferred to the B cell membrane are capable of triggering active calcium signaling in antigen-specific B cells (124). This led us to hypothesize that this may also be true for CD40L signaling at the immunological synapse. Transfer of CD40L in a vesicle to antigen-specific B cells would allow those B cells expressing more antigen to outcompete others expressing less antigen. This would explain how antigen-specific B cells are able to quantify the signal they receive from T cells as proposed by Michael Dustin in a recent review (125). This hypothesis is investigated in Chapter 3 of this dissertation. Discoveries in this chapter could greatly modify our ideas about how active CD40L signaling occurs within the immunological synapse, and would be particularly important in the germinal center where interactions are thought to occur over brief periods of time, allowing short interactions to translate into sustained signaling events in B cells resulting in selection of high affinity B cells for differentiation into long-lived plasma cells and memory B cells.

Figure:



This figure is from Thauland, T.J., Y. Koguchi, S.A. Wetzel, M.L. Dustin, and D.C. Parker. 2008. Th1 and Th2 cells form morphologically distinct immunological synapses. J. Immunol. 181:393-399

Figure 1. Th1 and Th2 cells have distinct immunological synapse structures. A, Th1 cell have a bull's eye synapse structure. B, Th2 cells have a multifocal synapse structure. The left panels show 3 Th1 cell synapses (A) and 4 Th2 cell synapses (B, 2 in each frame). The three panels to the right in A and B show a single Th1 (A) or Th2 cell synapse (B). The panel labeled I-E^k shows the accumulation of the fluorescently labeled I-E^k (MHC) molecules within each synapse. The panel labeled ICAM-1 shows the accumulation of fluorescent ICAM-1 within the synapse of Th1 (A) and Th2 (B) synapses. The panel labeled merged shows both fluorescent ICAM and I-E^k within the same synapse.

Chapter 2: Cyclosporine-resistant, Rab27a-independent Mobilization of Intracellular Preformed CD40L Mediates Antigen-specific T Cell Help In Vitro

This chapter was published in the July 15, 2011 issue of the Journal of Immunology (126). I am second author and performed and analyzed experiments which resulted in Figures 2, 3, 4A, 5, and 6 as numbered in this dissertation. I was also involved in the editorial process prior to acceptance of the final manuscript.

Abstract: CD40L is critically important for the initiation and maintenance of adaptive immune responses. It is generally thought that CD40L expression in $CD4^+$ T cells is regulated transcriptionally and made from new mRNA following antigen recognition. However, recent studies with two-photon microscopy revealed that the majority of cognate interactions between effector $CD4^+$T cells and APCs are too short for de novo synthesis of CD40L. Given that effector and memory $CD4^+$T cells store preformed CD40L (pCD40L) in lysosomal compartments and that pCD40L comes to the cell surface within minutes of antigenic stimulation, we and others have proposed that pCD40L might mediate T cell-dependent activation of cognate APCs during brief encounters in vivo. However, it has not been shown that this relatively small amount of pCD40L is sufficient to activate APCs, owing to the difficulty of separating the effects of pCD40L from those of de novo CD40L and other cytokines in vitro. Here we show that pCD40L surface mobilization is resistant to cyclosporine or FK506 treatment, while de novo CD40L and cytokine expression are completely inhibited. These drugs thus provide a tool to dissect the role of pCD40L in APC activation. We find that pCD40L mediates selective

activation of cognate but not bystander APCs in vitro and that mobilization of pCD40L does not depend on Rab27a, which is required for mobilization of lytic granules. Therefore, effector $CD4^+$T cells deliver pCD40L specifically to APCs on the same time scale as the lethal hit of CTLs but with distinct molecular machinery.

Introduction:

CD40L (CD154), a member of the TNF superfamily, mediates T cell help for APCs during humoral and cellular immune responses (127-130). It is generally thought that CD40L is synthesized from new mRNA (de novo CD40L) and delivered while effector $CD4^+$ T cells are engaged in intimate interactions with cognate APCs in the time frame of a few hours. However, we and others have demonstrated that human and mouse effector and resting memory $CD4^+$ T cells retain preformed CD40L (pCD40L) intracellularly, and that pCD40L can come to the cell surface within a few minutes of antigenic stimulation (75, 76).

Given that the interactions between effector $CD4^+$T cells and APCs are typically brief in vivo (46, 51, 131-133), we propose that pCD40L is rapidly delivered to cognate APCs on a time scale of minutes. Our previous study (76) showed that pCD40L is stored in lysosome- related organelles known as secretory lysosomes, a category of secretory vesicles which includes the lytic granules in CTLs and NK cells (134). Lytic granules are secreted through the center of adhesion ring of the immunological synapse formed between the T cell and target cell (135), indicating that the immunological synapse may serve to ensure efficient delivery of effector functions to specific targets but not to bystanders (114, 136). Although the findings mentioned above suggest that effector $CD4^+$ T cells may selectively activate cognate APCs by directional delivery of pCD40L, it remains to be shown that pCD40L is delivered to the cell surface in sufficient amounts to activate APC, and is not merely in the process of being degraded in lysosomes following

CD40 engagement and internalization (137). Also, it has not been addressed whether compartments containing pCD40L use the same trafficking machinery used by lytic granules in CTLs and NK cells, such as the small GTPase, Rab27a (138).

In the present study, taking advantage of selective suppression of de novo CD40L and cytokine expression by the calcineurin inhibitors, cyclosporine A (CsA) and FK506, we show that pCD40L from effector $CD4^+$T cells is sufficient to activate APCs and provide help selectively to cognate APCs. We also show that the delivery of pCD40L by effector $CD4^+$T cells depends on distinct molecular machinery from that required for delivery of lytic granules to target cells by CTLs and NK cells.

Materials and Methods:

Mice:

Mice were housed under specific pathogen–free conditions. These studies were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University. BALB/c, C57BL/6, C.B-17 *(Igh^{b}), Cd40lg*, CD45.1 congenic, and DO11.10 mice were from Jackson Laboratories (Bar Harbor, ME). *Cd40lg$^{-/-}$* DO11.10 mice were bred in-house. DO11.10 *Rag2$^{-/-}$* and BALB/c *nu/nu* mice were obtained from Taconic Farms (Germantown, NY). Ashen mice on a C57BL/6 background were obtained from Dr. Miguel Seabra (Imperial College London, United Kingdom). SMARTA mice, which have a transgenic TCR specific for a lymphocytic choriomeningitis virus (LCMV) epitope (139), were obtained from Dr. J. Lindsay Whitton (The Scripps Research Institute). *ash*/+ or *ash/ash* SMARTA mice were bred in-house. *Cd40$^{-/-}$* spleens were provided by Dr. David Hinrichs (Veterans Affairs Medical Center, OR).

Antibodies and reagents:

PE-Cy7-anti-Fas was purchased from BD Biosciences (San Jose, CA). FITC-PNA was from Vector Lab. Inc. (Burlingame, CA). All other antibodies for flow cytometry were purchased from eBioscience (San Diego, CA). Recombinant cytokines were purchased from Peprotech (Rocky Hill, NJ). Anti-IL-4 was from Bio X Cell (West Lebanon, NH). Easy Sep mouse CD4^{+} enrichment and B cell enrichment kits were from Stemcell

Technologies (Vancouver, Canada). Papain was from Calbiochem (San Diego, CA). Endotoxin-free OVA protein was from Profos AG (Regensburg, Germany). OVA peptide (323-339) was from AnaSpec, Inc. (Fremont, CA). LCMV peptide gp67 was from New England Peptide (Gardner, MA). lipopolysaccharides (LPS [L6761]), phorbol myristate acetate (PMA), ionomycin, CyclosporinA (CsA), and FK506 were from Sigma-Aldrich (St Louis, MO).

In vitro-generated Th1 cells:

In vitro-generated Th1 cells were prepared by culturing spleen cells from DO11.10 mice in the presence of 1 μM of antigenic peptide (OVA 323-339) for 4 days in the presence of 1 ng/ml IL-12 and 10 μg/ml anti-IL-4. To prepare *ash*/+ and *ash/ash* polyclonal Th1 cells, purified $CD4^+$T cells were incubated with Mouse T-Activator CD3/CD28 beads (Invitrogen, Carlsbad, CA) at a 1:1 ratio for 4 days in Th1 conditions.

In vivo-generated effector $CD4^+$ T cells:

In vivo-generated effector $CD4^+$ T cells were obtained from the draining lymph nodes (dLNs) of BALB/c *nu/nu* recipients which had received 5 x 10^5 naïve purified $CD4^+$ T cells from DO11.10 *Rag2*$^{-/-}$ mice followed by subcutaneous immunization with OVA protein (50 μg) plus papain (50 μg) (140). For Fig.7, antigen-specific in vivo Th1 cells were recovered on day 8 post-infection from LCMV (i.p. infection with 2 x 10^5 PFU of LCMV Armstrong 53b)-infected recipient C57BL/6 mice given 2 x 10^4 *ash*/+ or *ash/ash* SMARTA cells (76).

Flow Cytometry:

The surface mobilization assay was described previously (76). Briefly, in the surface mobilization assay, fluorochrome-labeled anti-CD40L mAb is included in the culture during the activation of cells at 37 °C. Compared to the "snap shot" nature of conventional staining at 4 °C after completion of stimulation, the mobilization assay captures CD40L that has been delivered to the cell surface during stimulation while blocking CD40- dependent internalization, thereby providing the "long exposure" view of CD40L expression. By limiting the stimulation period to 30 minutes, we were able to exclude the expression of de novo CD40L as shown previously (76). Data were obtained with an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

In vitro and ex vivo T helper assay for B cells:

In vitro Th1 cells and in vivo effector $CD4^+$ T cells were generated as described above. WT or *Cd40*$^{-/-}$ spleen cells were differentially labeled with CFSE (141) or the CellTrace Violet dye (Invitrogen) to distinguish cognate (peptide-pulsed) and bystander (unpulsed) populations. Antigenic peptide was pulsed at 1 μM concentration at 37 °C for 2 hours followed by extensive washes. WT or *Cd40lg*$^{-/-}$ *in vitro*-generated Th1 or in vivo-generated effector $CD4^+$ T cells were pretreated with 1 μM CsA at 37 °C for one hour and co-incubated with a mixture of cognate and bystander spleen cells from WT or *Cd40*$^{-/-}$ mice in the presence of CsA throughout the assay. After the indicated hours of incubation, B cell activation was evaluated by staining for CD86, MHC class II, ICAM-1, CD62L, and IL-21 receptor. For analysis of cell division, cognate and bystander B cells were labeled with the same concentration of CFSE and were distinguished by IgM allotype (IgM^a versus IgM^b), and cell division was measured by CFSE dilution. For

analysis of differentiation, cognate and bystander B cells were labeled with the same concentration of CFSE and were distinguished by the CD45 congenic allelic marker (CD45.1 versus CD45.2). The T helper assay was conducted in the presence of IL-4 for 3 days to assess proliferation by CFSE dilution, and in the presence of IL-4 (day 0-4) plus IL-21 (day 2-4) to assess B cell differentiation by staining for Fas, GL7, and CD138. Alternatively, cognate and bystander B cells were distinguished by differentially labeling them with CFSE or CellTrace Violet dye.

In vitro T helper assay for dendritic cells (DCs):

Bone marrow-derived DCs (BMDCs) were generated as previously described (142). Drug pretreated or untreated WT or *Cd40lg*$^{-/-}$ in vitro-generated Th1 cells were co-cultured with BMDCs in the presence or absence of antigenic peptide, CsA (1 μM) or FK506 (0.1 μM), and isotype control Ab or anti-CD40L for 18 hours. The level of CD70 on DCs was analyzed by flow cytometry. IL-6 production from DCs was analyzed by ELISA (eBioscience).

Statistics:

p values were determined by the unpaired Student's t test. All results are shown as the mean and the standard deviation of the mean. A p value of < 0.05 was considered significant.

Results:

Calcineurin inhibitors block de novo CD40L and cytokine synthesis but do not interfere with pCD40L surface mobilization

We proposed that pCD40L has a physiological role to selectively activate cognate APCs during brief encounters often observed in vivo. Because de novo CD40L and cytokines can cause general activation of APCs regardless of antigen-specificity *in vitro*, we sought culture conditions in which *de novo* CD40L and other cytokines made by Th1 cells are blocked, but surface mobilization of pCD40L is preserved. Although we used cycloheximide to block *de novo* CD40L and cytokines but to preserve pCD40L in the previous study (76), cycloheximide could not be used in the present study because it inhibited the upregulation of activation markers on APCs, as expected (data not shown). We also conducted experiments with the irreversible protein synthesis inhibitor, emetine. However, we found that T cells pretreated with emetine and extensively washed released sufficient emetine to inhibit APC activation (data not shown). We then tested the calcineurin inhibitors, CsA or FK506, known to inhibit *de novo* CD40L expression (143). CsA does not interfere with CD40-mediated B cell activation by fixed, activated $CD4T^+$ cells (144) or agonistic anti-CD40 (data not shown). These drugs also inhibit T cell production of IFN-γ and IL-4 (145). Upon stimulation with PMA plus ionomycin, Th1 cells showed evident pCD40L mobilization at 30 minutes (Fig. 2A, left) as well as robust induction and surface expression of de novo CD40L at 120 minutes (Fig. 2A, right). CsA or FK506 pre-treatment completely inhibited activation-induced expression of de novo CD40L but had no effect on surface mobilization of pCD40L (Fig. 2B). The efficacy of

CsA and FK506 was confirmed by complete suppression of IFN- γ production throughout this study as shown in Fig. 2C.

pCD40L in Th1 cells selectively activates cognate B cells and promotes their proliferation and differentiation *in vitro*

To determine whether surface mobilization of pCD40L is sufficient for antigen- specific T cell help for B cells, we measured B cell activation. Cognate (antigenic peptide-pulsed) and bystander (unpulsed) B cells were differentially labeled with CFSE, and were cultured with Th1 cells in the presence of CsA. *Cd40*$^{-/-}$ B cells were used as a negative control in separate cultures. CsA was maintained in the culture medium throughout the incubation period. CD40L-dependent up-regulation of CD86, MHC class II, and ICAM-1 (CD54), and down-regulation of CD62L were observed in cognate butnot in bystander B cells after overnight culture (Fig. 3A). Increased size (forward scatter) and granularity (side scatter) were also observed in cognate B cells only (data not shown). By day 2, cognate but not bystander B cells up-regulate IL-21 receptor (IL-21R) in a CD40-dependent manner (Fig. 3B). By these measures of B cell activation, the stimulatory capacity of pCD40L (with CsA) was comparable to that of *de novo* CD40L (no drug). We also observed a weak but noticeable level of bystander activation of B cells by *de novo* CD40L, but not by pCD40L, when assessed by ICAM-1 and IL-21R levels.

To determine whether pCD40L is sufficient to initiate T cell-dependent B cell proliferation, IL-4 was added to WT or *Cd40lg*$^{-/-}$ Th1 cells co-cultured with B cells for 3 days in the presence of CsA, and CFSE dilution was analyzed for both cognate and bystander B cells. Cognate and bystander B cells were differentiated by IgM allotype.

The data show that Th1 cells promote proliferation of cognate but not bystander B cells in a CD40L-dependent manner in the presence of CsA (Fig. 4A). We obtained the same result when the IgM allotypes of cognate and bystander B cells were switched (data not shown). Both cognate and bystander B cells responded well with anti-CD40 plus IL-4 stimulation (Fig. 4A far right). When antibody was used to neutralize CD40L instead of using *Cd40lg*$^{-/-}$ Th1 cells, we obtained a similar result (data not shown). The ability of pCD40L to induce B cell proliferation in the presence of IL-4 is slightly inferior to that of de novo CD40L (no drug). We also tested whether pCD40L is sufficient to induce B cell differentiation in cultures containing IL-4 (added on day 0) and IL-21 (added on day 2). Cognate B cells but not bystander B cells acquired surface markers of germinal center (GC) B cells (Fas^{+}GL7^{+}) and plasma cells (CD138^{+}) in a CD40-dependent manner (Fig. 4B,C). The ability of pCD40L to induce germinal center and plasma cell markers is comparable to that of de novo CD40L. These results clearly show that pCD40L in Th1 cells is sufficient to mediate antigen-specific B cell activation and promote proliferation and differentiation of cognate B cells in concert with IL-4 and IL-21.

In vivo-generated effector CD4^{+}T cells can selectively activate cognate B cells via pCD40L

Although we observed that pCD40L in Th1 cells generated *in vitro* can mediate cognate B cell activation, we were concerned that this finding could be an artifact of T cells activated in vitro. Therefore, we conducted a similar experiment using in vivo-generated effector CD4^{+}T cells. To generate effector CD4^{+}T cells in vivo, purified naïve CD4^{+} T cells from DO11.10 *Rag2*$^{-/-}$ mice were transferred into nude mice, followed by subcutaneous immunization with papain plus OVA. Six days after immunization, cells

from dLNs showed a robust expansion of effector $CD4^+$T cells as well as a vigorous GC reaction in an immunized mouse (Fig. 5A) compared to an unimmunized nude mouse (Fig. 5B), indicating that in vivo-generated effector $CD4^+$T cells are capable of helping B cells in vivo. The expanded $CD4T^+$ cells are $CD44^{hi}$ effector cells and possess abundant pCD40L (Fig. 5C and data not shown). Highly purified (> 95%) in vivo-generated effector $CD4^+$T cells were used in an *ex vivo* T helper assay in the presence of CsA. Like in vitro-generated Th1 cells, *in vivo*-generated effector $CD4^+$T cells are capable of activating cognate, but not bystander, B cell activation in a pCD40L-dependent manner as shown by increased ICAM-1 expression (Fig. 5D).

pCD40L can trigger DC activation

Another important function of CD40L is licensing of DC, which is crucial for generating effective $CD4^+$ and $CD8^+$ T cell responses (130). Taking advantage of the fact that in vitro-generated Th1 cells do not produce detectable IL-6 (data not shown), we measured IL-6 production from bone marrow-derived DCs (BMDCs) to evaluate the ability of pCD40L to activate DCs. WT or *Cd40lg*$^{-/-}$ DO11.10 Th1 cells were co-cultured with BMDCs in the presence or absence of antigenic peptide and CsA or FK506 for 18 hours. Although the magnitude of IL-6 production is significantly lower in the CsA- or FK506-treated groups than the untreated group, treated Th1 cells still clearly induce IL-6 production from BMDCs in a peptide dose- and CD40L-dependent manner (Fig. 6A). CsA or FK506 treatment did not inhibit IL-6 production from DCs upon LPS stimulation (data not shown). To exclude potential differences between WT and *Cd40lg*$^{-/-}$ Th1 cells other than CD40L itself, we conducted similar experiments using WT *in vitro*-generated Th1 cells in the presence of either control Ab or neutralizing anti-CD40L. This

experiment yielded a similar result (Fig. 6B). We also evaluated expression of CD70, which is known to be upregulated by a CD40 signaling and facilitates primary and secondary $CD8^+$ T cell responses (146). We observed that Th1 cells induced upregulation of CD70 on DCs in the presence of antigenic peptide in a CD40L-dependent fashion in the presence of CsA or FK506 to levels comparable to the drug untreated group, suggesting that pCD40L is sufficient to efficiently induce CD70 upregulation on DCs (Fig. 6C). The efficacy of CsA and FK506 was confirmed by complete suppression of T cell IFN-γ production induced by DCs in the presence of antigenic peptide (Fig. 6D). Together, these results show that pCD40L can also mediate T cell-dependent DC activation.

The mobilization of pCD40L uses distinct machinery from that of lytic granules

Our previous study indicated that pCD40L exists in secretory lysosomes, a category of secretory vesicles which includes the lytic granules of CTLs and NK cells (76). To investigate the molecular mechanism of pCD40L mobilization from cytoplasm to cell surface, we used ashen mice, which are defective in release of lytic granule contents due to a mutation in the small GTPase Rab27a (138). First, we compared the surface mobilization of pCD40L with CD107a in polyclonal Th1 cells generated with $CD4^+$T cells from ash/+ or ash/ash mice. The mobilization of CD107a to the cell surface is an indication of the release of lytic granules (147). As expected, ash/ash Th1 cells have defective CD107a mobilization following all three stimuli tested (Fig. 7A). However, the mobilization of pCD40L is unimpaired in *ash/ash* Th1 cells (Fig. 7B). To confirm this phenotype using *in vivo*-generated effector $CD4^+$T cells, ashen mice were bred with

SMARTA TCR transgenic mice. WT recipient mice of *ash*/+ or *ash/ash* SMARTA $CD4^+$ T cells were infected with LCMV. On day 8 post-infection, splenocytes from infected animals were used *ex vivo* for evaluation of pCD40L mobilization (Fig. 8A, B). While the mobilization of CD107a is clearly impaired in *ash/ash* SMARTA effector $CD4^+$ T cells following antigenic stimulation (Fig. 8C), pCD40L mobilization is maintained (Fig. 8D). We conclude that effector $CD4^+$ T cells use distinct machinery for the mobilization of pCD40L from that of lytic granules.

Discussion:

In vivo studies using CD40L knockout mice and neutralizing anti-CD40L antibody have firmly established that CD40L mediates T cell help (128). In the classic model, effector $CD4^+$T cells deliver de novo CD40L, along with cytokines, to cognate APCs during prolonged interactions lasting hours. However, recent imaging studies with two-photon microscopy show clearly that effector $CD4^+$ T cells do not usually have enough time to synthesize *de novo* CD40L. Given that effector $CD4^+$ T cells also store intracellular pCD40L and mobilize it to the cell surface immediately after TCR stimulation, we proposed that pCD40L can mediate cognate APC activation during the brief antigen-specific interactions that dominate *in vivo*. In the current study, we blocked the synthesis of *de novo* CD40L and soluble cytokines while preserving the surface mobilization of pCD40L in effector $CD4^+$ T cells, and demonstrated that pCD40L can selectively deliver T cell help to cognate B cells and trigger DC activation.

We speculate that effector $CD4^+$ T cells deliver help to antigen-specific B cells using pCD40L at the T zone-B zone boundary, at extrafollicular sites, and in the GC (129). *In situ* staining studies have detected CD40L staining in all of the locations listed above (75, 148). Studies with neutralizing anti-CD40L clearly show that CD40L is indispensable not only for the initiation but also for the maintenance of GCs, as well as the differentiation and affinity maturation of GC B cells (149, 150). However, we think that pCD40L plays a minor role during stable interactions between effector $CD4^+$ T cells and cognate B cells owing to the opportunity for abundant delivery of *de novo* CD40L and cytokines. B cells undergoing stable interaction with T cells seem to be poised to differentiate into plasma

cells. Several studies have shown that high affinity B cells preferentially become plasma cells (1, 151, 152). It is believed that high affinity B cells gather, process, and present more antigen (38, 153). The immunological synapse formed by T cells is a versatile structure which can be re-oriented from APCs bearing fewer peptide/MHC to those bearing more peptide/MHC (154). A study with two-photon microscopy estimated that each cognate GC B cell encounters as many as 50 T cells per hour (46), allowing T cells to form stable interactions with rare high affinity GC B cells (37). During stable interaction, GC B cells would receive a prolonged CD40 signaling as well as cytokines including IL-21 (155). Since IL-21 induces both the transcriptional factors necessary for maintaining GC B cell phenotype (Bcl-6) and plasma cell differentiation (Blimp-1) (156-158), and these two transcriptional factors counter-regulate each other (159), it is likely that a prolonged CD40 signaling owing primarily to *de novo* CD40L would promote plasma cell differentiation of GC B cells by repressing Bcl-6 via upregulation of IRF-4 expression (160).

We propose that pCD40L plays a major role in facilitating the affinity maturation process during brief interactions between low affinity GC B cells and T cells in the light zone of GCs, as suggested previously (75). Compared to high affinity B cells, low affinity B cells preferentially seed GC reactions (1, 151). Increased peptide/MHC expression on GC B cells regardless of BCR affinity via DEC205-mediated Ag delivery enhanced plasma cell differentiation and compromised affinity maturation (49). This result may be explained partly by prolonged CD40 signaling owing to forced stable interactions between low affinity GC B cells and T cells. Similarly, heightened CD40 signaling caused by agonistic anti-CD40 or a CD40L transgene in B cells also resulted in skewed short-lived plasma

cell differentiation of GC B cells accompanied by premature termination of GC reactions, compromised affinity maturation, and diminished generation of long-lived plasma cells (161-163). A recent study suggested a model that explains how transient CD40 signaling facilitates affinity maturation of GC B cells by deletion of cells with damaged DNA (164). Brief CD40 signaling can induce temporary disruption of Bcl-6 repressor functions, promoting rapid, transient expression of DNA checkpoint genes and subsequent apoptosis of GC B cells with unrepaired DNA lesions (164). In fact, one quarter of T cells in GCs are associated with blebs from dead GC B cells (46). In this scenario, surviving GC B cells may undergo further affinity maturation by rapidly recovering Bcl-6 function (165). Whether a low avidity T cell-B cell interaction is sufficient to induce the mobilization of pCD40L, as has been reported for preformed Fas ligand (pFasL) in CTLs (79), needs to be addressed experimentally. A recent study showed that a transient BCR signaling increases B cell sensitivity to CD40L (166). This mechanism may also play an important role for optimizing a pCD40L-mediated selection process. Enrichment of both BCR and CD40 signaling signatures in the light zone compared to the dark zone of GC by microarray analysis further supports this notion (49). Although our data showed that pCD40L could promote acquisition of plasma cell markers by cognate B cells, this may simply reflect favorable conditions for T cell help *in vitro* owing to the lack of the germinal center environment that enforces intermittent CD40 signaling. Nevertheless, our data reveal the competence of pCD40L for B cell activation.

Another important function of pCD40L may be DC licensing. Two-photon microscopy shows predominantly brief interactions of effector memory $CD4^+$ T cells with APCs in target tissues (132), suggesting a role for pCD40L during the effector phase of

inflammation, probably through promoting cytokine secretion and upregulation of costimulatory molecules in DCs. Importantly, pCD40L has been implicated in the pathogenesis of rheumatoid arthritis (167). In this study, peripheral blood $CD4^+$ T cells and synovial fluid $CD4^+$ T cells from patients with rheumatoid arthritis, but not healthy donor $CD4^+$ T cells, stored and mobilized pCD40L. Why healthy donor $CD4^+$ T cells lacked pCD40L in this study is unknown, although it is possible that the sensitivity of the assay and/or human-mouse differences are contributing factors. The authors further showed, using stimulated and fixed patient $CD4^+$ T cells, that the amount of pCD40L that came to the cell surface upon stimulation with PMA plus ionomycin was sufficient to trigger IL-12 production from DCs. CsA-insensitive CD40L, presumably pCD40L, has been detected on $CD4^+$ T cells from systemic lupus erythematosus patients (168, 169). These findings suggest a role for dysregulation of pCD40L in autoimmune settings. Possible regulatory functions of pCD40L (for example, induction of IL-10 production from macrophages (170) or dampening innate immune responses by inhibiting inflammasomes (171) should also be included in considering pCD40L as a therapeutic target for autoimmune or inflammatory diseases.

The mobilization of pCD40L is Rab27a-independent, indicating that pCD40L is stored in compartments distinct from lytic granules. Since the acquisition of lytic granules is limited to effector T cells (134), our recent finding of pCD40L expression in CD4 single positive thymocytes, naïve $CD4^+$ T cells, and resting memory $CD4^+$ T cells provides further evidence that the pCD40L-containing compartment is distinct from lytic granules (76)Koguchi et al., manuscript in preparation). Although pFasL has been observed in lytic granules (172), recent reports indicate that pFasL also resides in compartments

distinct from lytic granules because surface expression of pFasL is resistant to a microtubule inhibitor that blocks release of lytic granules (77) and occurs at a lower stimulation threshold than that of lytic granules (79). Our previous study showed that pCD40L is colocalized strongly with FasL in Th1 cells (76). Together, these results suggest the existence of a distinct trafficking mechanism for rapid delivery of pCD40L and other TNF family members to the cell surface in $CD4^+$ T cells. Evidence is accumulating that other TNF superfamily members are stored in secretory compartments in hematopoietic cells, including CD70 and CD40L in DCs (173, 174) and pCD40L and LIGHT in platelets (175, 176). Investigation of this trafficking machinery will provide methods to dissect the functions of preformed versus newly synthesized CD40L *in vivo* through the generation of knockout and transgenic mice lacking one or the other. It might also offer insight into the regulation of the many biological functions mediated by other TNF family members. Such efforts will provide new therapeutic targets for immunosuppression, including methods to further increase the efficacy of calcineurin inhibitors for established inflammatory conditions by blocking residual T cell-dependent APC activation triggered by pCD40L.

Figures:

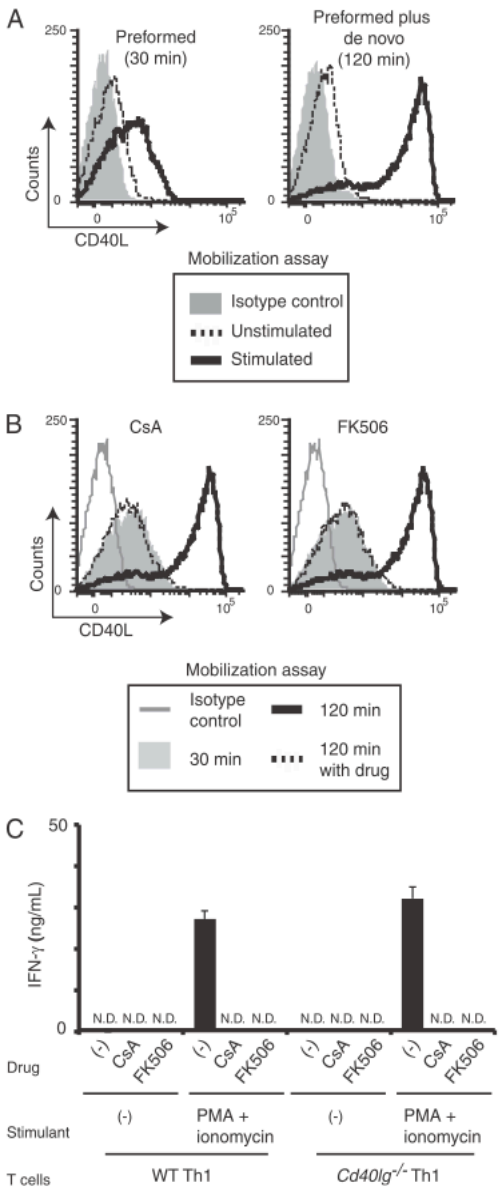


Figure 2. Calcineurin inhibitors block *de novo* CD40L expression and cytokine secretion but not surface mobilization of pCD40L. A, Levels of CD40L mobilization during a 30-min incubation (left, preformed CD40L) and a 120-min incubation (right, preformed plus *de novo* CD40L) upon PMA plus ionomycin stimulation. B, The effect of CsA (left) or FK506 (right) on the levels of CD40L mobilization upon 30- or 120-min PMA plus ionomycin stimulation. C, WT or *Cd40lg*$^{-/-}$ Th1 cells were incubated with or without PMA plus ionomycin overnight in the presence or absence of CsA or FK506. IFN-g production was measured by ELISA with a detection limit of < 0.06 ng/ml. Each bar represents the mean 6 SD for triplicates. Data are representative of three independent experiments (A, B) or at least five independent experiments (C). N.D., not detected.

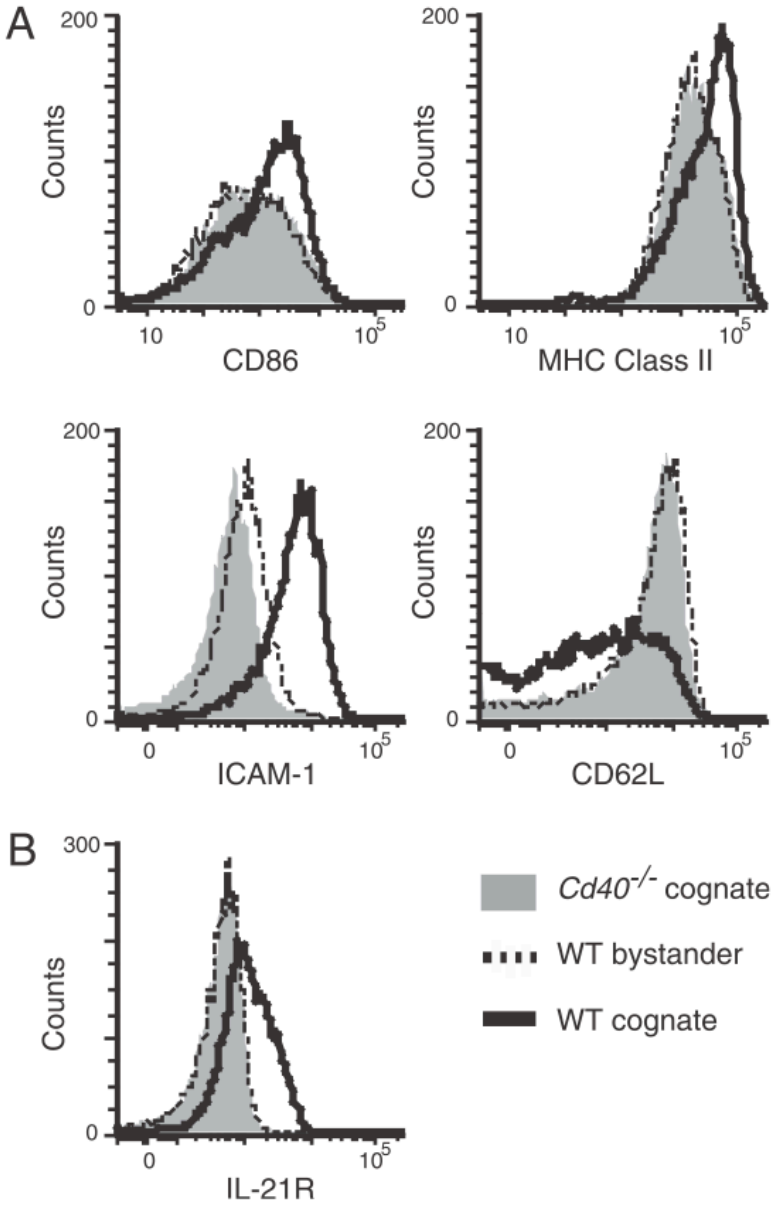


Figure 3. pCD40L in Th1 cells is sufficient to mediate selective activation of cognate B cells. For the *in vitro* T helper assay, CsA-pretreated Th1 cells were mixed with cognate (peptide-pulsed) and bystander (unpulsed) spleen cells for 18 h (A) or 2 d (B). As a control, *Cd40*$^{-/-}$ spleen cells were used. The levels of CD86, MHC class II, ICAM-1 (CD54), CD62L (A), and IL-21R (B) in control, bystander, and cognate B cells are shown. Data are representative of five independent experiments.

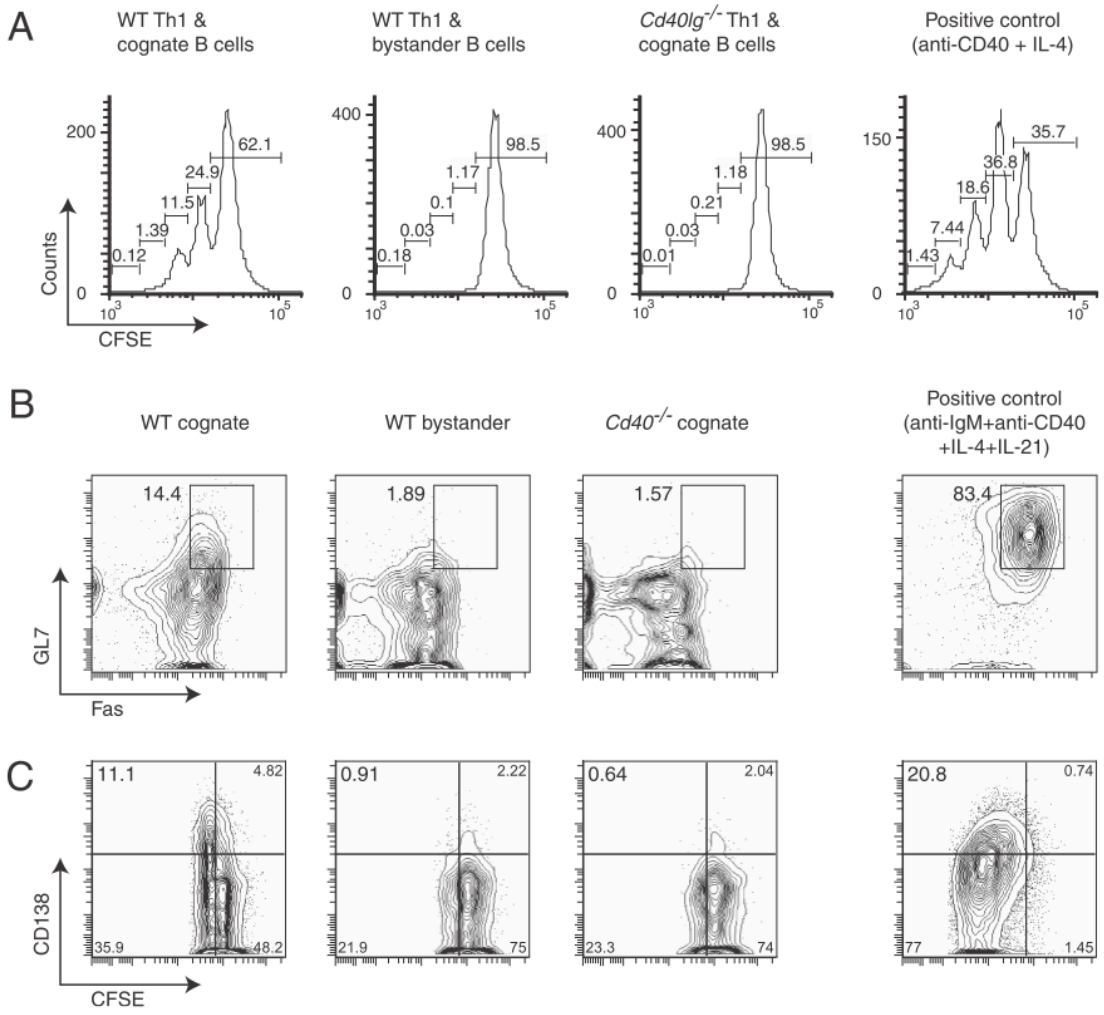


Figure 4. pCD40L in Th1 cells can mediate T cell-induced selective proliferation and differentiation of cognate B cells. Exogenous helper cytokines were added to assess the potential of pCD40L to support B cell proliferation and differentiation. A, CsA- treated WT or *Cd40lg*$^{-/-}$ Th1 cells were incubated with CFSE-labeled bystander (IgM^{a}) plus cognate (IgM^{b}) B cells in the presence of IL-4. CsA was added daily throughout the culture period. As a positive control, B cells were stimulated with anti-CD40 plus IL-4 in the presence of CsA. On day 3, CFSE dilution was evaluated. B and C. CsA-treated Th1 cells were incubated with CFSE-labeled bystander (CD45.2^{+}) and cognate (CD45.1^{+}) B cells in the presence of IL-4 (days 0–4) and IL-21 (days 2–4). As a control, *Cd40*$^{-/-}$ B cells were used. CsA was added daily throughout the culture period. As a positive control, B cells were stimulated with anti-IgM, anti- CD40, IL-4, and IL-21 in the absence of CsA. On day 4, GC markers (GL7 and Fas, B) and the plasma cell marker (CD138, C) were evaluated. Data are representative of four independent experiments.

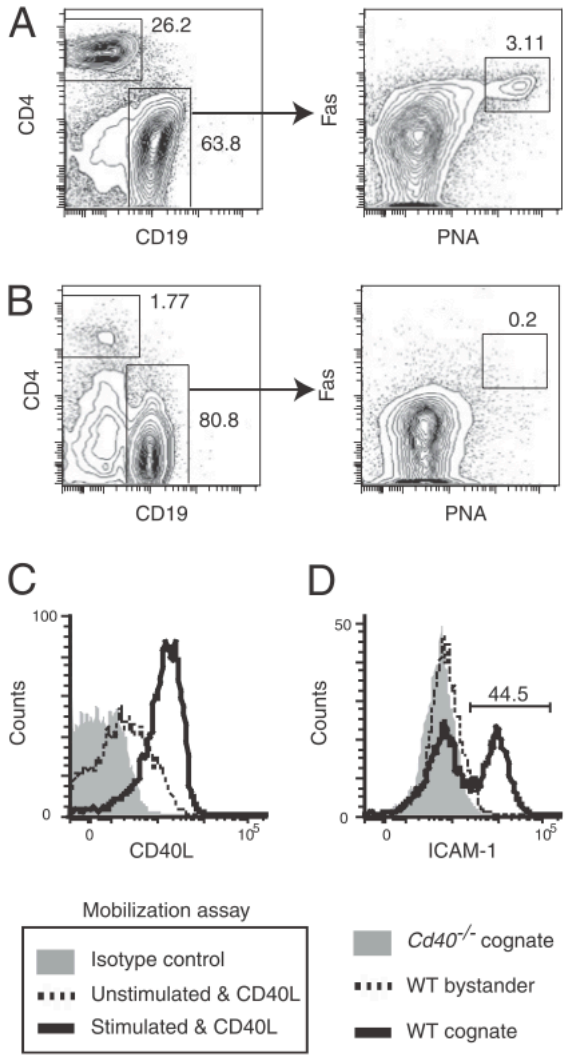


Figure 5. pCD40L in *ex vivo* effector CD4$^+$T cells is sufficient to mediate selective activation of cognate B cells. A and B, Naive CD4$^+$T cells from DO11.10 *Rag2*$^{-/-}$ mice were transferred into BALB/c *nu/nu* recipient mice followed by s.c. immunization with papain plus OVA to obtain *in vivo*-generated effector CD4$^+$T cells. The percentages of CD4$^+$T cells, B cells, and GC (Fas$^+$, PNA$^+$) B cells in dLNs from a BALB/c *nu/ nu* immunized recipient on day 6 after immunization (A) or from a naive BALB/c *nu/nu* mouse (B) are shown. C. The mobilization assay for pCD40L in donor CD4$^+$T cells from a BALB/c *nu/nu* immunized recipient. D, Purified CD4$^+$T cells from BALB/c *nu/nu* immunized recipients were incubated overnight in the presence of CsA with cognate and bystander WT B cells, or cognate *Cd40*$^{-/-}$ B cells, differentially labeled with CFSE. ICAM-1 expression on each B cell population was analyzed. Numbers in histograms indicate the percentage of cognate B cells that upregulate ICAM-1. Data are representative of two independent experiments.

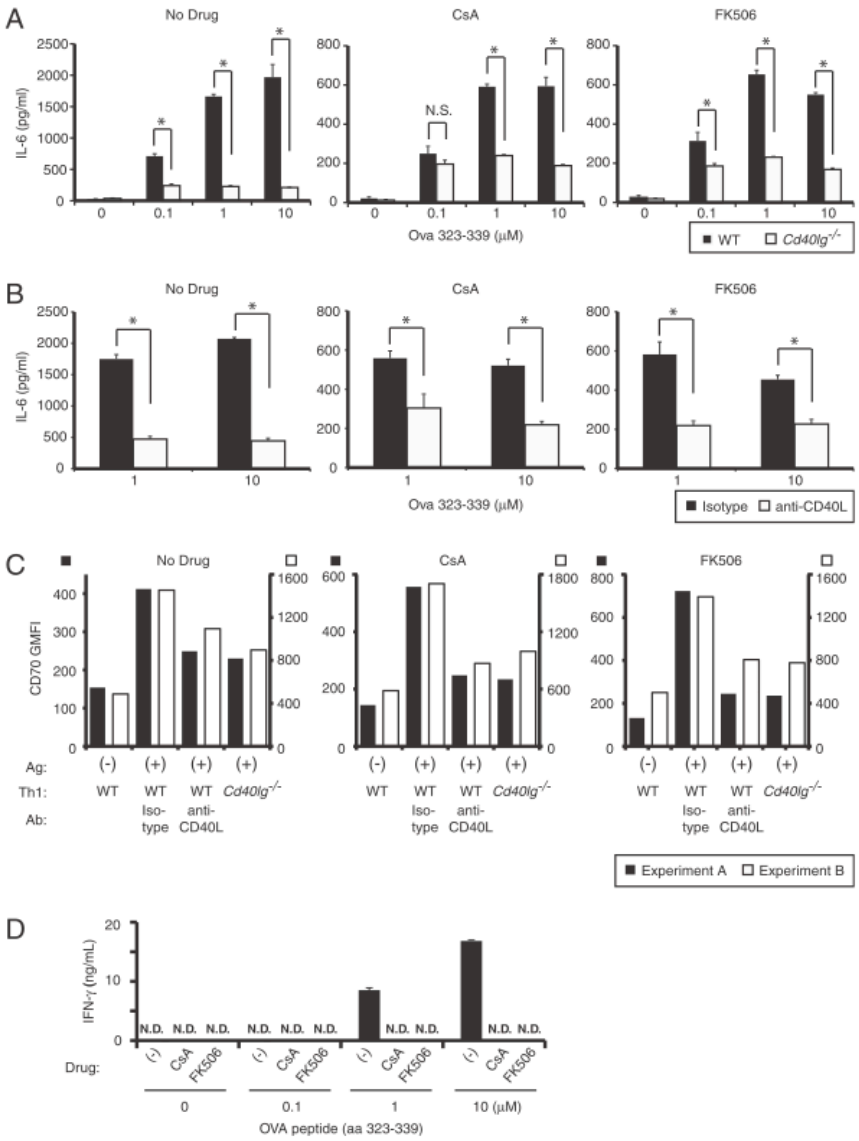


Figure 6. pCD40L in Th1 cells is sufficient to activate DCs. A and B. IL-6 production from BMDCs cocultured with *in vitro*-generated WT or *Cd40lg*$^{-/-}$ Th1 cells for 18 h in the presence or absence of antigenic peptide, CsA or FK506, and isotype Ab or anti-CD40L was measured by ELISA. Note that different y-axes were used for the groups with drug (CsA or FK506) and without. C, CD70 upregulation was assessed after overnight culture of DCs with Th1 cells in the presence or absence of antigenic peptide, CsA or FK506, and isotype Ab or anti-CD40L. Raw geometric mean fluorescent intensity (GMFI) of CD70 staining from two representative experiments is shown. D, WT Th1 cells were incubated with BMDCs in the presence or absence of OVA peptide overnight. CsA and FK506 were kept in the media throughout the culture. IFNγ production was measured by ELISA with a detection limit of 0.06 ng/ml. Each bar represents the mean 6 SD for triplicates. Data are representative of three independent experiments. *p , 0.01. N.D., not detected.

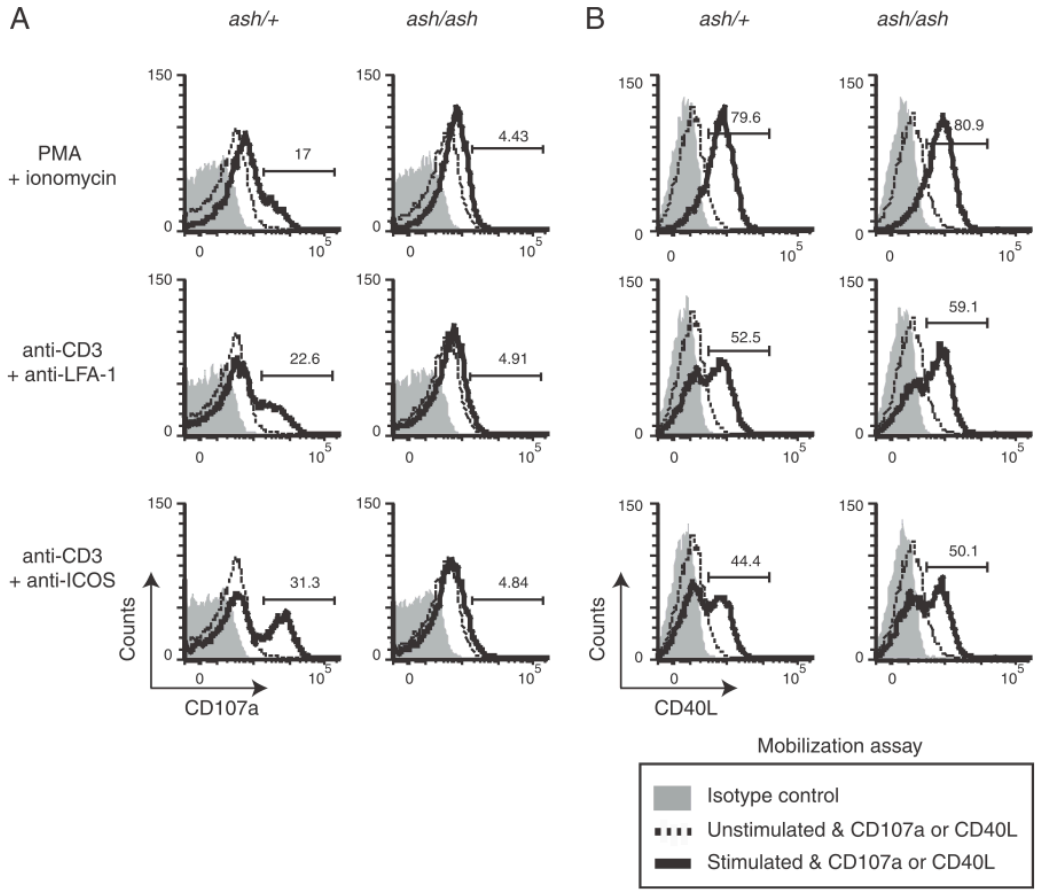


Figure 7. Surface mobilization of pCD40L in Th1 cells is Rab27a-independent. *In vitro*-generated polyclonal Th1 cells from *ash*/+ and *ash/ash* $CD4^+$ T cells were analyzed for mobilization of CD107a (A) and pCD40L (B) upon stimulation with PMA plus ionomycin, anti-CD3 plus anti–LFA-1, or anti- CD3 plus anti-ICOS for 30 min. Numbers in histograms indicate the percentage of stimulated cells that mobilize CD107a or CD40L. The mobilization assay was conducted in the absence of either CsA or FK506 treatment. Data are representative of three independent experiments (n = 2–3/group).

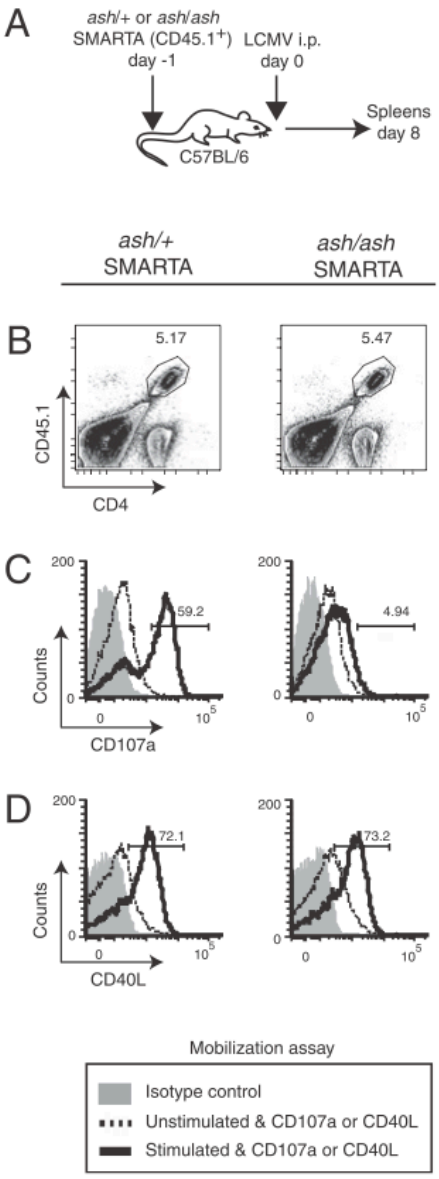


Figure 8. Surface mobilization of pCD40L in *ex vivo* effector CD4$^+$ T cells upon antigenic stimulation is Rab27a-independent. A, CD4$^+$ T cells from *ash*/+ or *ash/ash* SMARTA TCR transgenic mice were transferred into WT mice. Recipients were i.p. infected with 2 x 10^5 PFU LCMV. On day 8 postinfection, spleen cells were collected for the mobilization assay. B, Gating strategy for *in vivo*-generated *ash*/+ and *ash/ash* SMARTA effector CD4$^+$ T cells (CD4$^+$CD45.1$^+$). C and D, *In vivo*-generated *ash*/+ and *ash/ash* SMARTA effector CD4$^+$T cells were analyzed for mobilization of CD107a (C) and pCD40L (D) upon incubation with Ag-unpulsed APCs (unstimulated) or Ag-pulsed APCs (stimulated) *ex vivo* for 30 min. Numbers in histograms indicate the percentage of stimulated cells that mobilize CD107a or CD40L. The mobilization assay was conducted in the absence of either CsA or FK506 treatment. Data are representative of two independent experiments (n = 2–3/group).

Chapter 3: Antigen-specific transfer of CD40L to B cells by helper T cells

Jennifer Gardell and David Parker planned submission.

Abstract: It has been known for decades that the delivery of T cell help for B cells is antigen-specific, MHC-restricted, and depends on CD40L. However the mechanisms by which CD40L, a transmembrane cytokine, is delivered to the T cell surface and engages CD40 on antigen-presenting B cells remain to be determined. Huse et al. (105) showed that a subset of cytokines is delivered directionally to the point of contact between the T cell and the antigen-presenting cell, also known as the immunological synapse, but CD40L was not among the cytokines investigated in that study. Although CD40L, like other cytokines, is made *de novo* following T cell activation, Koguchi et al. (76, 177) showed that CD40L protein is stored in effector and memory CD4 T cells in intracellular vesicles that come to the cell surface rapidly following antigen recognition in sufficient amounts to activate antigen-presenting B cells. Productive signaling has been proposed to be the result of T cell recognition of an antigen-presenting B cell, CD40L expressed on the T cell surface engages with CD40 on the surface of B cells for a period long enough to lead to productive signaling. Here I show for the first time that CD40L does not remain on the surface of the T cell, but is actually transferred to the B cells. This transfer is nearly absent from bystander B cells that are not presenting antigen, and may be partially dependent upon the presence of CD40 on the antigen-presenting B cells. My data suggests that CD40L might be deposited in some type of membrane vesicle on antigen-presenting B cells. This transfer may allow for sustained CD40L-CD40 signaling after a very brief (46, 48, 49) interaction with helper T cells in the germinal center. If delivery of

CD40L is proportional to the amount of antigen on the B cell, transfer of CD40L could be the mechanism by which B cells with higher affinity for antigen following somatic hypermutation are selected in the germinal center reaction, as recently proposed by Dustin (125).

Introduction:

T cell/ B cell collaboration is essential to the development of an effective antibody response. CD40L is the central cytokine produced by T cells and delivered to B cells that contributes to changes in B cells that promote cell division and production of higher affinity antibodies, allowing for more rapid pathogen recognition and removal. Germinal centers are specialized areas in secondary lymphoid organs where follicular B cells that recognize and present antigen become activated and receive T cell help signals necessary for survival, differentiation, and antibody maturation. CD40L is the limiting factor delivered by helper T cells in germinal centers, determining which B cells will be chosen to survive and proliferate versus those B cells that do not receive this signal and die (178, 179).

Upon recognition of antigen on an antigen-presenting B cell, a T cell undergoes extensive subcellular reorganization (180). The microtubule organizing center or centromere polarizes along with all the cellular machinery necessary for secretion of proteins towards the area of T cell/B cell interaction, or immunological synapse. As described in the introduction of this dissertation, some cytokines produced by T cells, IL-2 and IFNγ, are secreted towards the immunological synapse, while others are released multidirectionally, even towards the pole opposite the synapse (105). CD40, which engages with CD40L to initiate B cell signaling, has been shown to accumulate on the B cell within the immunological synapse (181), and effective CD40L signaling to DC requires T cell polarization (112, 182). However the molecular details of CD40L delivery to the T cell

surface and what happens next pre- and post-engagement with CD40 on B cells has not been determined. CD40L is downmodulated from the T cell surface after interaction with CD40 on B cells (137), but to what extent the CD40L is internalized and degraded (183) or recycled by the T cell, cleaved by metalloproteases (184) and released as a soluble protein (185), or internalized by the B cell in a signaling complex in an endosomal compartment as shown for endothelial cells (186), is unknown.

Michael Dustin has recently shown that within the immunological synapse, TCRs are released from the plasma membrane in microvesicles for delivery to APCs (124). These TCRs are engaged with MHC and the transferred microvesicles initiate calcium signaling in B cells by direct signaling through MHC class II, a signaling pathway described years ago by Cambier and colleagues (187). This led us to hypothesize and investigate whether CD40L may be delivered in a similar manner to antigen-specific B cells. This delivery would allow B cells to receive a quantifiable sustained signal from T cells during brief antigen-specific interactions, as was proposed by Dustin in a recent review (125). B cells are selected in the germinal center by the limited signals that they receive from T cells (178, 179), but how the B cell quantifies signals during these interactions and the exact molecular mechanisms involved in this selection are not known and are further investigated in this section of the dissertation.

Materials and Methods:

Mice:

B10.A and B10.A-*Rag2tm1Fwa H2-T18a* Tg (5CC7 TCR transgenic mice on a B10A.RAG knock-out background), specific for a pigeon cytochrome c peptide, amino acids 81-104, and reactive against moth cytochrome c were purchased from Taconic (188). B6.129P2-*Cd40tm1Kik*/J (CD40 knock-out mice on a B6 background) were purchased from Jackson and bred to B10.A mice (63). Heterozygous mice from this breeding were crossed to produce I-E^k homozygous CD40 knock-out mice. 5CC7 TCR transgenic male mice were crossed to female B6.129S2-*Cd40lgtm1Imx*/J (65) (CD40L knock-out mice on a B6 background, purchased from Jackson) to produce 5CC7 CD40L knock-out male mice. Mice were housed in specific-pathogen free conditions at Oregon Health and Science University according to institutional standards.

Antibodies and labels:

The antibodies and reagents used for flow cytometry experiments were as follows: anti-CD4 PeCy7, anti-CD19 PerCP, anti-CD19-FITC, ICAM-biotin, ICAM-APC Streptavidin-APC, anti-CD40L-PE, anti-CD40L-APC, hamster IgG-APC and hamster IgG-PE. All were from eBiosciences. Cells were differentially labeled with carboxyfluorescein succinimidyl ester (CFSE) or Cell Trace Violet (Invitrogen) to mark Ag-pulsed or bystander cells in individual assays.

***In vitro* T cell polarization:**

Th1 conditions:

Spleens were harvested from 5CC7 TCR transgenic mice. Red blood cells were removed by hypotonic lysis. Cells were suspended in RPMI 1640 medium, 1% glutamine/penicillin/streptomycin, 10% Tumor Cocktail pH 7.0 (S-MEM (630 ml, Gibco 11385036), Dextrose (7.5 g, Sigma G7021), Essential Amino Acids (75 ml, Invitrogen 11130-05), Non-Essential Amino Acids (140 ml, Gibco 10001), Sodium Pyruvate (100ml, Gibco 03844), Sodium Bicarbonate (8.5g, Mallinckrodt 7412), Penicillin G (600mg, Sigma P-3032), Gentamycin (10ml, Gibco 3755), Streptomycin Sulfate (1g, Gibco 1010574), and 2-Mercaptoethanol (34 µl, Sigma M-3148)), 10% Fetal Bovine Serum (FBS), and 1% 2-Mercaptoethanol (2-ME) (Sigma M-3148) at 5 x 10^6 cells/ml. For polarization to Th1, cells were incubated with .1 µM moth cytochrome c peptide (MCC) [ANERADLIAYLKQATK] and 10 µg/ml anti-IL-4 (11B11 [eBioscience]) for four days.

Th2 conditions:

Spleens were harvested from 5CC7 TCR transgenic mice. CD4+ T cells were purified using the EasySep system (STEMCELL technologies). CD4+ T cells were suspended in RPMI medium with 1% glutamine/penicillin/streptomycin, 10% Tumor Cocktail, 10% FBS, and 1% 2-ME at 1.7 x 10^6 cells/ml (2x). Splenocytes were prepared from B10A mice and irradiated at 15 Gray using a cesium irradiator. These irradiated splenocytes

were suspended at 8.3 x 10^6 cells/ml (10x) to act as APCs for the CD4+ T lymphocytes. CD4+ T cells and irradiated APCs were mixed together and pulsed with MCC peptide at 2.5 μM and polarizing cytokines and antibodies: IL-4 (50 ng/ml eBioscience) and anti-IFNγ (20 μg/ml XMG 1.2[BioXcell]). On day two of culture, IL-2 (derived from supernatant of T cell hybridoma cell line) was added to these cells at 80 U/ml. On day 4 of culture, cells were centrifuged with Lympholyte M (Cedarlane) to remove dead cells and restimulated with new APCs. During this secondary culture, anti-IL-4 was added at 10 μg/ml instead of IL-4 to ensure expression of preformed CD40L in these cells (177).

Intracellular CD40L stain:

After overnight T cell/B cell incubation at 37 °C, cells were fixed (BD Biosciences Cytofix/Cytoperm)and permeabilized (BD Biosciences Perm/Wash Buffer) and stained with PE-labeled CD40L or hamster IgG-PE control antibody (eBioscience). These samples were then analyzed on LSRII flow cytometer (Becton Dickinson).

***In vitro* overnight assay:**

Th1 and Th2 cells cultures were harvested on day four after stimulation, or restimulation in the case of Th2 cells. Lympholyte M was used to remove dead cells. Cells were then counted and resuspended at 2 x 10^6 cells/ml either with or without 2 μM Cyclosporin A (CsA) (Sigma-Aldrich) to block *de novo* TCR-induced cytokine production. Spleens were harvested from 5CC7 CD40L knock-out, B10-A and B10-A CD40 knock-out mice and

target splenocytes were prepared by hypotonic lysis. Antigen-bearing splenocytes were labeled with CFSE or Cell Trace Violet (following manufacturer's protocol) and pulsed with 2.5 μM MCC peptide for two hours at 37° C. The splenocytes were then were washed three times and combined with 1 x 10^6 T cells, either with or without CsA (final concentration 1 μM) treatment. Overnight assays were performed in the presence or absence of unlabeled or fluorescently labeled anti-CD40L antibody and with or without addition of Fc receptor (FcR) blocking antibody anti-CD16/CD32, clone 2.4G2 from BioLegend. After overnight incubation, cells were stained with antibodies for CD4, CD19, and ICAM, and analyzed on the LSRII flow cytometer. Proliferation was assessed based on the dilution of CFSE or CTV.

Transwell Experiment:

1 x 10^6 Th1 cells and 8 x 10^5 target splenocytes (4 x 10^5 Ag+ and 4 x 10^5 Ag-, differentially labeled) were placed in the top chamber of the transwell system separated by a 0.4 μM cell impermeable membrane from 8 x 10^5 splenocytes (Ag+ and Ag-). Following overnight incubation, cells were stained with antibodies for CD4, CD19, and ICAM and analyzed by flow cytometry.

Fluorescent Cell Microscopy:

Imaging of CD40L on antigen-pulsed and bystander cells after overnight incubation with T cells in the presence of APC labeled anti-CD40L antibody was performed on a Zeiss

LSM 780 laser-scanning confocal microscope. The Zeiss LSM 780 is mounted on a fully motorized AxioObserver Z1 inverted microscope stand and has a 32 channel GaAsP detector for capturing spectral information for linear unmixing. The LSM 780 stage is motorized in X, Y to acquire images of area larger than the field of view through tiling of individual image stacks. Overnight cultured T and B cells were stained with CD19-FITC, fixed, and placed into a single chamber of a 1 % poly-l-lysine (Sigma #P8920) treated 8 chambered glass slide (Lab-Tek®). Eleven z stack images were acquired for each microscopy field analyzed. An image was acquired every 0.25 μm movement in the z axis, allowing for the entire cell to be visualized from the point of cell attachment to the glass to the cell surface opposite the point of attachment. Images and movies were developed and analyzed using ImageJ.

Selection criteria and quantification of CD40L on B cells in microscopy images:

B cells were selected for quantification based on positive CD19-FITC staining. Cells were excluded from quantification if they were not attached to the glass surface and mobile at any point during acquisition of z stacks, if it was not possible to distinguish which cell in the image was positive for CD40L (cells were too close in proximity or overlapping), if cells appeared dead, or CD19 staining did not correspond to only the cell surface. The CD19 surface stain was used to differentiate the B cell surface from inside the B cell, serving as a marker to distinguish between surface CD40L and intracellular CD40L on selected B cells.

Results:

All effector and memory CD4 helper T cells with the exception of T regulatory cells express a small amount of intracellular CD40L that can be brought to the cell surface within minutes of T cell receptor (TCR) engagement (177). Only during longer periods of antigen-specific TCR engagement, for instance during T cell priming, are large amounts of CD40L produced *de novo*. Intravital, two-photon microscopy of murine lymph nodes has determined that germinal center T cell/B cell interactions are brief and last only a few minutes (46, 47). Koguchi et al. showed that the small amount of intracellular CD40L in DO11.10 T helper cells (BALB/c TCR transgenic T cells specific for OVA peptide in the context of I-A^d) is capable of inducing upregulation of activation markers as well as proliferation of peptide-pulsed B cells as measured by dilution of CFSE (126). In order to further investigate delivery of CD40L during antigen specific interactions, I performed preliminary experiments to determine if these results hold true for a different TCR transgenic mouse line and different MHC restriction. 5CC7 Th1 or Th2 cells were incubated with Ag-pulsed and bystander B cells overnight at 37 degrees (Figure 9A). In order to differentiate between the Ag-pulsed and bystander B cells they were differentially labeled with different concentrations of CFSE. I could then gate on either cell population following incubation and determine expression of activation markers by each B cell population (Figure 9B).

5CC7 TCR transgenic mice are on a Recombination Activating Gene (RAG) knock-out background, therefore all their T cells express a TCR that recognizes the MCC peptide in

the context of the MHC class II molecule, I-E^k. After *in vitro* polarization, 5CC7 Th1 and Th2 cells were treated with cyclosporine A (CsA) to block *de novo* CD40L synthesis as shown in Chapter 2 and prevent accumulation of large amounts of surface CD40L after stimulation with PMA/ionomycin. Similar to the DO11.10 T cells, both CsA-treated 5CC7 Th1 and Th2 incubated overnight with antigen-pulsed and unpulsed bystander B cells resulted in upregulation of ICAM-1 only on the antigen-pulsed B cells (Figure 10A and B). The bystander B cells had ICAM levels equal to that of B cells in a well to which peptide was not added. Upregulation of ICAM by Th2 cells varied more among experiments when compared to Th1 cells. This may have been due to variable reversal of inhibition of CD40L expression by the high levels of IL-4 added during the first culture under Th2 polarizing conditions (189). These results show that CD40L is delivered in a directed manner specifically to antigen-pulsed B cells under conditions in which *de novo* CD40L synthesis is blocked and only stored CD40L protein is available, as shown previously in the DO11.10 system in Chapter 2.

Upregulation of ICAM on B cells by CsA-treated T cells was also evaluated in culture conditions more favorable for bystander activation, in which the cells were forced into close contact. I combined the CsA-treated Th1 or Th2 cells with peptide-pulsed and bystander B cells in wells of a 96 well plate, and pelleted these cells by centrifugation prior to overnight incubation. Despite the forced close contact between T and B cells, only the peptide-pulsed B cells upregulated ICAM when compared to the B cells in the control well in which no antigen was added, similar to previous results (Figure 10C and 10D). The upregulation of ICAM by Th1 and Th2 cells is caused by CD40-CD40L

engagement, and not just the presence of antigen on these B cells or other molecules on the T cells, because Ag-pulsed CD40 knock-out B cells did not upregulate ICAM when cultured with helper T cells (Figure 10D).

Helper T cells produce large amounts of CD40L *de novo* during antigen-specific interactions when the engagement is longer than 30 minutes (76). Although it has been shown *in vivo* that germinal center interactions between B cells and T cells are brief, the significance of the large amount of CD40L produced during long TCR stimulation is not known. It is thought that CD40L may be constantly downmodulated from the surface of T cells after interactions with $CD40^{+}$ cells (137). Therefore, the large amount of CD40L that accumulates on the surface of T cells after two hours of TCR or PMA/Ionomycin stimulation may be an *in vitro* artifact due to the paucity of CD40+ B cells in the four day polarized Th1/Th2 cell cultures. I also wanted to evaluate delivery of CD40L in the more physiological situation when CD40L synthesis is not blocked and production of new protein is possible. In order to further study the specificity of delivery of CD40L by helper T cells, I wanted to determine if there were differences in induction of ICAM upregulation on antigen-presenting and bystander B cells by helper T cells that were capable of production of *de novo* CD40L. Therefore I performed the *in vitro* overnight assay with Th1 cells that had not been treated with CsA and could produce large amounts of CD40L upon TCR stimulation (Figures 11-26). Under these conditions, both bystander and antigen-pulsed B cells upregulate ICAM (Figure 11A and 11B). In each experiment, and in the case of both Th1 and Th2 cells, upregulation of ICAM on the antigen-pulsed B cells was greater than that of bystanders, indicating some preference towards antigen-

specific delivery of CD40L. This antigen-specific ICAM upregulation increases with increasing number of Th2 cells in culture with B cells (Figure 12). Delivery of help is also dependent upon T cell contact. When the antigen-pulsed and bystander B cells were separated from the T cells by a protein-permeable membrane in a Costar Transwell, the B cells did not upregulate ICAM (Figure 13). Only the cells in the upper chamber that were able to contact T cells received T cell help, indicating that upregulation of ICAM on bystander B cells is dependent upon their ability to contact T cells, and not owing to a soluble factor. This contact dependent help is also dependent upon the presence of CD40 on these B cells, because Ag-pulsed CD40 knock-out B cells did not upregulate ICAM upon interaction with T cells, although they were able to stimulate the T cells to activate CD40-sufficient bystander B cells (Figure 13). In summary, when *de novo* synthesis of CD40L by Th1 or Th2 cells is not blocked, both antigen-presenting and bystander B cells receive contact- and CD40-dependent T cell help, although the response of the antigen-presenting B cells is greater.

Further evidence of the directional delivery of CD40L was seen upon addition of a CD40L blocking antibody to these cultures. At 10 μg/ml, a concentration that was more than sufficient to eliminate bystander activation, antigen-specific ICAM upregulation by Th1 (Figure 14A) and Th2 (Figure 14B) cells was still present, though attenuated. This difference between the antigen-presenting and bystander B cells becomes more apparent upon titration of the anti-CD40L blocking antibody. Antigen-specific help is much less affected while bystander activation is completely eliminated at 1μg/ml of anti-CD40L (Figure 15). These results suggest that there is some fundamental difference in the

delivery of CD40L to the antigen-pulsed cells that makes this help less sensitive to antibody inhibition. Upregulation of ICAM on antigen-pulsed and bystander B cells by Th2 cells did not reach statistically significance in these experiments, but this may be due to inhibition of CD40L expressed by Th2 cells due to IL-4 production. It is possible that the CD40L that helps the bystander cells may need to accumulate on the T cell surface and is more readily available for neutralization by the anti-CD40L antibody in comparison to directed delivery of CD40L during antigen-specific interactions.

Engagement of CD40 via CD40L on B cells initiates signaling which in the presence of soluble T cell cytokines, such as IL-4 or IL-21, induces B cell proliferation (61, 115). I next investigated antigen-specific delivery of CD40L in terms of B cell function by measuring proliferation following 48 hours of incubation with Th1 or Th2 cells. In this assay, the antigen pulsed B cells were labeled with CFSE, while the bystanders were labeled with Cell Trace Violet or vice versa. After 48 hours in the presence of Th1 or Th2 cells, antigen-pulsed B cells had a larger proportion of cells that divided compared to the bystanders (Figure 16). Antigen-pulsed CD40 knock-out B cells had almost no cells that divided following incubation with Th1 or Th2 cells (Figure 16). These results indicate that B cell proliferation in this assay requires CD40-CD40L engagement, and the presence of antigen on CD40-sufficient B cells causes them to receive a larger CD40L signal than the bystander B cells. When blocking antibody is added to the T/B cell cultures at the low concentration of 0.1 μg/ml, bystander proliferation is completely inhibited, while antigen-specific proliferation is largely unaffected (Figure 16A and 16

B). Therefore CD40L seems to be delivered in a directed manner towards antigen-presenting B cells, and when these cells express CD40, it is capable of inducing proliferation.

I hypothesized that CD40L may be deposited on antigen-bearing B cells during interactions with helper T cells. To test my hypothesis, I investigated the possible presence of CD40L protein on antigen-pulsed and bystander B cells in an overnight *in vitro* assay. Following overnight incubation with helper T cells, I surface stained both antigen-pulsed and bystander B cells for CD40L. I found that there was a small amount of CD40L staining on the surface of a small percentage of antigen-pulsed B cells that was not present on bystander or antigen-pulsed CD40 knock-out B cells (Figure 17). However since B cells are capable of producing CD40L (190), it was necessary to determine if this CD40L was being produced by the B cells or actually originating from the helper T cells. Therefore I set up the overnight assay with CD40L knock-out antigen-pulsed target B cells. These CD40L knock-out B cells did not display the same small amount of CD40L on their cell surface after overnight incubation. This indicated that the small amount of CD40L present on the surface of the antigen-pulsed B cells was actually produced by the B cells (190) or that the CD40L knock-out cells were deficient in other ways necessary to detect this transfer.

It has been proposed that CD40L engages with CD40 on the B cell surface long enough to initiate signaling in the B cell and then is downmodulated from the T cell surface (137, 191). However the specific mechanisms of CD40L downmodulation from the T cell

surface are not known. One group suggested that CD40L engagement with CD40 may trigger endocytosis along with recruitment of downstream signaling molecules in endothelial cells (186). If endocytosis of CD40L-engaged CD40 sustains B cell signaling, it may provide a way in which B cells are able to quantify the CD40L signal they receive from brief encounters with T cells in the germinal center, and thereby determine which cells are selected for affinity maturation (125). I performed intracellular staining on the different B cell populations after *in vitro* overnight incubation with Th1 cells to determine if the engaged CD40L is internalized by the antigen-pulsed B cells. Intracellular staining of CD40L knock-out antigen-pulsed B cells shows an increase in intracellular CD40L above bystander B cells and hamster IgG control stained antigen-pulsed B cells (Figure 18), indicating that they do receive more CD40L than B cells not pulsed with antigen. Internalized CD40L also depends on CD40 on the B cells (Figure 18). This result suggests that during antigen-specific T-B cell interactions, T cell-derived CD40L engages with CD40 and is internalized which may induce signaling in that B cell.

When measuring CD40L expressed by CD4 T cells in the presence of CD40+ cells following antigenic or PMA/Ionomycin stimulation, more CD40L is detected when the fluorescently labeled anti-CD40L antibody is added during the stimulation at 37 degrees (76, 191-193). Therefore, I set up an overnight *in vitro* assay in which I included the fluorescently labeled anti-CD40L antibody during the overnight T/B incubation. Due to the differential CFSE labeling of the antigen-pulsed and bystander cells in this assay, I was able to detect CD40L transferred to each B cell population within the same well. After overnight incubation with T cells, a large percentage of Ag-pulsed B cells were

brightly CD40L positive, while bystander B cells were mostly CD40L negative (Figure 19A, 19B, and 19D). This transfer was dependent upon CD40L expression by T cells because when the B cells were incubated with CD40L knock-out T cells, Ag-pulsed B cells did not obtain CD40L after overnight incubation (Figure 19A and 19B). This signal was not entirely dependent on CD40 expression by B cells, as antigen-pulsed CD40 knock-out B cells had about a third of the number of CD40L positive cells after overnight incubation with CD40L- sufficient T cells when compared to CD40-expressing antigen-pulsed B cells (Figure 19C and 19D). However there does seem to be a difference in the fluorescence intensity of CD40L staining between CD40-sufficient and CD40 knock-out cells, indicating that CD40-sufficient cells may obtain more CD40L on a per cell basis, so effective transfer to antigen-specific B cells may still depend upon CD40-CD40L interaction (Figure 19C). This CD40L was made by the T cells because CD40L knock-out B cells had almost equal transfer of CD40L compared to CD40L-sufficient B cells (Figure 20A). B cells are known to express Fc receptors (FcRs), therefore it is important to determine that the transfer of CD40L to the antigen-specific B cells was not entirely due to FcR-mediated internalization of the fluorescently labeled antibodies. This was not the case, because when a blocking anti-FcR antibody was added during overnight culture, Ag-pulsed B cells were still largely positive for CD40L expression (Figure 20B). However, the antigen-specific transfer to CD40 knock-out B cells appears to be more dependent upon FcRs because when the FcR blocking antibody is added, antigen-specific transfer is more severely reduced compared to CD40-sufficient cells (Figure 20B). Therefore, it is possible that transfer or persistence of transferred CD40L complexed with fluorescent anti-CD40L may depend on the presence of either CD40 and/or FcR on the B

cell. However, I was able to perform this experiment with CD40 knock-out antigen-specific B cells with addition of FcR blocking antibody only once, and I will need to do it a few additional times to see if this conclusion holds.

As discussed in the introduction of this dissertation, most T/B cell germinal center interactions last only a few minutes (46-49). Therefore I wanted to investigate CD40L transfer to antigen-pulsed B cells with a shorter T/B cell incubation time. I evaluated CD40L transfer after only two hours of T/B cell incubation. As expected, two hours of T cell/B cell interaction was not long enough to induce antigen-specific ICAM upregulation (Figure 21A). T cells transferred CD40L to antigen-specific B cells and not bystanders during this time period (Figure 21B and 21C). CD40 knock-out antigen-pulsed cells obtained CD40L to the same extent as the antigen-pulsed cells during this time period. Therefore it appears that the transfer at two hours as detected by inclusion of fluorescently labeled anti-CD40L antibody during the two hour T/B cell incubation is largely CD40-independent (Figure 21B and 21C). The CD40L present on the antigen-specific B cells was not produced by the B cells because CD40L knock-out B cells had just as many CD40L positive cells after the two hour incubation (Figure 21D). However further experimentation with a FcR blocking antibody during the two hour T/B incubation revealed that much of this early Ag-specific transfer is FcR dependent, 40% in the CD40 sufficient B cells and over 75% in the CD40 knock-out Ag-pulsed cells (Figure 21E). This experiment will have to be repeated a few additional times to enable a thorough statistical analysis. As shown in Figure 20B, the antigen-specific transfer to

CD40-sufficient B cells after overnight incubation also seems to be less dependent upon the presence of available FcR.

Due to the large amount of CD40L transfer during this short period of 2 hours, I was interested in determining if the CD40L transferred by T cells is functional in the antigen-specific B cells. I performed experiments to try to determine if the CD40L transferred at this early time point is capable of inducing proliferation. CD19+CD4-CD40L+ and CD19+CD4-CD40L- antigen-pulsed B cells were sorted after two hours of incubation with T cells and exogenous IL-4 was added to these cells. 48 hours after the separation of these two B cell subsets from the T cells, proliferation was assessed by dilution of cell trace violet. If the cells had divided, the violet dye would dilute out from the original concentration according to the number of divisions of B cells. This experiment was performed two times, but despite CD40L transfer to the antigen-pulsed cells, I was unable to detect cell division over background levels in the Ag-pulsed B cells (data not shown).

To further characterize the CD40L that is transferred to the antigen-pulsed B cells, I used confocal laser scanning microscopy. Antigen-pulsed splenocytes were labeled with a cell permeable dye (cell trace violet) and expression of CD40L on these cells was visualized through the use of an APC-labeled antibody added during the overnight culture of these cells with unpulsed splenocytes and T helper cells. B cells were surface stained with a CD19-FITC labeled antibody following overnight incubation. I then assessed the presence of CD40L on antigen-pulsed versus bystander B cells by laser scanning

microscopy using CD19 and cell trace violet. For each image I took, I numbered the B cells (CD19 positive) and then determined if they were antigen-pulsed or bystander by positive or negative cell trace violet staining and scored them as CD40L (APC) positive or negative (Figure 22 and 23). I then used the surface CD19 stain and z-stack images through the entire cell to determine if the CD40L was surface or intracellular. Many more of the Ag pulsed B cells had CD40L compared to the bystander cells (Figure 22-25). Figures 23 and 24 depict cells under high magnification. Antigen-pulsed B cells had both intracellular and surface CD40L as assessed by z-stacks and CD19-FITC surface staining (Figure 23 and 25). Microscopy results were consistent with samples from the same cultures analyzed by flow cytometry (Figure 26). Cells incubated overnight with a control hamster IgG APC labeled antibody did not have positive APC fluorescence after overnight incubation. As expected, Ag-pulsed B cells had a large population of cells that had transferred CD40L, while bystanders did not. There were very few CD40 knock-out cells that were seen to be CD40L-APC positive via microscopy compared with flow cytometry: one of these, a CD40L-APC positive CD40 knock-out cell, is shown in Figure 24. The difference between the percentage of CD40 knock-out CD40L-APC positive cells detected by microscopy as compared to flow cytometry could be due to a difference in detection sensitivity using the microscope versus flow cytometer, as the mean fluorescent intensity of the CD40L label in the CD40 knock-out B cells is lower that than of the CD40-sufficient B cells (Figure 26).

Additional experiments were performed incubating the T cells with fluorescent anti-CD40L to label CD40L in the T cells prior to incubation with antigen-pulsed B cells.

These labeled T cells and antigen-pulsed B cells were imaged for two hours using wide field microscopy to try to capture live delivery of CD40L. However only a small amount of CD40L was labeled on the unstimulated T cells, as expected, and although T cells could be seen to interact with B cells, I was unable to capture live transfer of CD40L. Most of the CD40L staining was in the trailing edge of the T cells as they moved and interacted with B cells, away from the area of immunological synapse formation (data not shown).

Discussion:

In this chapter I analyze the antigen-specific delivery of CD40L to B cells in an *in vitro* overnight assay. I hypothesize that CD40L is delivered in vesicles to the outer membrane of antigen-specific B cells where it engages with CD40 and initiates signaling in these B cells. Prior work suggests that CD40L engages with CD40 on the B cell surface for a period long enough to induce B cell signaling and then is downmodulated from the T cell surface, however the exact subcellular mechanism by which this occurs is not known. In this chapter I show that CD40L is actually transferred to and present in and on antigen-specific B cells following overnight incubation. If this CD40L is engaged with CD40, it could be that the transferred CD40L provides the signal necessary for antigen-specific proliferation, although I have not been able to demonstrate this. The transferred CD40L may be the way in which antigen-specific B cells quantify the signal they receive from T cells. The ability to quantify T cell signal may be particularly important in the germinal center where antigen-specific B cells with higher affinity antibodies outcompete those of lower affinity. Future work will be needed to further characterize the transfer of CD40L and determine if the transferred CD40L engages with CD40 to promote activation and proliferation of the antigen-specific B cells. This may be accomplished by staining and visualization of CD40 together with CD40L, as well as downstream signaling components, on the antigen-specific B cells by microscopy.

Although Michael Dustin recently proposed that CD40L may be delivered to antigen-specific B cells in a review article (125), this theory was not tested experimentally prior

to the experiments performed in this chapter. This antigen-specific delivery would allow B cells to quantify the signals that they receive from T cells during limited interactions within the germinal center. Overnight incubation of T and B cell cultures while including a fluorescently labeled anti-CD40L antibody allows both for accumulation of labeled CD40L on the T cell surface, as shown previously (191), and, in this dissertation, a method for detection of transferred CD40L in and on the helped B cells. When the fluorescently labeled anti-CD40L antibody is added to the overnight cultures, CD40L, rather than being downmodulated by CD40+ cells may accumulate on the T cell surface. The presence of the fluorescently labeled antibody may also allow for detection of CD40L transfer to antigen-presenting B cells during formation of an immunological synapse. CD40L transfer did not occur in the absence of antigen, indicating that despite intermittent T cell interaction with bystander cells *in vitro*, CD40L transfer occurs only during productive T cell/B cell interaction. I propose that upon immunological synapse formation following T cell recognition of peptide in the context of MHC on a B cell, CD40L is deposited in vesicles on antigen-presenting B cells. After the CD40L engages CD40, it is internalized and productive signaling leads to activation and proliferation of the B cell.

Presence of the anti-CD40L antibody during the overnight *in vitro* assay may also allow for longer persistence of CD40L in or on the antigen-specific B cell following transfer explaining the large increase in CD40L positive antigen-specific B cells following overnight incubation with the antibody compared to traditional methods of staining post overnight incubation. B cells may need to accumulate a certain threshold amount of

CD40L prior to induction of signaling downstream of the CD40L-CD40 interaction (194). Since the anti-CD40L antibody is a blocking antibody, much more CD40L may be required to initiate signaling downstream of CD40 in the presence of anti-CD40L. This suggests that the some delivered CD40L is in a trimeric form and available for active signaling, while other CD40L is engaged by the antibody and incapable of engaging with CD40. Alternatively it may be that the fluorescent label (APC or PE) on the CD40L antibody is more resistant to degradation by the B cell than CD40L allowing the CD40L signal to be detected for a much longer period of time. However antigen-specific transfer was further verified by fluorescent microscopy and the more traditional method of staining intracellular CD40L in antigen-specific B cells that were fixed and permeabilized following overnight incubation with T cells. Future work will be needed to characterize the compartment in B cells that stores CD40L following antigen-specific interactions, and whether or not transferred CD40L is engaged with CD40. CD40L bound by the fluorescently labeled anti-CD40L blocking antibody may be destined for degradation through the lysosomal pathway. This could be determined by measuring CD40L on antigen-specific B cells with and without addition of bafilomycin, an inhibitor of lysosomal acidification, by flow cytometry. If the CD40L is destined for degradation, then upon addition of the inhibitor, the presence of CD40L on the antigen-presenting B cells should increase.

Antigen-specific transfer of CD40L was also shown to be partially dependent on CD40. CD40 knock-out antigen-presenting B cells accumulate a smaller amount of CD40L following overnight incubation with T cells compared to CD40-sufficient B cells, and

T/B interaction did not result in upregulation of ICAM on the B cells. CD40L engagement with CD40 may be required for CD40L persistence on the B cell surface in order to block internalization and degradation of unbound CD40L or even removal by other CD40+ cells. Transfer of CD40L to CD40 knock-out cells also seems to be much more sensitive to the addition of a FcR blocking antibody during overnight culture than CD40 sufficient antigen-bearing B cells, indicating that fluorescently labeled anti-CD40L uptake to CD40 knock-out cells could be through FcRs, dependent also on CD40L from the T cell. The questions regarding transfer dependence upon B cell FcRs will be addressed in additional experiments with the blocking antibody and potentially with a FcR knock-out mouse.

Many questions remain regarding the form of the transferred CD40L. Although the transferred CD40L appeared as punctate spots in and on B cells via microscopy, further resolution, perhaps using electron microscopy, will be needed to further classify the structures containing CD40L. It is not known whether the CD40L is delivered to antigen-presenting B cells in microvesicles, exosomes, patches of T cell membrane, or through B cell trogocytosis or transendocytosis. Since it is known that peptide-MHC engaged TCR is delivered in microvesicles to antigen-presenting B cells (124), I stained antigen-specific B cells for both TCR and CD40L to see if these two proteins colocalized within the same cells by flow cytometry. Preliminary results suggest that delivery of TCR microvesicles is distinct from that of CD40L (data not shown).

These *in vitro* results suggest a new perspective on how antigen-specific B cells secure signaling from limiting CD40L available from T cells. Transferred CD40L may provide a means of sustained signaling in antigen-specific B cells, despite short germinal center T/B cell interaction times. It is therefore important to determine if this transfer is associated with a biological function in B cells in vivo. Future experiments with the ability to distinguish between active signaling downstream of CD40 in CD40L positive compared to CD40L negative antigen-specific B cells will be needed to address this hypothesis. Active signaling could be evaluated by assessing processing of p100 in the NF-κB2 pathway in these two B cell populations (195), or by measuring nuclear localization of NF-κB components (196). Transfer of ligands during antigen-specific interactions may be a common means of B cell activation, or even inhibition, during antigen specific interactions. In addition to CD40L, there are other tumor necrosis family members involved in both T cell proliferation and death, including Fas Ligand, which could potentially be delivered to antigen-specific B cells by a similar mechanism (197).

Figures:

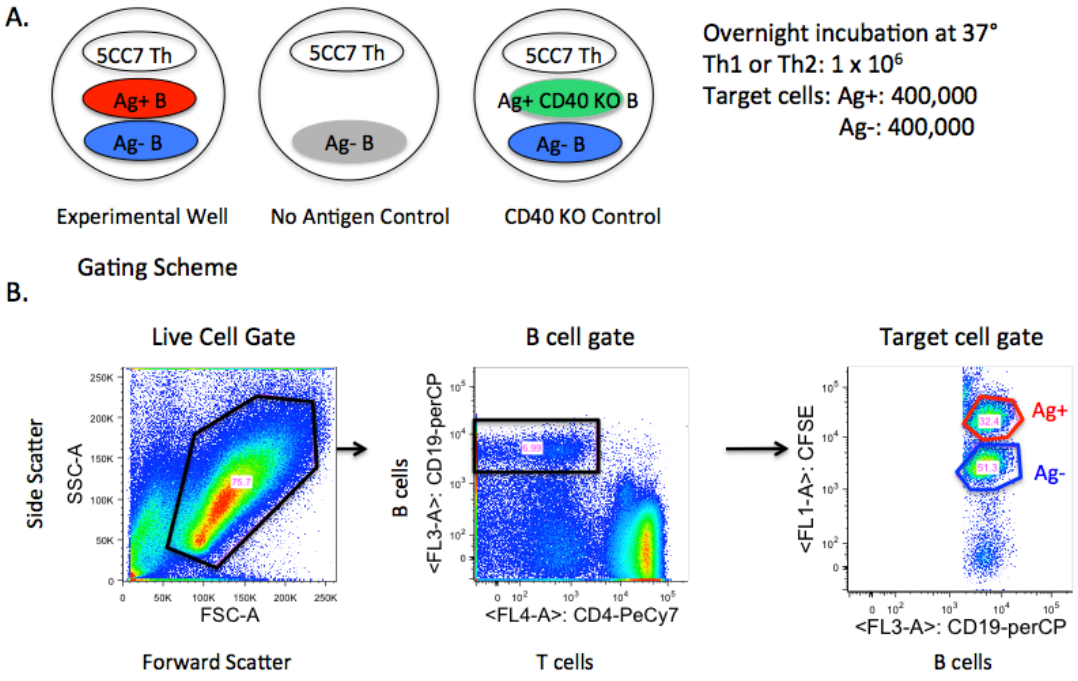


Figure 9. Experimental set-up and gating scheme used for the *in vitro* overnight assay. A, 5CC7 Th1 or Th2 cells on day four of polarization were incubated overnight with target B cells (Ag-pulsed (Ag+) or bystander (Ag-)) from B10A mice that were differentially labeled with CFSE. A, The set up for no Ag control and CD40 knock-out control wells are show to the right of the experimental well set-up. B, The gating scheme used in the analysis to differentiate between Ag-pulsed (Ag+) and bystander (Ag-) in each assay is shown.

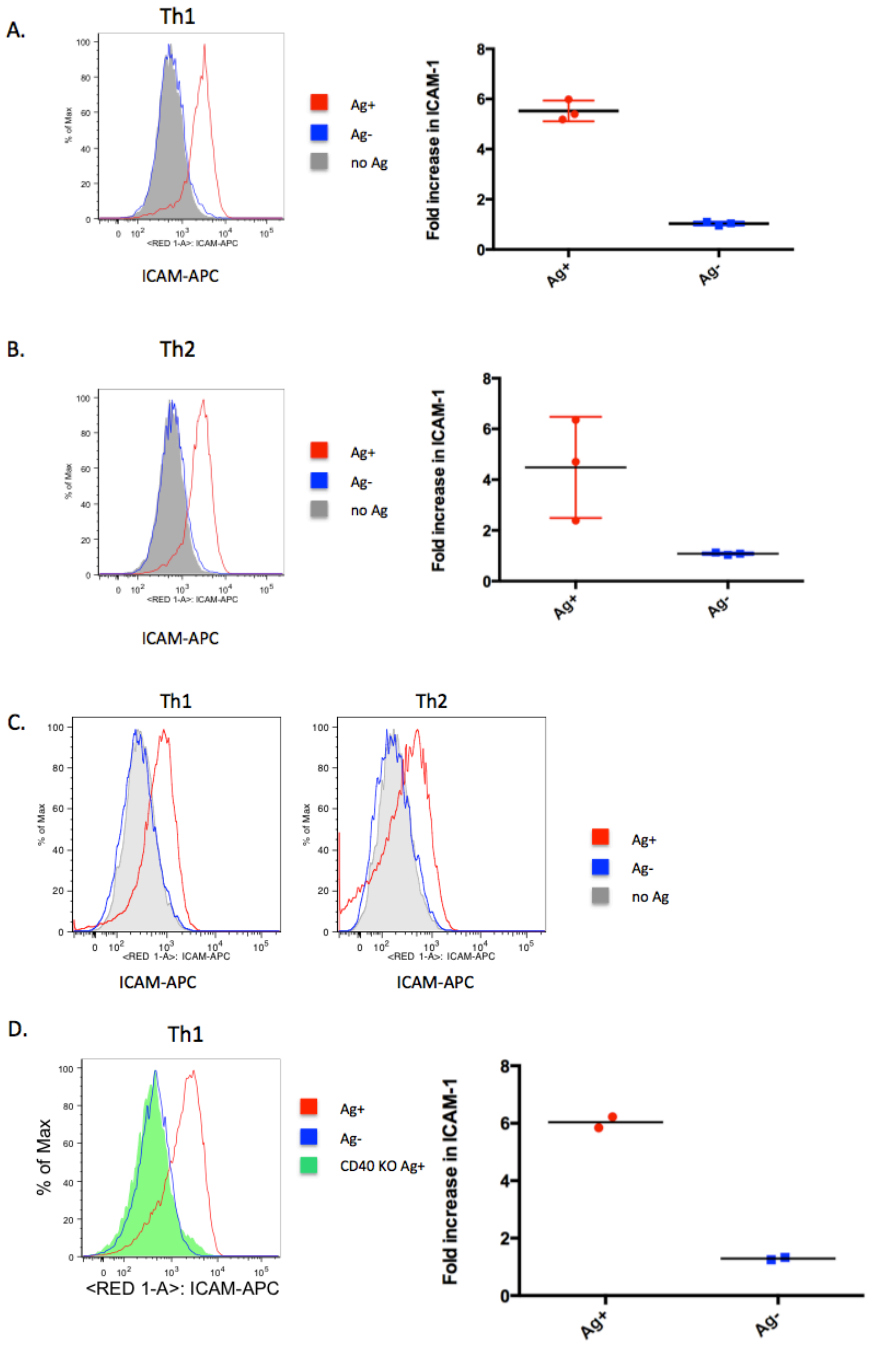


Figure 10. CsA-treated 5CC7 Th1 and Th2 cells upregulate ICAM on Ag-pulsed B cells and not bystanders after overnight incubation. A-B, Representative histograms of ICAM upregulation on Ag+, Ag-, and no Ag control B cells after overnight incubation with CsA-treated 5CC7 Th1 cells (A) or Th2 cells (B). C-D, Representative histograms of ICAM upregulation on Ag+, Ag-, and no Ag control B cells (C) or Ag+ CD40KO control B cells (D) after pelleting and overnight incubation with CsA-treated 5CC7 Th1 or Th2 cells in a 96 round bottom plate. Graphs show the fold increase in ICAM MFI (+/- SD) on each of the B cell populations for individual experiments. The fold increase in MFI for the well with no antigen (A and B) or Ag+ CD40KO (D) was set to 1.

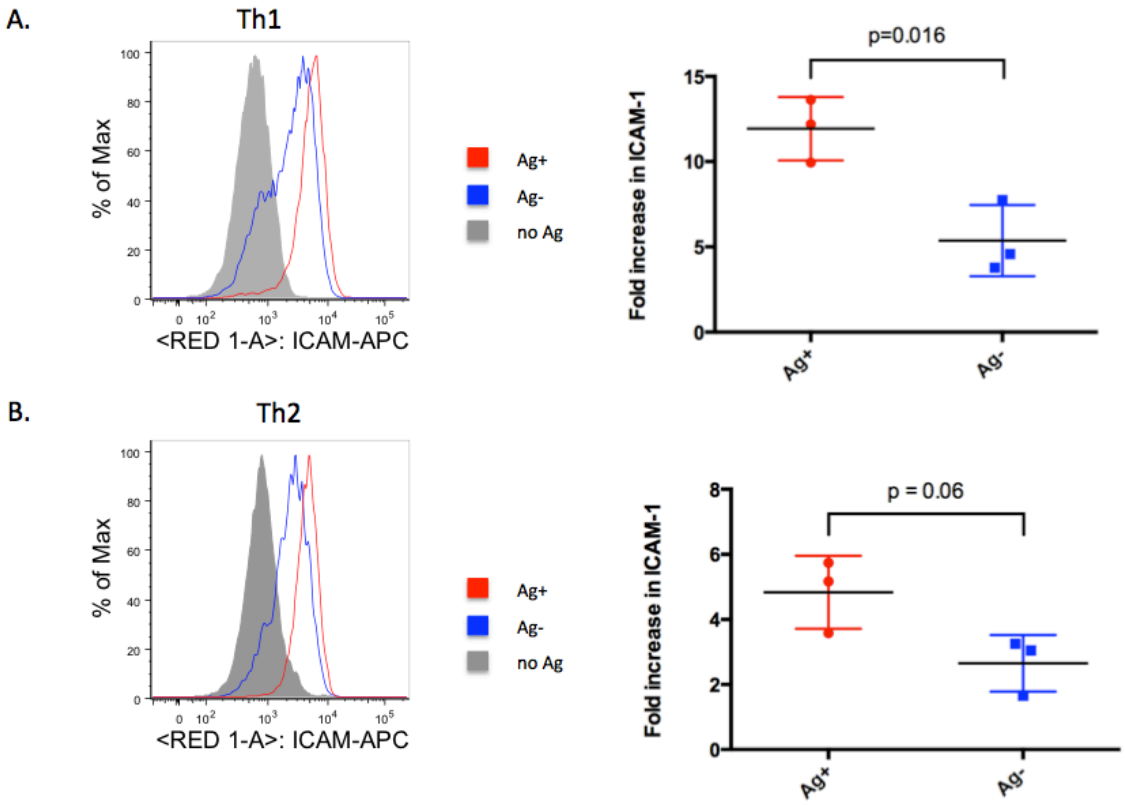


Figure 11. Non-CsA treated 5CC7 Th1 and Th2 cells upregulate ICAM on Ag-pulsed and bystander B cells. A and B, Representative histograms of ICAM upregulation on Ag+, Ag- and no Ag control B cells after overnight incubation with 5CC7 Th1 (A) and Th2 (B) cells. Graphs show the fold increase in ICAM MFI after overnight incubation with helper T cells in each B cell population for three individual experiments (+/- SD). The fold increase in ICAM MFI for the no Ag control B cell was set to 1.

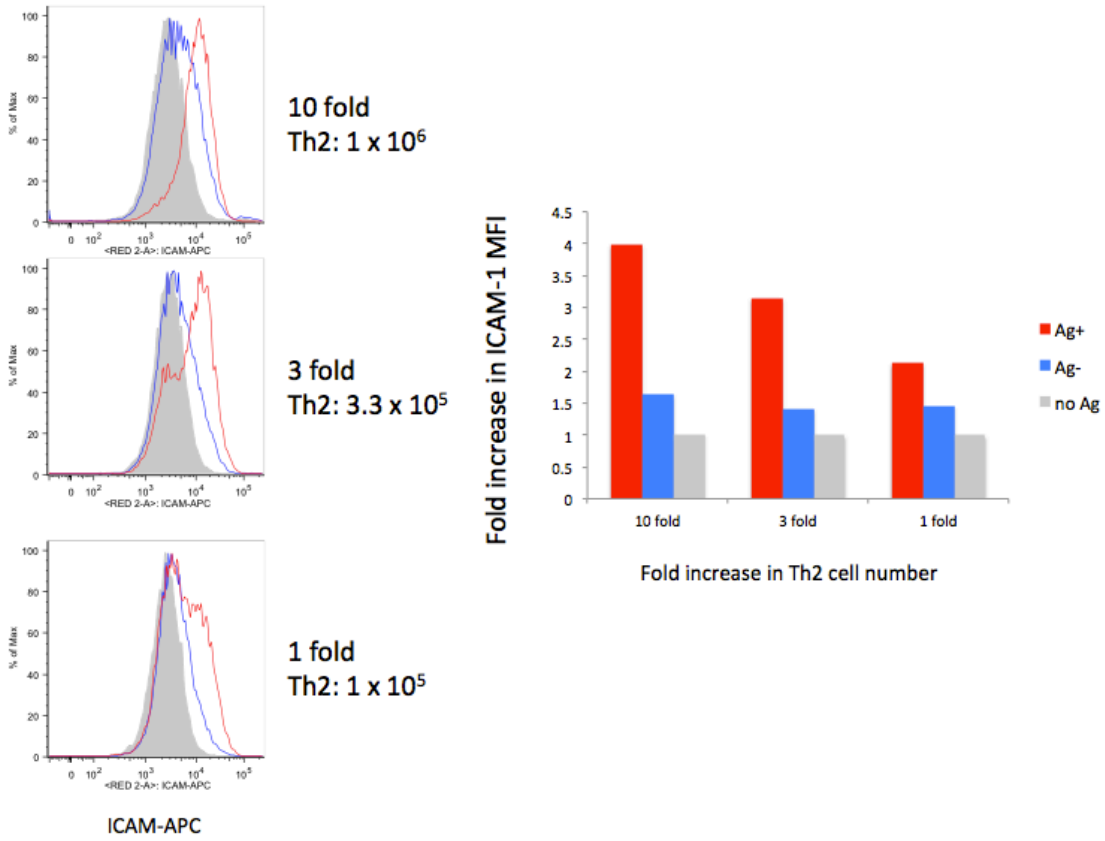


Figure 12. Ag-specific help increases with increasing 5CC7 Th2 cell number. ICAM upregulation was measured on Ag-pulsed (Ag+) and bystander (Ag-) B cells following overnight incubation with non-CsA treated 5CC7 Th2 cells. Histograms show ICAM expression on each B cell population following overnight incubation with the number of 5CC7 Th2 cells listed to the right of the histogram. The bar graph shows the fold increase in ICAM MFI on each B cell population (Red- Ag+ and Blue- Ag-) compared to no antigen control well (grey, set to 1).

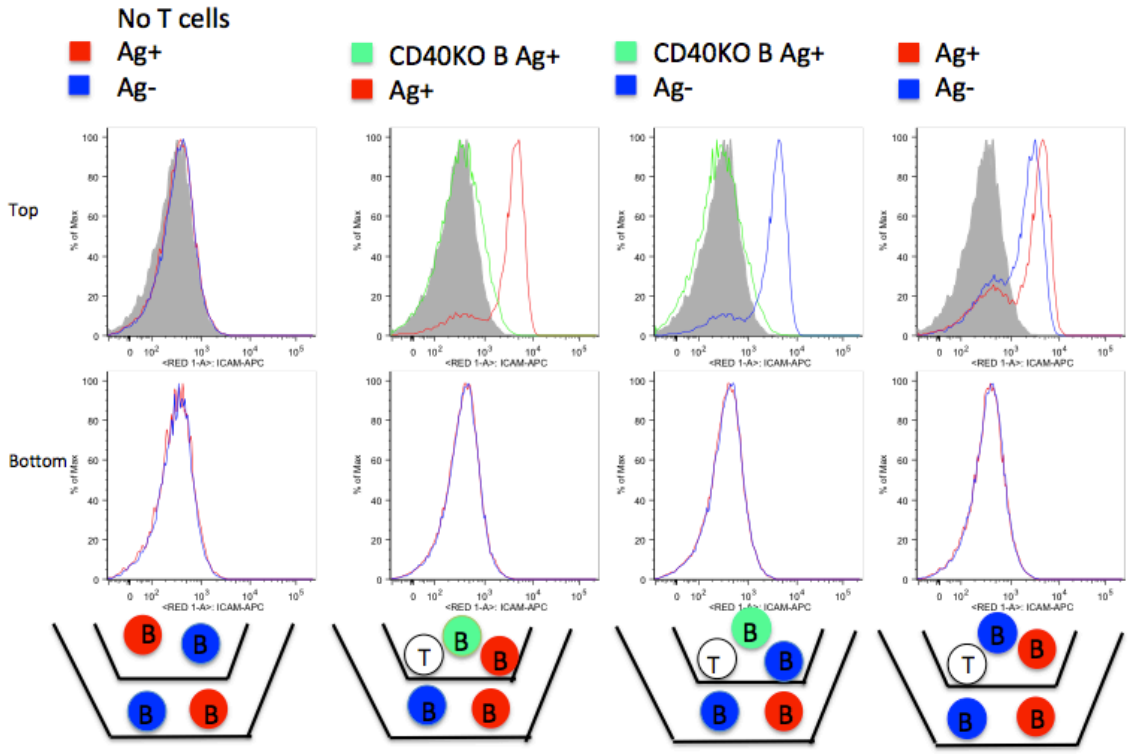


Figure 13. Antigen-specific (Ag+) and bystander (Ag-) T cell help is dependent on CD40L and direct cell-cell contact. Non-CsA treated 5CC7 Th1 cells were added along with the B cell populations depicted below the histograms to the top and bottom chambers of the transwell overnight. ICAM upregulation was measured on the B cell populations in both the top and bottom chambers of the transwell. Listed on top of the histograms are the cell populations in the top chamber of the transwell. Both antigen-pulsed (Ag+) and bystander (Ag-) B cells were added to the bottom chamber of the transwell for each experiment and their corresponding ICAM histograms are shown below the histograms for the B cells in the top chamber of the transwell. The grey histogram on each graph is the no antigen control. n= 3 independent experiments.

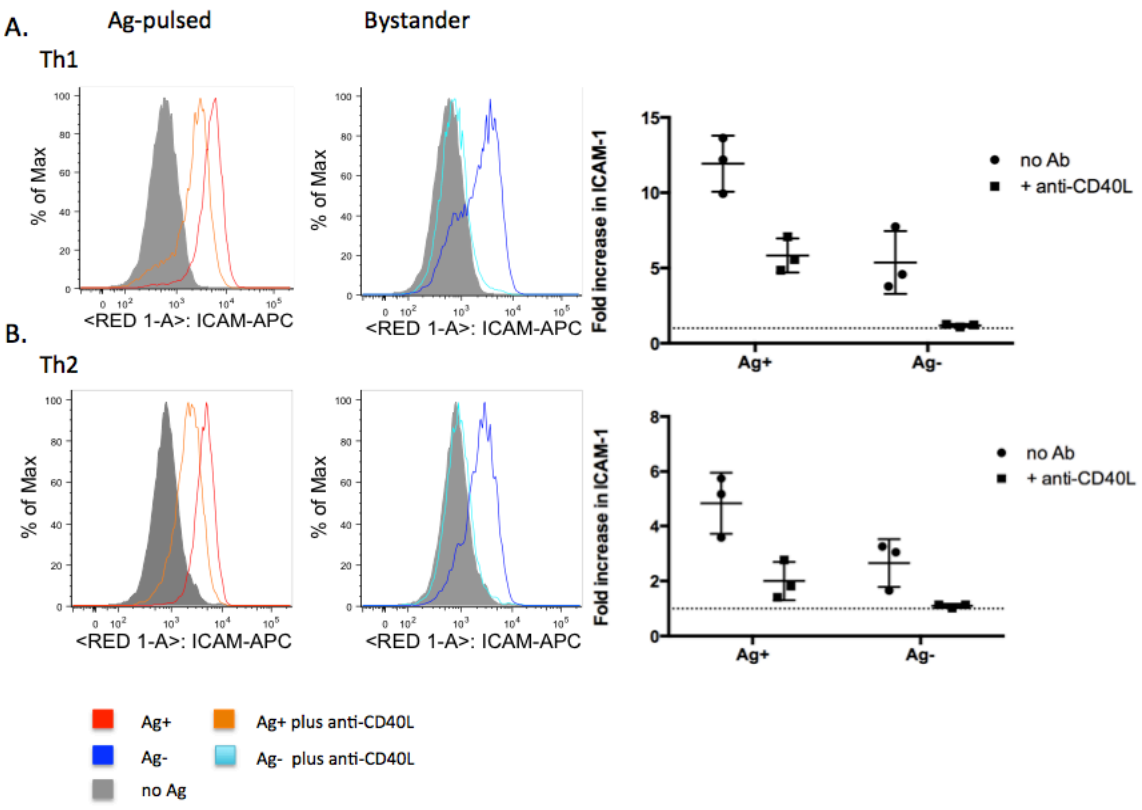


Figure 14. Antigen-specific help but not bystander help is resistant to neutralizing anti-CD40L blocking antibody. A and B, Non-CsA treated 5CC7 Th1 and Th2 cells were incubated with antigen-pulsed (Ag+) and bystander (Ag-) B cells in the presence of absence of neutralizing anti-CD40L antibody (10 µg/ml). Activation of Ag+ (left histogram) and Ag- (right histogram) B cells was accessed by ICAM upregulation. Representative histograms show the levels of ICAM for indicated B cells. A and B, Graphs shows the fold increase in ICAM MFI for each B cell population (Ag+ and Ag-) in the presence of absence of neutralizing anti-CD40L antibody compared to a no Ag control for each of three independent experiments performed (+/- SD). Dotted line on the graph indicates no upregulation of ICAM (y=1).

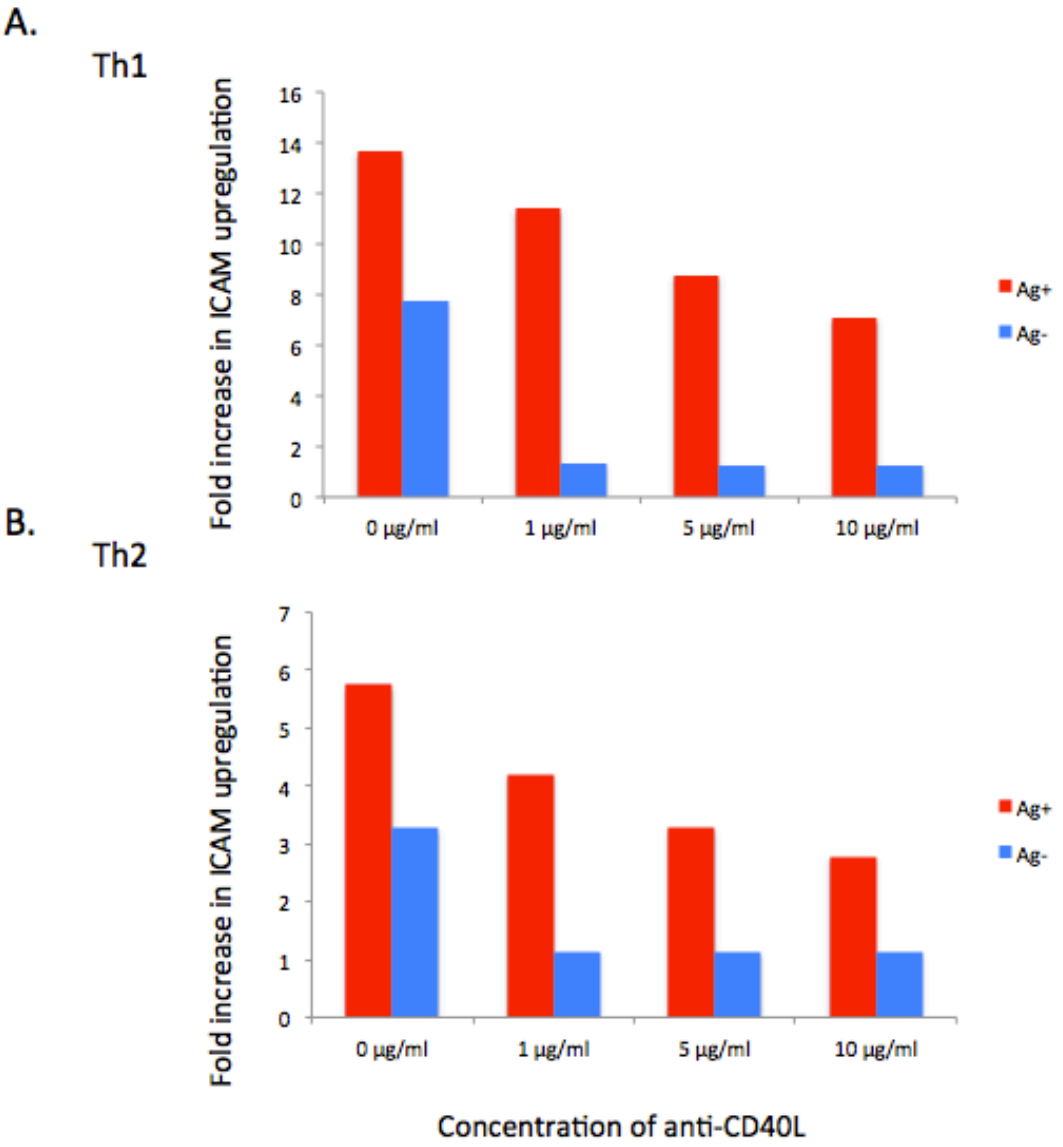


Figure 15. Bystander help is sensitive to low concentrations of anti-CD40L that only partially inhibit antigen-specific help. Graphs show the fold increase in ICAM on antigen-specific (Ag+) and bystander (Ag-) B cells after overnight incubation with non-CsA treated 5CC7 Th1 (A) and Th2 (B) cells with the addition of different concentrations of anti-CD40L. For each experiment the ICAM MFI on B cells in a well with no antigen was set to 1.

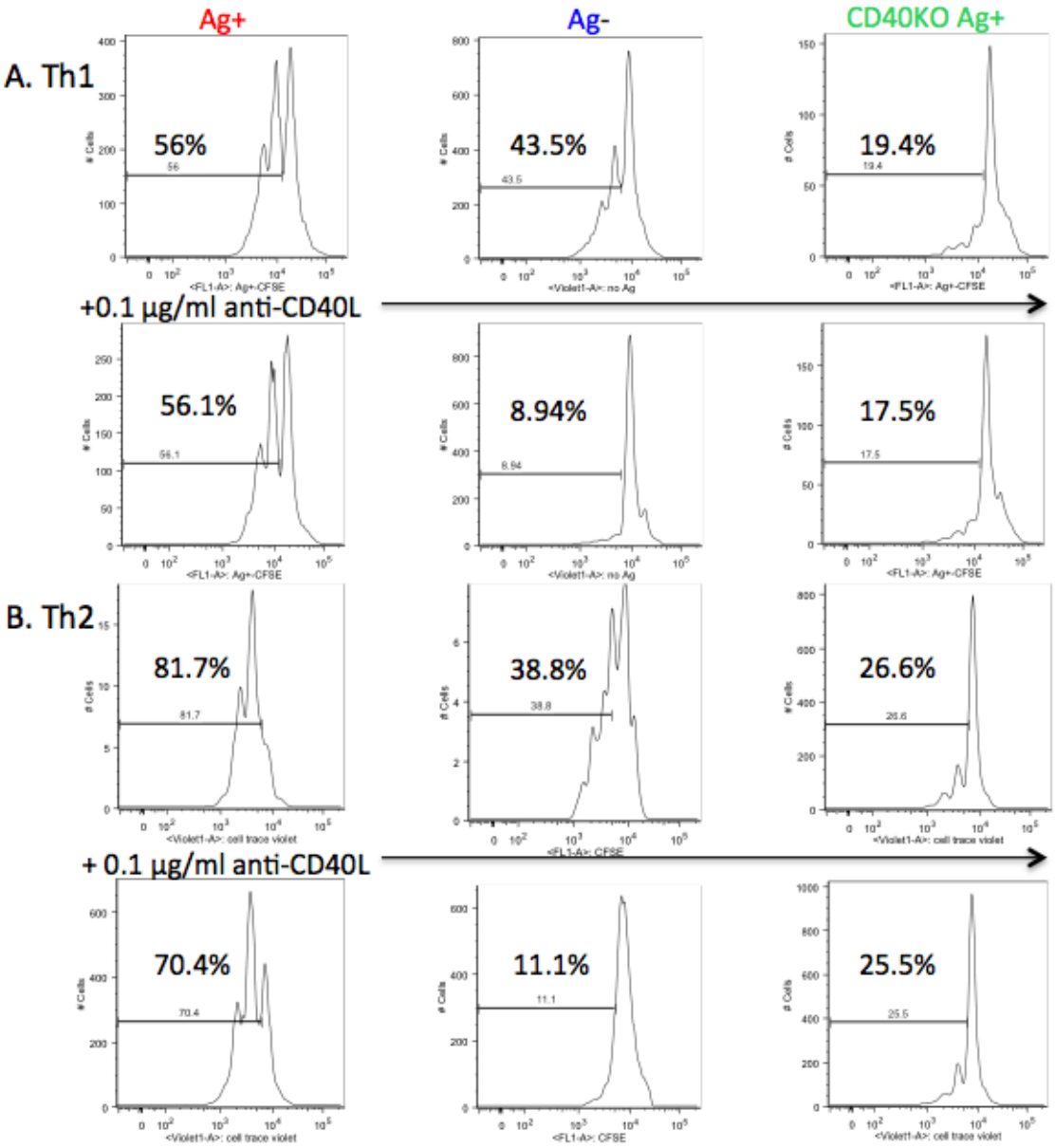


Figure 16. Bystander proliferation is completely blocked at low concentrations of anti-CD40L that have little effect on proliferation of antigen-pulsed B cells. A and B, Differentially labeled (Cell Trace Violet or CFSE) Ag-pulsed (Ag+) and CD40KO Ag+ along with Ag- B cells were incubated with 5CC7 Th1 (A) or Th2 (B) cells and proliferation was assessed after 48 hours by dilution of these dyes. The top row of histograms in A and B show B cell proliferation without the addition of anti-CD40L antibody. The bottom rows in A and B show proliferation of B cell populations with the addition of 0.1 µg/ml anti-CD40L blocking antibody. The percent of divided cells is labeled on each histogram.

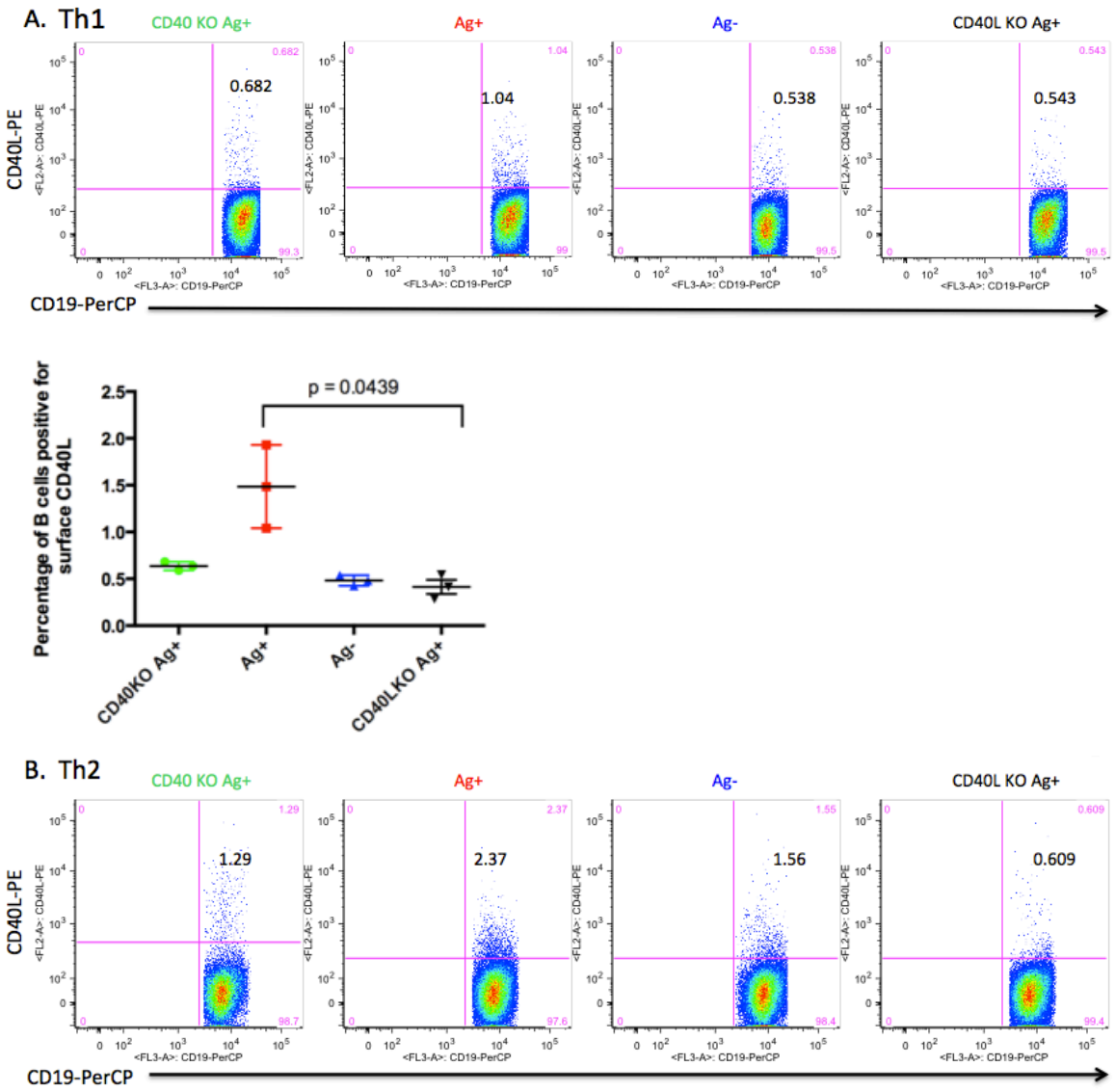


Figure 17. Limited levels of CD40L are detected on the surface of antigen-pulsed B cells after overnight incubation with 5CC7 Th1 or Th2 cells. A and B, The presence of CD40L detected by surface staining of target B cells following overnight incubation with 5CC7 Th1 or Th2 cells appears dependent on the target B cell's ability to produce CD40L. Dot plots show surface CD40L on the target B (CD19+) cell populations listed above the plots following overnight incubation with 5CC7 Th1 (A) or Th2 (B) cells. The graph shows percentage of each B cell population that is positive for surface CD40L following overnight incubation with 5CC7 Th1 cells for 3 independent experiments (+/- SD). Th2 experiment was not quantified because it was only performed once.

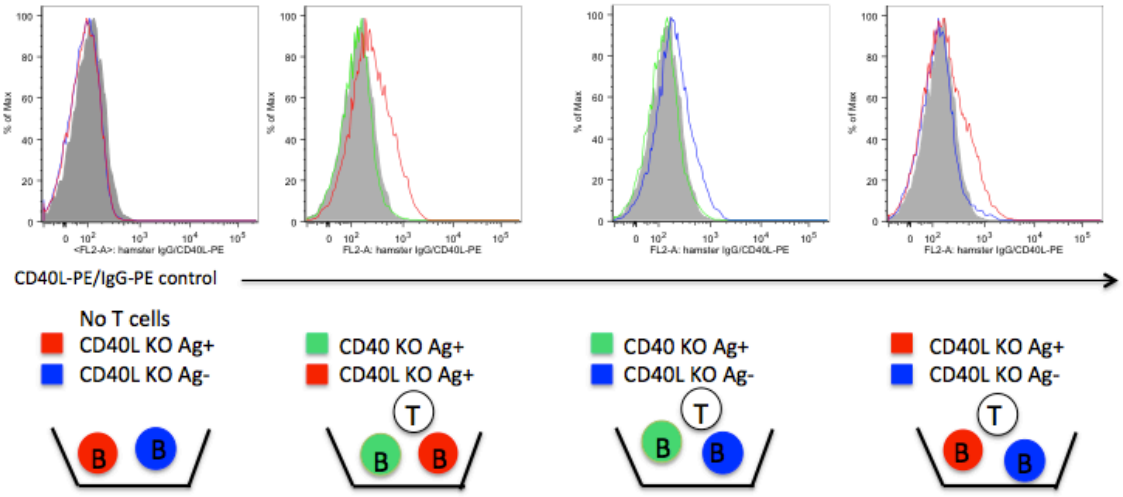


Figure 18. Ag-pulsed B cells (Ag+) internalize CD40L following overnight incubation with 5CC7 Th1 cells. CD40L KO Ag+, CD40 KO Ag+, and CD40L KO Ag- B cell populations were incubated with 5CC7 Th1 cells overnight, then fixed, permeabilized, and stained with anti-CD40L-PE antibody. Listed and depicted below each histogram are the cells added to corresponding well. The control histogram (solid grey) and is obtained from intracellular staining with a hamster IgG-PE antibody of CD40LKO Ag+ B cells incubated with Ag- B cells and 5CC7 Th1 cells. The experiment with CD40L KO Ag+ and CD40 KO Ag- B cells was performed twice. The experiment with CD40L KO Ag+ and CD40L KO Ag- B cells was performed 3 times.

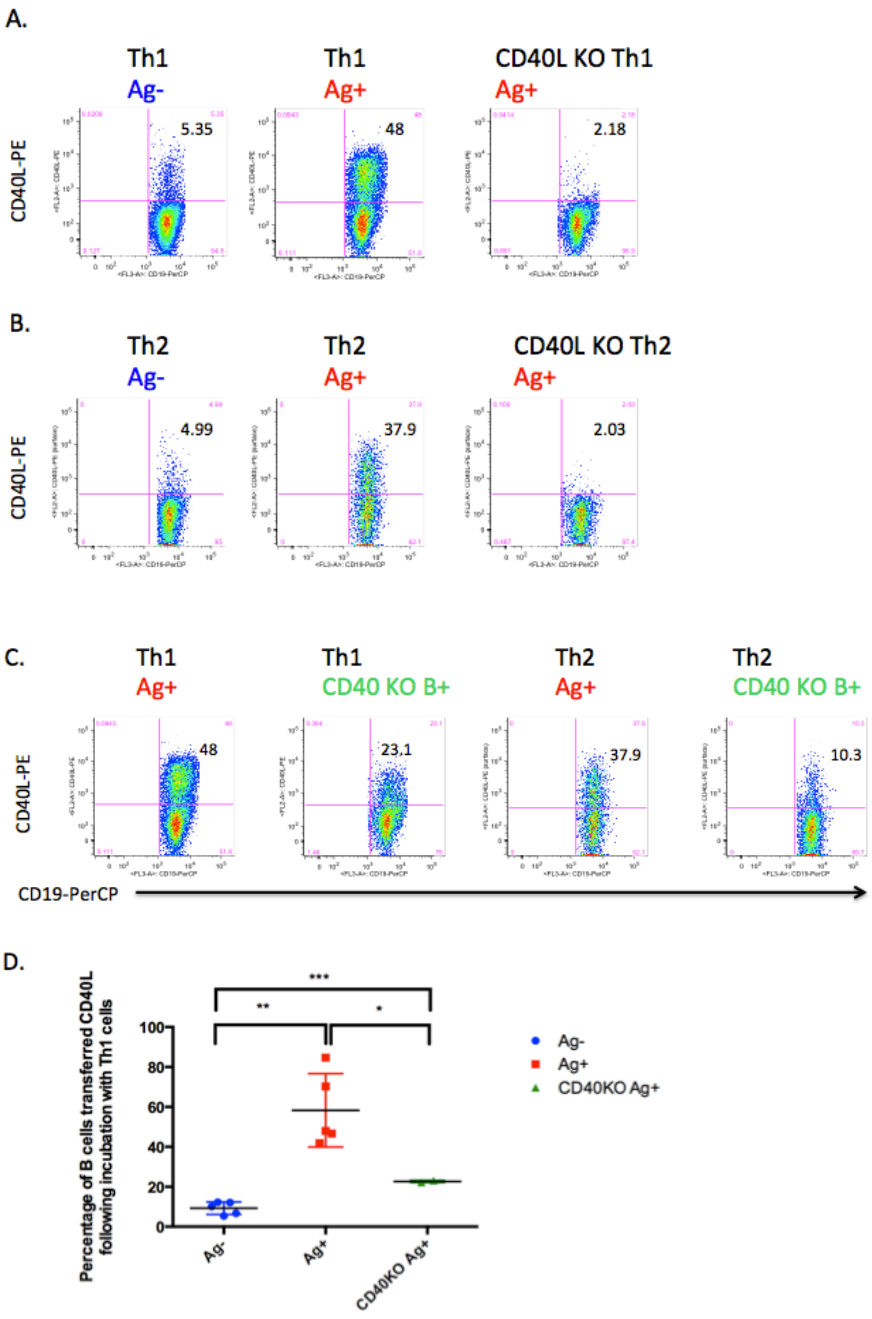


Figure 19. CD40L is transferred to antigen-pulsed B cells and not bystander B cells following overnight incubation with 5CC7 Th1 or Th2 cells. A and B, Each FACS plot shows the percentage of B cells that have acquired CD40L following overnight incubation with 5CC7 Th2 (A) or Th1 (B) cells in the presence of fluorescently labeled anti-CD40L antibody. Th1 results are representative of 5 experiments and Th2 results are representative of 2 experiments. C, Percentage CD40 KO Ag+ and Ag+ B cells positive for CD40L following overnight incubation with 5CC7 Th1 or Th2 cells in the presence of

anti-CD40L-PE. Th1 (n=2) Th2 (n=1) D, Graph shows the results from individual experiments with Th1 cells. Th1 Results were statistical analyzed by an unpaired, two-tailed t-test. P values were calculated as follows: Ag+ vs. Ag- p = .0035, Ag+ vs. CD40KO Ag+ p = 0.0122, and Ag- vs. CD40KO Ag+ p = 0.0004.

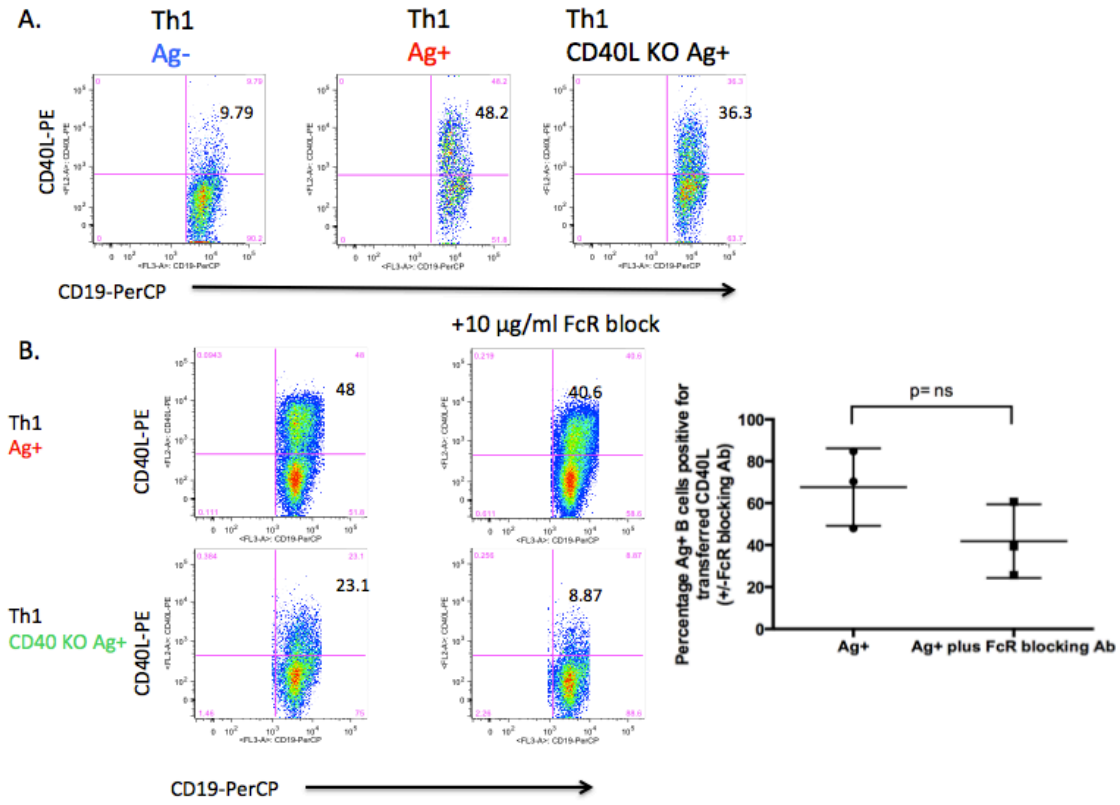


Figure 20. Antigen-specific CD40L transfer is not dependent on B cell-derived CD40L and is mostly independent of FcR on B cells. A, Dot plots show CD40L transferred to Ag-pulsed (Ag+), bystander (Ag-), and CD40L KO Ag+ B cells following overnight incubation with 5CC7 Th1 cells in the presence of fluorescently labeled anti-CD40L antibody. B, Percentage of B cells (Ag+ or CD40 KO Ag+) transferred CD40L following incubation with 5CC7 Th1 cells in the presence or absence of FcR blocking antibody (10 µg/ml). Graphs shows the percentage of B cells transferred CD40L in the presence of absence of FcR blocking antibody for three individual experiments (+/- SD). Addition of FcR blocking antibody with CD40 KO Ag+ cells was only performed once.

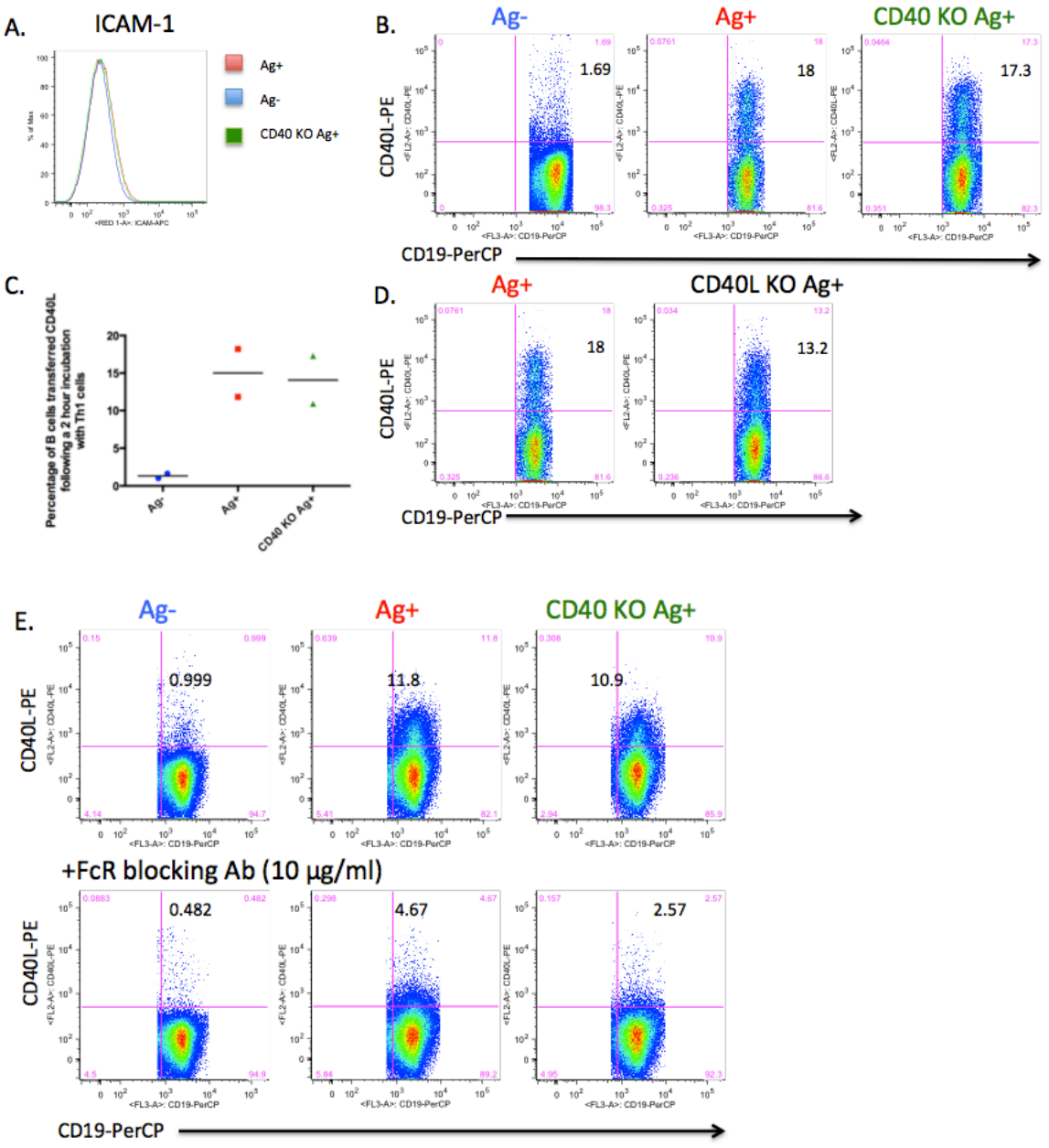


Figure 21. CD40L is transferred equally to antigen-pulsed B (Ag+) and CD40 knock-out antigen-pulsed B cells (CD40 KO Ag+) B cells following a two-hour incubation in the presence of PE-anti-CD40L with 5CC7 Th1 cells. A, Histogram shows ICAM levels on the B cell populations listed in the legend after two hours of T/B cell incubation. B and D, CD40L transfer detected during two hours on B cell populations listed above FACS plot. C, Graph shows the percentage of B cells positive for CD40L transfer following 2 hour incubation with 5CC7 Th1 cells in the presence of PE-anti-CD40L for two individual experiments. E, Transfer of CD40L to the B cell populations listed above FACS plots in the absence (top) or presence (bottom) of FcR blocking antibody (10 µg/ml) during the two-hour incubation time. This experiment was only performed once.

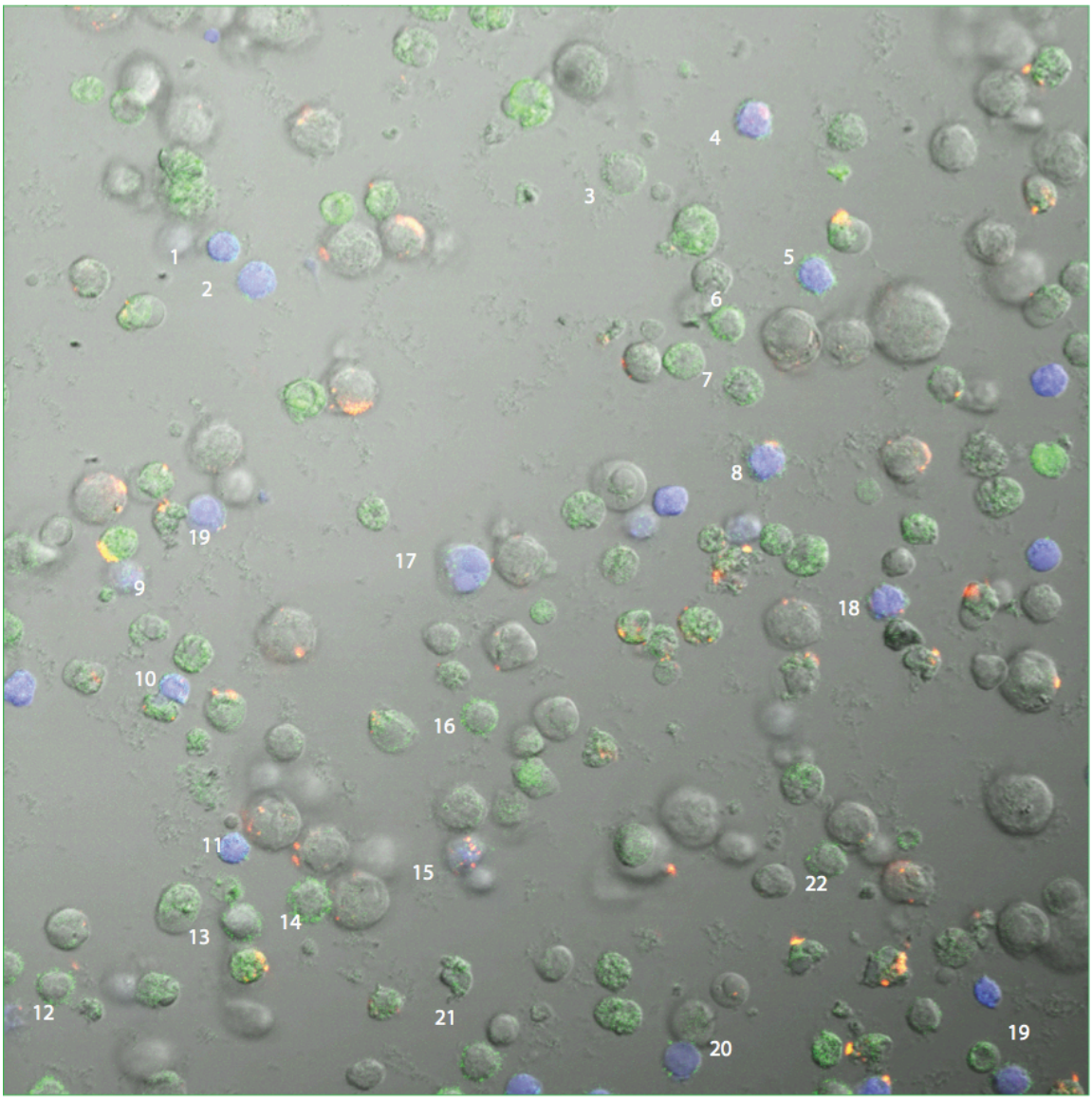


Figure 22. Antigen-pulsed B cells and not bystander B cells are positive for CD40L following overnight incubation with 5CC7 Th1 cells in the presence of anti-CD40L antibody as assessed by laser scanning microscopy. The microscopy image shows B and T cells following overnight incubation in the presence of anti-CD40L-APC antibody that were then stained for CD19-FITC (B cell marker, shown in green), fixed, and added to a single chamber of a poly-l-lysine treated 8 chambered dish. Prior to overnight incubation Ag+ B cells were Cell Trace Violet (CTV) labeled, shown in blue. CD40L presence on both T and B cells is detected by anti-CD40L-APC and is shown in red in the image. Numbers label B cells in the image that fit requirements for quantification as described in the methods of this chapter.

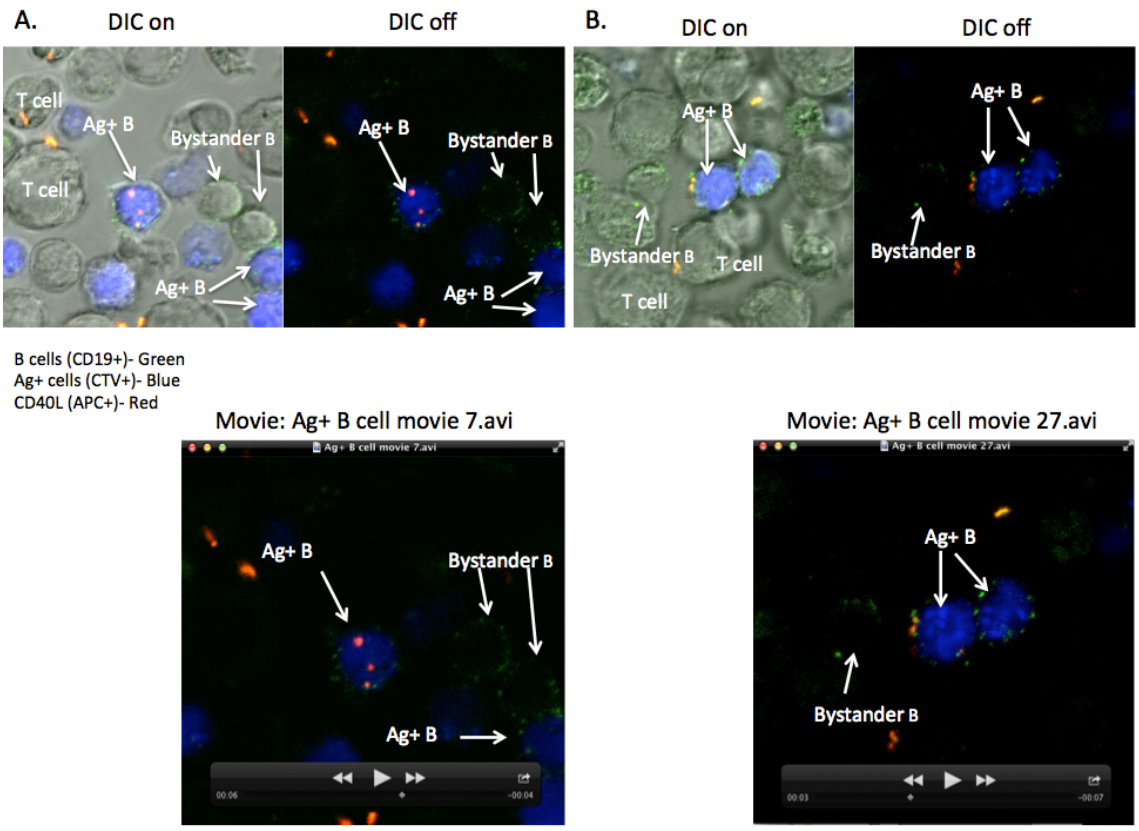


Figure 23. Transferred CD40L is found intracellularly and on the surface of Ag-pulsed B (Ag+ B) cells following overnight incubation with 5CC7 Th1 cells. Representative images of high magnification Ag+ B cells (green (CD19) and blue (CTV)) and bystander B (Ag-) cells (green only (CD19)) together with T cells are shown in the top of A. and B. with and without DIC following overnight incubation. White arrows point to each B cell and a few CD40L expressing T cells are labeled in the DIC image. Shown in the lower images in A. and B. is a frame from a movie created from 0.25 µM z stacks through the cells shown in the images above. CD40L-APC positive cells are shown in red in these images. Intracellular CD40L is detected in the Ag+ B cell in A. An example of surface CD40L is shown in B.

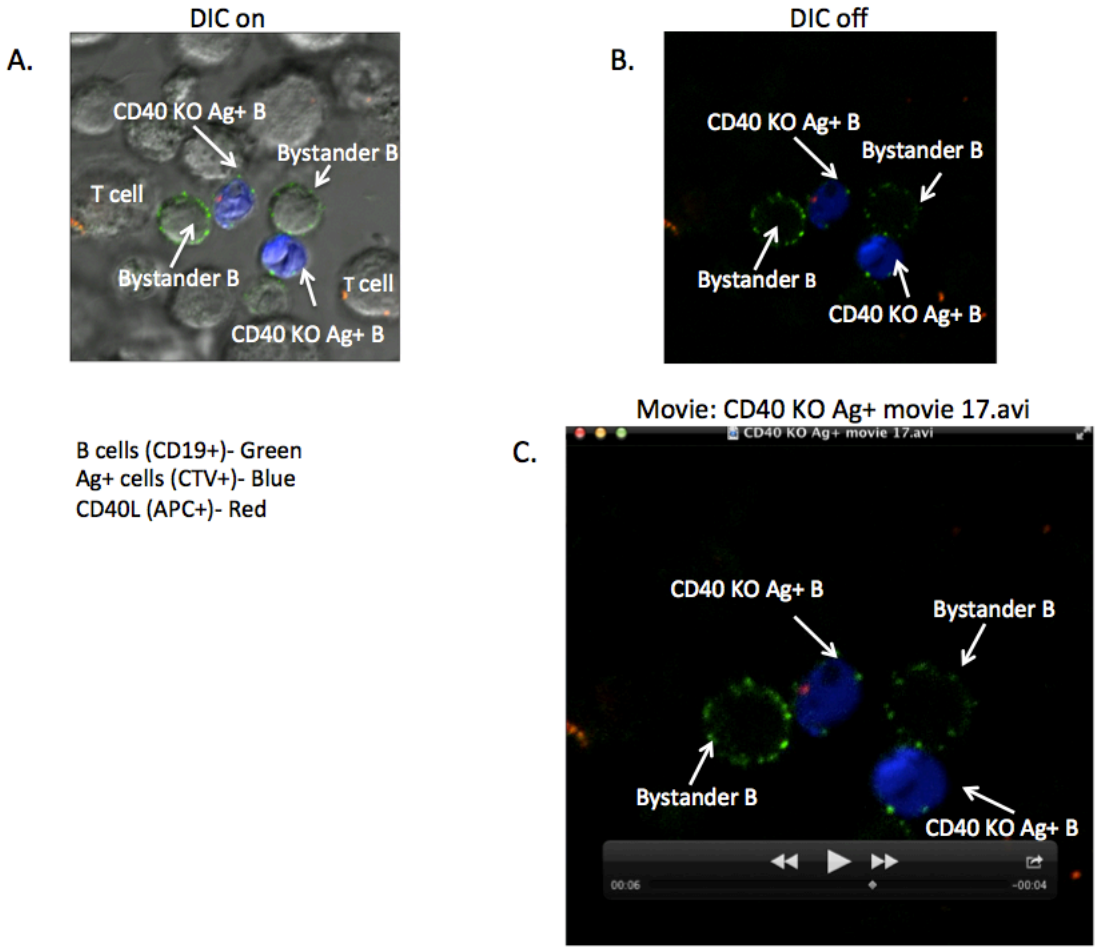


Figure 24. CD40L is present on Ag-pulsed CD40 knock-out (CD40 KO Ag+) B cells following incubation with 5CC7 Th1 cells. A, Representative high magnification image of T and B cells following overnight culture in the presence of anti-CD40L-APC antibody, stained with CD19-FITC, and placed into a poly-l-lysine treated glass chamber. A, White arrows point to a CD40 KO Ag+ containing CD40L (shown in red) and a CD40 KO Ag+ B cell negative for CD40L following overnight culture. Bystander B cells (Ag-) are stained with CD19 (green only) and CD40 KO Ag+ B cells are both CTV (blue) and CD19 (green) positive. A few T cells are also labeled in the image. B, Same image as in A but with the DIC turned off in order to better display fluorescent labeling. C, A frame from a movie created from 0.25 μM z stacks through the cells in A and B.

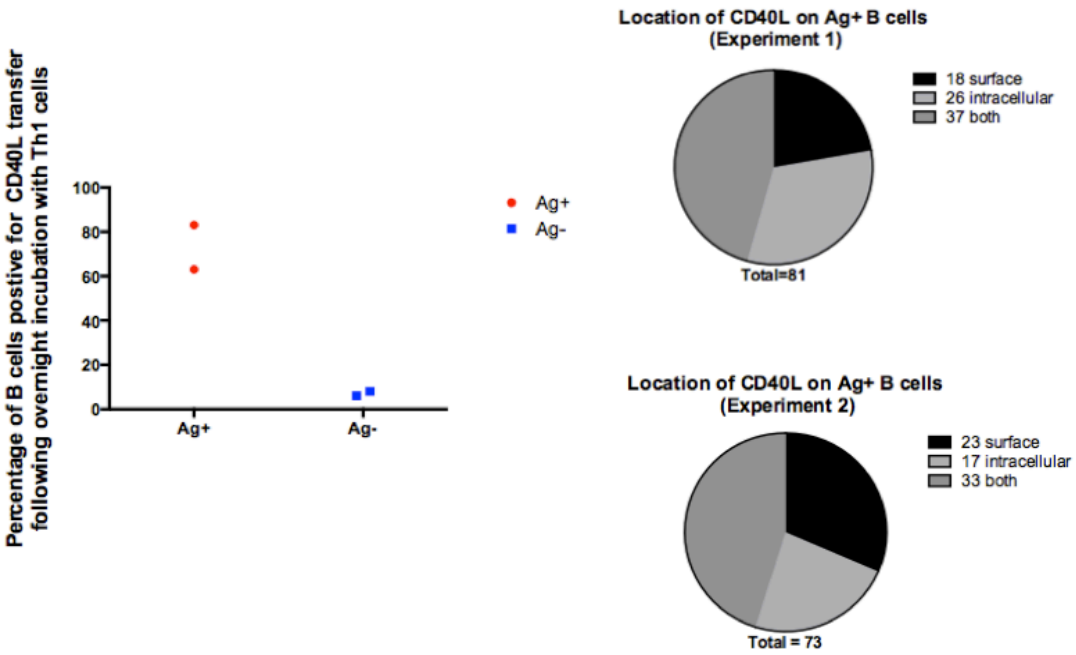


Figure 25. Transferred CD40L is detected both at the surface and intracellularly on antigen-pulsed B cells. Quantification of data from the Ag+ and Ag- B cells from two microscopy experiments as shown in Figures 15 and 16. The graph shows the percentage Ag+ and Ag- B cells positive for presence of CD40L following incubation with 5CC7 Th1 cells in the presence of anti-CD40L-APC antibody for two individual experiments. Pie charts show the relative numbers of B cells positive for surface, intracellular, or both surface and intracellular CD40L following overnight incubation with 5CC7 Th1 cells. Intracellular versus surface CD40L was assessed via z-stacks using CD19 as a surface marker on B cells. There were 281 B cells analyzed in Experiment 1 (128 Ag+ and 153 Ag-) and 193 total B cells for Experiment 2 (88 Ag+ and 105 Ag-).

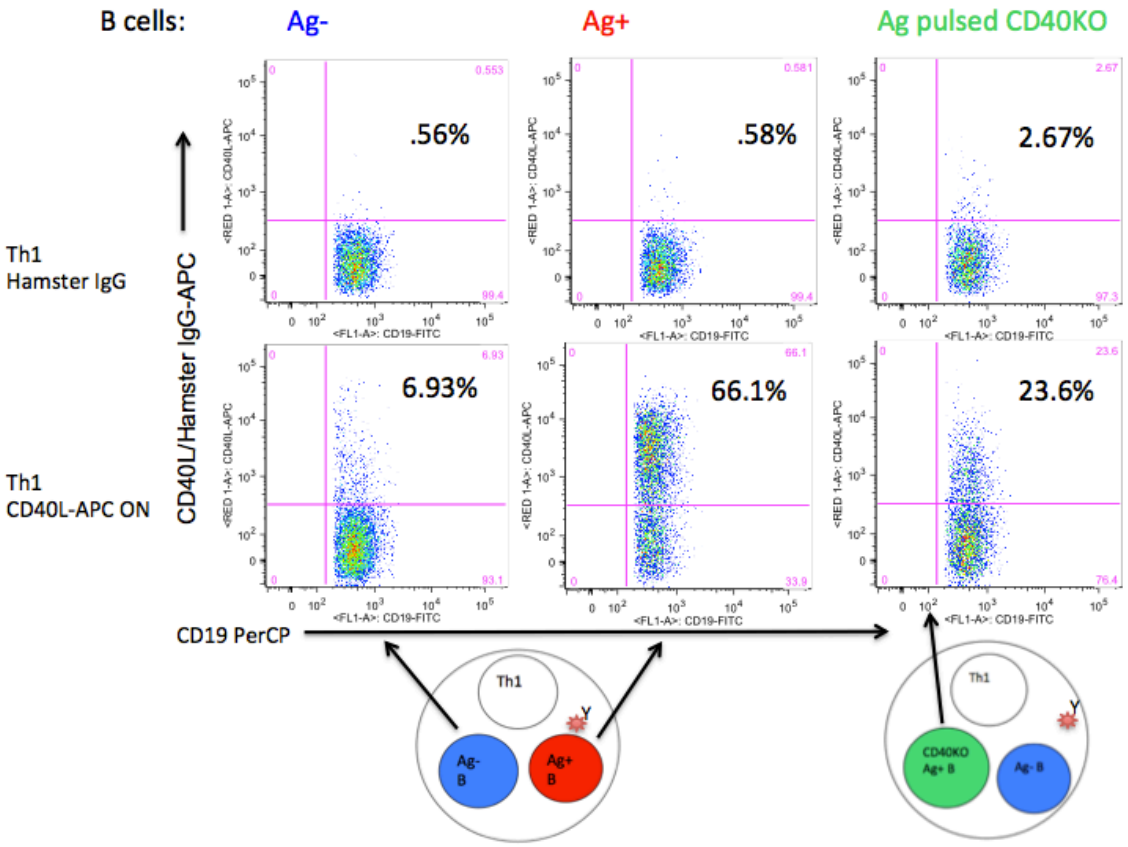


Figure 26. Microscopy cultures analyzed by flow cytometry confirm percentages of CD40L transfer to antigen-pulsed B cells by 5CC7 Th1 cells after overnight incubation in the presence of APC-anti-CD40L antibody as determined by microscopy. The top row shows B cell populations stained with control hamster IgG-APC during overnight incubation with 5CC7 Th1 cells in the presence of this antibody. The bottom row shows B cell populations following overnight incubation of T and B cells with addition of an APC-labeled CD40L antibody. Results are comparable to the statistics determined from laser scanning microscopy experiments and quantified in the graph in Figure 18.

Chapter 4: Despite disorganized synapse structure, Th2 cells deliver CD40L to antigen-expressing B cells with the same efficiency as Th1 cells

Jennifer Gardell and David Parker planned submission.

Abstract: Th1 and Th2 cells, upon recognition of peptide displayed on MHC molecules, form distinct immunological synapse structures (25, 114). Th1 cells have a bull's eye synapse structure with the TCR and MHC interactions occurring central to adhesion molecules, while Th2 cells have a multifocal synapse with small clusters of TCR/MHC interactions throughout the area of T cell/APC interaction. The function of this difference in immunological synapse structure has yet to be determined. Abraham Kupfer was the first to describe the immunological synapse which he observed in natural killer cells, cytotoxic T lymphocytes, and helper T cells interacting with antigen-presenting target cells (198). These studies were originally pursued investigating the question of how NK and CTLs could lyse their targets cells after antigen recognition while leaving their own cellular membranes intact. Kupfer saw that upon interaction with antigen-presenting cells there was a reorganization of microtubule organizing center in the T cells followed by an accumulation of actin at the area of interaction with the APC. He proposed that the immunological synapse structure may be important for the delivery of cytotoxic granules, as well as T cell help in the case of T helper cells. Therefore I hypothesized that the Th1/Th2 difference in immunological synapse structure maintains functional differences in the delivery of cytokines between these two T cell subsets. CD40 ligand (CD40L) is an essential membrane bound cytokine delivered by helper T

cells after recognition of foreign antigens displayed on the MHC molecules of antigen-presenting cells. It has been shown that CD40 accumulates at the immunological synapse (181), but whether or not CD40L is mobilized to the cell surface at this point of contact to induce changes in the antigen-presenting cell is not known. In order to evaluate the delivery of CD40L within different immunological synapse structures, I used the *in vitro* overnight assay described in Chapter 3 in which Th1 and Th2 cells were incubated with antigen-specific and bystander B cells. Results from these experiments suggest that despite differences in immunological synapse structure, Th1 and Th2 cells preferentially activate antigen-specific APCs. When Th1 and Th2 cells synthesize large amounts of CD40L, both the antigen-specific and bystander APCs are activated. Despite significant differences in immunological synapse structure between Th1 and Th2 cells, differences in bystander and antigen-specific activation and inhibition of activation by anti-CD40L were not revealed by the methods employed in this Chapter.

Introduction:

CD40L is essential to the development of an adaptive immune response. Once an effector T cell enters a secondary lymphoid structure, it first interacts with a B cell at the boundary between the T cell zone and B cell zone (199). Some T cells are then brought into the follicle or B cell area by the B cells and these T/B interactions lead to the formation of a germinal center. The T helper cells involved in T/B interactions expresses CD40L on their surface which can engage with CD40 and activate B cells to proliferate and produce antibodies specific for the immunizing antigen. If CD40L is lacking during this response, as in CD40LKO mice or after addition of anti-CD40L antibody, antibody formation is suppressed, and germinal centers do not develop (64, 149). Due to the essential nature of this cytokine in development of adaptive immunity, it is important to learn more about how this cytokine is delivered during the initiation and course of this response.

Cytokines are known to be released using two different pathways upon interaction with an APC. Some cytokines, including IL-2 and IFNγ are delivered towards the center of the immunological synapse, while others, including TNFα, are delivered multidirectionally, and actually away from the immunological synapse (105). Delivery of CD40L to the cell surface was not investigated in this study, so it remains to be determined if this cytokine uses one of these two pathways and/or alternative means (granule exocytosis, microvesicles, exosomes) to reach the cell surface upon interaction of a T cell with an APC. I hypothesize that delivery of CD40L would be tightly regulated and directed

towards the synapse. This directed delivery would ensure only antigen-specific B cells would become activated and receive the signals necessary to proliferate in the germinal center. This targeted release of CD40L is comparable to cytotoxic T lymphocyte's (CTL's) delivery of lytic granules to induce killing of only virally infected target cells, and not uninfected bystanders (99, 100). Similarly, targeted delivery of CD40L by helper T cells could limit help to only the antigen-specific B cells, and thereby aids in the selection process necessary to develop high-affinity antibodies against foreign pathogens.

T cells express CD40L on the T cell surface with two different kinetics (76). First, there is a small amount of preformed, intracellular CD40L expressed in all T helper cell subsets, excluding T regulatory cells, that can be brought to the cell surface rapidly following brief TCR stimulation (177). Additionally, like other cytokines, CD40L can also be produced *de novo* upon longer interaction with an APC. This interaction induces a signaling cascade in which CD40L's transcription factors, AP-1 and NFAT, translocate to the nucleus initiating transcription of CD40L mRNA (17, 18). Since *in vivo* imaging of germinal centers has proven that most T cell/B cell interactions are brief and not long enough for production of *de novo* protein (46-49), I was interested in the delivery of preformed, intracellular CD40L, and how this delivery might be affected by differences in immunological synapse structure.

Th1 cells have a more organized bull's eye synapse structure, while Th2 cells have a less organized, multifocal synapse (94). I hypothesized difference in immunological synapses may allow for differences in delivery of effector cytokines between these two subsets.

Th1 and Th2 cells play very different roles in adaptive immunity. Th1 cells specifically activate dendritic cells in a process called dendritic cell licensing, a maturation process necessary for upregulation of costimulatory molecules and production of cytokines necessary for CD8 T cell activation and survival. Th1 cells are also involved in specific activation of antigen-presenting B cells and have the ability to differentiate to a more CTL-like cell and induce the killing of target cells through the release of cytotoxic granules. Th2 cells, on the other hand, are involved in more diffuse inflammatory responses like allergies and asthma (200). Therefore Th1 cells need a way to deliver their cytokines in a restricted manner, while Th2 cells need to send signals to activate many cells in a tissue at once. Delivery of effector molecules to the immunological synapse is dependent on the extent of actin clearance in this interaction zone (110, 201). Th2 cells, when compared to Th1 cells, appear to have less actin clearance within the center of the immunological synapse (94). Therefore I proposed that Th2 cells may have less directed delivery of CD40L and be less efficient at activating solely antigen-pulsed cells, inducing bystander B cells activation as well. Unfortunately techniques to polarize the most relevant subset involved in B cell help, T follicular helper cells, have not been developed (202), so the immunological synapse structure of Tfh cells could not be investigated in these studies.

Materials and Methods:

Mice:

AD10 TCR transgenic mice on a B10.BR background, specific for pigeon cytochrome c 88-104 and reactive against moth cytochrome c 88-103 were provided by S. Hedrick (University of California at San Diego, La Jolla, CA) by way of P. Marrack (National Jewish Center, Denver, CO) B10A, 5CC7 TCR transgenic, and B10A CD40 knock-out mice were purchased and generated as stated in Chapter 3 of this dissertation.

Antibodies and labels:

The antibodies and reagents used for flow cytometry experiments were as follows: anti-CD4 PeCy7, anti-CD19 PerCP, ICAM-biotin, Streptavidin-APC, anti-CD40L-PE, anti-CD40L-APC. All were from eBiosciences. Cells were differentially labeled with carboxyfluorescein succinimidyl ester (CFSE) or Cell Trace Violet (CTV) (Invitrogen) to mark Ag-pulsed and bystander cells.

In vitro T cell polarization:

Th1 and Th2 cells were generated using the same conditions as stated in Chapter 3.

Intracellular Cytokine Stain:

Th1 cells and anti-IL-4 treated Th2 cells were tested for production of IL-4 and IFNγ by intracellular cytokine stain. One million T cells were suspended in 100µl media treated with brefeldin A (eBioscience) and phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) for 5 hours in a 96 well plate at 37 °C. Cells were fixed and permeabilized (eBiosciences kit) and stained with anti-IL-4-PE (eBioscience) and anti-IFN γ-APC (eBioscience). These samples were then analyzed on BD LSRII flow cytometer.

***In vitro* overnight assay:**

Assay was performed using the same conditions as stated in Chapter 3 of this dissertation.

Supported planar lipid bilayers:

GPI-linked forms of Oregon Green 488 labeled I-E^k (200 molecules/ μm^2) and Cy5-labelled ICAM-1 (300 molecules/ μm^2) were incorporated into dioleoylphatidylcholine bilayers as described (84, 85). 100 µM MCC was loaded to the bilayers for 24 hours in a PBS/citrate buffer pH 4.5 at 37° C. These bilayers were supported on a coverslip in a

Bioptechs flow cell (http://www.bioptechs.com/Products/FCS2/fcs2.html) closed chamber system.

Live Cell Microscopy:

10^7 Th2 or anti-IL-4 treated Th2 in 1ml HBS buffer with 1% human serum albumin were injected onto the bilayers at 37°C. Imaging was performed with a 60 X objective using the DeltaVision CoreDV Widefield Deconvolution System. This system includes an Olympus IX71 inverted fluorescent microscope, Nikon CoolPix HQ cooled CCD camera, and a motorized stage is controlled by XYZ nanomotors, and the DeltaVision SoftWorx software package. Images were analyzed and developed using ImageJ, and differences in immunological synapse structure were evaluated by a blinded observer.

Results:

Prior work in the Parker lab showed that Th1 and Th2 cells have very different immunological synapse structures (94). Whether this was due to the artificially high levels of IL-4 in *in vitro* culture of Th2 cells or truly reflective of a difference between these cell subsets had not been investigated. I compared the immunological synapses generated by the Th2 cells that received anti-IL-4 upon secondary stimulation to those that had been restimulated in the presence of IL-4 on the planar lipid bilayer. I found that both the IL-4 treated and anti-IL-4 treated Th2 cells displayed the characteristic multifocal synapse structure (Figure 27A and 27C). This allowed me to further investigate the delivery of CD40L within two distinct immunological synapse structures generated by the two helper T cell subsets.

All CD4 helper T cell subsets, with the exception of T regulatory cells, have an intracellular compartment of preformed CD40L, which can be expressed rapidly on the cell surface following TCR activation (177). Expression of preformed CD40L is inhibited in *in vitro* generated Th2 cells due to the concentrated IL-4 in the *in vitro* cultures (189). After secondary culture with anti-IL-4, the expression of preformed CD40 ligand was restored in these cells. These experiments were performed with I-A^d-restricted DO11.10 T cells. In order to further investigate delivery of CD40L using the lipid bilayer system, I had to determine if I-E^k-recognizing Th2 cells also expressed performed CD40L upon secondary restimulation and treatment with anti-IL-4. AD10 Th2 cells were polarized with IL-4, anti-IFNγ, irradiated splenocytes, and MCC for their primary stimulation. Four

days after this primary simulation, cells were restimulated with anti-IL4 instead of IL-4 to restore preformed CD40L expression. As with the DO11.10 Th2 cells (Chapter 2, (177)) I saw restoration of CD40L expression (Figure 28A). Now that CD40L expression had been restored in these cells, I had to determine that Th2 cells, after culture in anti-IL-4, continued to express IL-4, the main cytokine indicative of Th2 cell polarization. As expected, the anti-IL-4 treated Th2 cells did produce IL-4 and no IFNγ (Figure 28B).

The reason for the difference in synapse structure between Th1 and Th2 cells is not known. I hypothesized that the ring structure is required for specific delivery of CD40L to antigen-bearing B cells. Therefore Th2 cells, with a multifocal synapse, would be more likely to activate bystander B cells (B cells in the same cultures not bearing antigen) than Th1 cells. This hypothesis was tested in an *in vitro* overnight bystander assay. In this assay, ICAM is upregulated in those B cells that have received a CD40L signal. CD40 knock-out B cells cannot engage CD40L and therefore do not upregulate ICAM when compared to CD40 sufficient B cells (Chapter 3, Figure 10D). Some of the B cells in this assay were pulsed with antigenic peptide, while others (bystanders) were not pulsed.

I was particularly interested in the delivery of preformed CD40L, because it could be delivered in less than 30 minutes of TCR stimulation. *In vivo* imaging studies have shown that this is the time period of most T/B interactions in the germinal center (46-49, 203), where CD40L is know to be an essential player in the generation of high affinity antibodies necessary for the clearance of a pathogen (149, 178). Results show that when CD40L is limited to a small, intracellular amount equivalent to that of preformed CD40L

which can be delivered rapidly to B cells, both Th1 and Th2 cells provide solely antigen-specific activation (Chapter 3, Figure 10A and 10B). Thus, despite differences in immunological synapse structure, there is no difference in bystander activation between Th1 and Th2 cells. Even when T cells and APCs were spun down together in a round-bottom 96 well plate overnight, increasing the likelihood of bystander activation, both Th1 and Th2 cells only activated the antigen-pulsed APCs and not bystanders (Chapter 3, Figure 10C and 10D).

To further explore delivery of antigen specific help, I evaluated antibody inhibition using a CD40L specific blocking antibody. I thought antigen-specific help may be harder to block in the case of Th1 cells due to their bull's eye synapse structure, where the ring of the adhesion molecules or the mode or speed of delivery may limit exposure to the neutralizing antibody, while the Th2 cells have a much more disorganized synapse that may allow for more efficient blocking of the delivery of help. In the presence of CsA to prevent new CD40L synthesis, when only small amounts of intracellular CD40L is present, blocking antibody completely blocks all antigen-specific activation in the case of both Th1 and Th2 cells (Figure 29). Both antigen-specific and bystander help occur when T cells are not treated with CsA and CD40L is expressed in large amounts (Chapter 3, Figure 11A and 11B). The CD40L delivered from Th2 cells to the antigen-pulsed B cells may be more easily blocked, as shown by the slightly greater inhibition of ICAM upregulation on Ag-specific B cells (Chapter 3, Figure 14) at comparable anti-CD40L concentrations (Figure 15 and 30). However this result may be due to differences in the amount of CD40L produced by Th1 and Th2 cells in these experiments, so a difference

between these subsets in sensitivity to antibody inhibition or antigen-specific versus bystander activation was not ascertained in the experiments presented in this Chapter.

Discussion:

Despite their different roles in the development of an immune response, Th1 and Th2 cells both deliver CD40L in a directed manner to antigen pulsed cells (Results summarized in Figure 31). Although I did not detect differences in delivery of CD40L due to differences in immunological synapse structure among Th1 and Th2 cells, T cells produce a wide variety of other cytokines and proteins that influence the immune response at the point of T cell/APC contact. It may be that differences in immunological synapse structure are necessary for the proper release of other cytokines or chemokines. Specifically, the classic cytokine released by Th1 cells is IFNγ, while Th2 cells release IL-4. This difference in cytokine expression is a defining difference between these subsets, and perhaps differences in immunological synapse structure are to ensure differences in the delivery of other cytokines, and not CD40L. Huse et al. showed that IFNγ is released towards the point of T cell/APC contact, while IL-4 release was multidirectional (105). As stated in the introduction, Th2 cells are involved in more wide-ranging immune responses, like allergies and asthma, and therefore Th2 cells may release IL-4 within the multifocal synapse to activate many cells in a tissue at one time. Alternatively, Th1 have the potential under some circumstances to produce cytotoxic granules, so the function of the bull's eye synapse may be to direct the release of lytic granules and not cytokines. The release of CD40L and lytic granules were shown to involve different trafficking machinery by experiments with the ashen mouse in Chapter 2.

T follicular helper cells are the most relevant subset to evaluate when analyzing T/B interaction (179, 204, 205). This subset of T helper cells interacts with B cells in the specialized areas of lymph nodes and spleens called germinal centers. This is the area where the processes of somatic hypermutation and isotype switching occur, allowing for pathogen clearance. It has been shown that tumor infiltrating Tfh cells may help to maintain CTLs at tumor sites, though they do not differentiate into cells that produce cytotoxic granules (206, 207). Therefore if the bull's eye synapse is necessary to ensure delivery of cytolytic granules, I would predict that Tfh have a multifocal synapse structure like Th2 cells. I did not analyze T follicular helper cells specifically because effective conditions for inducing them *in vitro* have not been discovered (202). Future work will have to be completed to determine the immunological synapse structure of Tfh cells and delivery of CD40L within this structure.

Due to the essential role of CD40L in the development of an adaptive immune response, understanding the delivery of CD40L to antigen-presenting cells could provide a means to design better treatment for individuals with deficiencies in this protein. Deficiencies in CD40L results in hyper-IgM syndrome in humans, and the only treatments available to these patients are immunoglobulin replacement therapy, which does not restore resistance to macrophage-dwelling pathogens, or stem cell replacement (68, 69, 71). Identifying the trafficking molecules involved and pathways used by CD40L for expression on the T cell surface may offer an alternative means to manipulate CD40L expression and further inform the design of gene therapy for such individuals. It will be necessary to understand the regulation of CD40L, both at the level of protein and transcriptionally, to ensure

proper expression of a transgenic CD40L. Overexpression of CD40L in transgenic mice leads to disruption of thymic architecture and lymphoproliferative disease (208, 209). Transduction of a retroviral vector containing CD40L into CD40L deficient cells that resulted in constitutive, low level expression of CD40L transferred into CD40L knock-out mice also resulted in a lymphoproliferative disease (210).

Future studies will be needed to determine if Th1 and Th2 cells release CD40L within the cSMAC at the points of TCR-pMHC interactions. Using the planar lipid bilayer system, it should be possible to determine if CD40L is released only at the points of pMHC-TCR interaction, or whether it is also released into areas where the adhesion molecules are interacting. Further knowledge of where within the immunological synapse CD40L is released could allow for better targeting of CD40L in patients lacking or expressing too much CD40L. Similar to bystander killing, activation of the non-antigen specific cells may be harmful to the host, triggering non-specific inflammation or autoantibody formation which may lead to the destruction of healthy host tissues. Lupus patients' helper T cells have been shown to have increased expression of CD40L when compared to healthy controls leading to the production of pathogenic autoantibodies (211). It has been suggested that this is due to increased activation of signaling pathways downstream of TCR activation as well as increased CD40L mRNA stability. Further understanding these processes and the molecules involved in regulation of CD40L will allow us to better understand and manipulate the immune response in cases of immune deficiencies or overstimulation in the case of autoimmune diseases.

Figures:

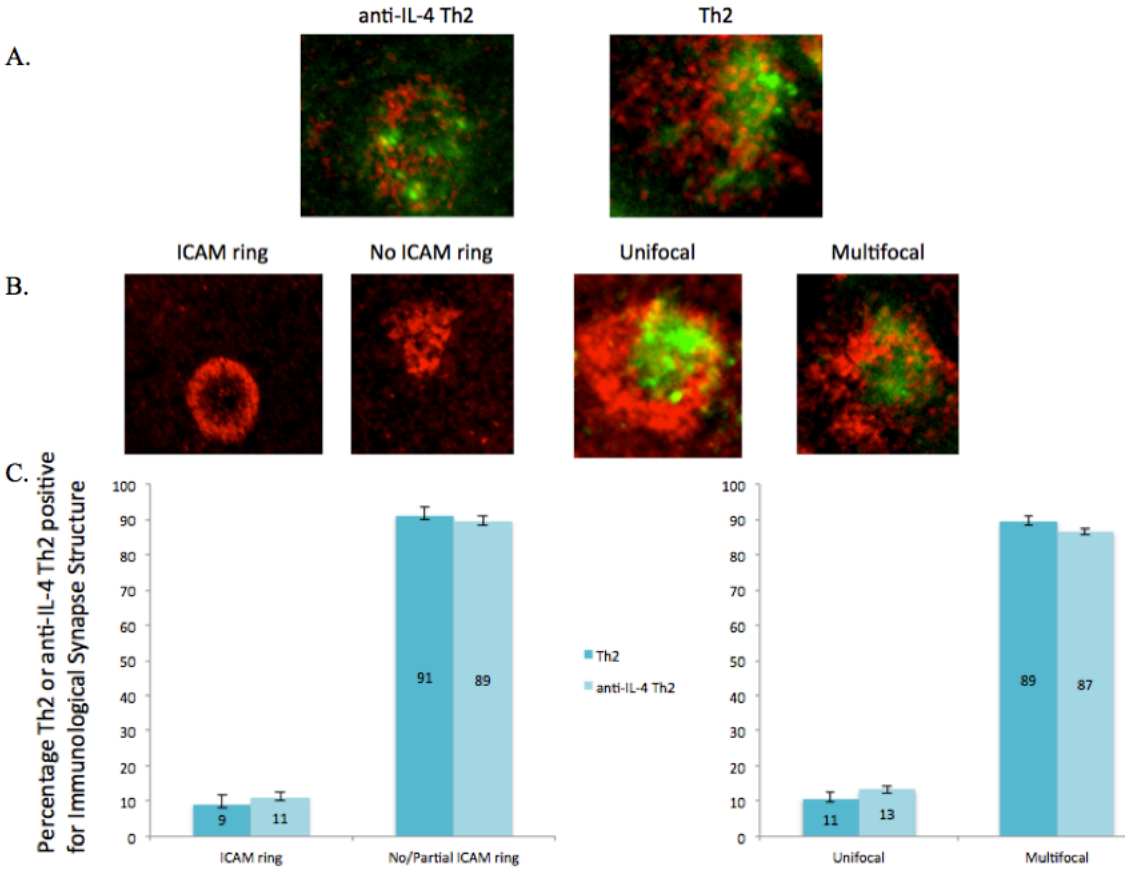


Figure 27. The anti-IL-4 treated Th2 cells retain a multifocal immunological synapse structure. A, Representative images of AD10 Th2 and anti-IL-4 treated AD10 Th2 immunological synapses. AD10 Th2 and anti-IL-4 treated AD10 Th2 cell synapses were imaged on a widefield microscope after addition to a lipid bilayer containing GPI-linked Oregon Green labeled I-E^k and Cy5 labeled ICAM. Cells were evaluated for presence of the structures depicted in B. C, Anti-IL-4 treated AD10 Th2, similar to non-anti-IL-4 treated AD10 Th2 cells, have immunological synapses without an ICAM ring and are multifocal. The percentage of ICAM ring verses no/partial ICAM ring and unifocal verses multifocal synapses in AD10 Th2 and anti-IL-4 treated AD10 Th2 cells are shown in the bar graphs in C. Cells were analyzed and pooled from three different experiments. (Experiment 1: 132 cells (Th2: 82, anti-IL-4 Th2: 50), Experiment 2: 928 cells (Th2: 650, anti-IL-4 Th2: 278), and Experiment 3: 492 cells (Th2: 255, anti-IL-4 Th2: 237))

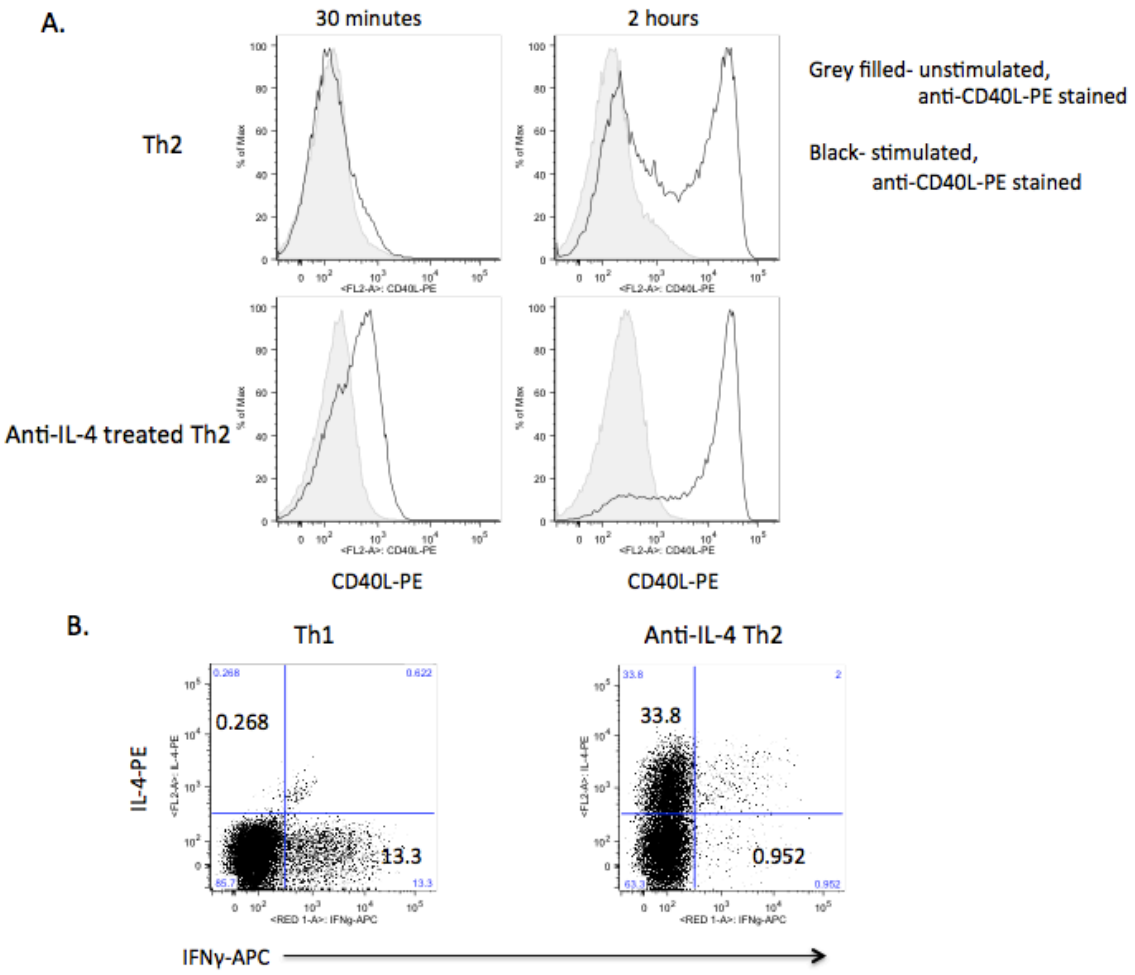


Figure 28. Anti-IL-4 treated 5CC7 Th2 cells express preformed CD40L and secrete IL-4 and no IFNγ upon PMA/Ionomycin stimulation. A, Upon secondary culture with anti-IL-4, *in vitro* generated 5CC7 Th2 cells express preformed CD40L in similar amounts as 5CC7 Th1 cells. 5CC7 Th2 or anti-IL-4 Th2 cells were stimulated (black) or not (grey) with PMA/Ionomycin for 30 minutes or 2 hours and expression of CD40L was measured by anti-CD40L-PE. B, Stimulated anti-IL-4 treated 5CC7 Th2 cells continue to express IL-4 and do not express IFNγ after 5 hours of PMA/Ionomycin stimulation by intracellular cytokine staining.

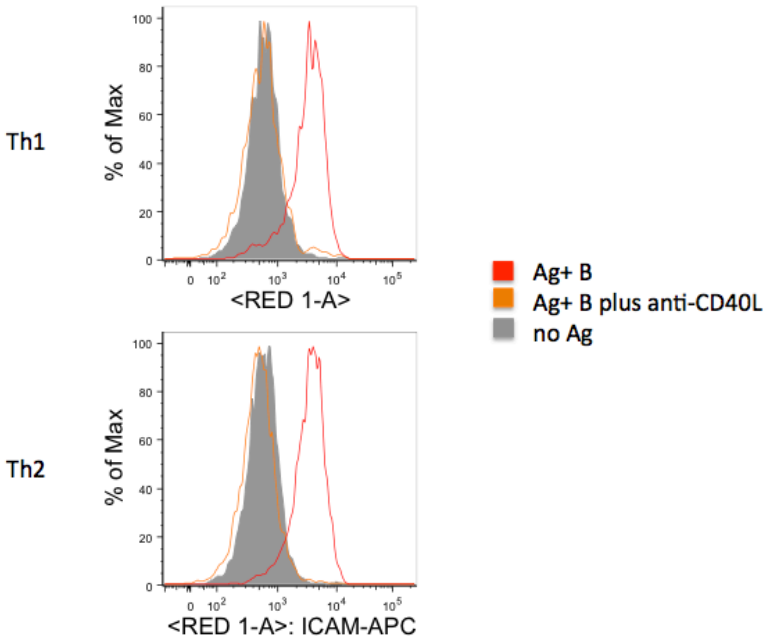


Figure 29. Activation of antigen-pulsed B cells by CsA-treated 5CC7 Th1and Th2 cells is completely blocked by addition of anti-CD40L blocking antibody. Histograms are representative of three independent experiments and show ICAM uregulation on each B cell population listed following overnight incubation with 5CC7 Th1 or Th2 cells in the presence or absence of 10 μg/ml anti-CD40L blocking antibody.

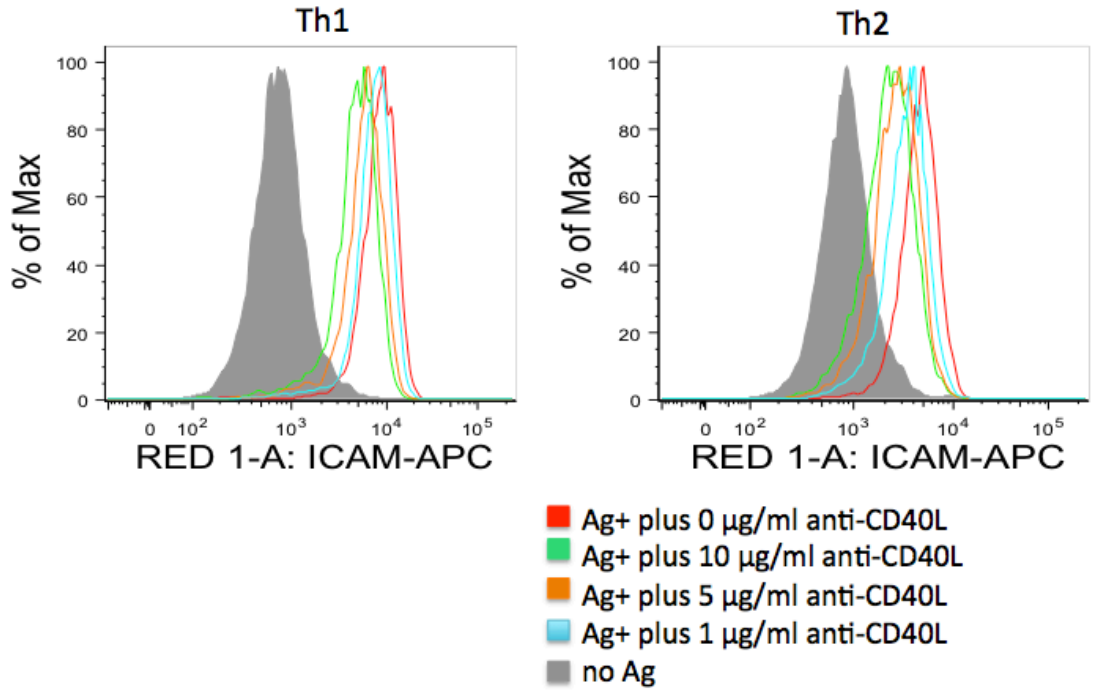


Figure 30. Th1 help for antigen-pulsed B cells is not more resistant to antibody inhibition than help provided by Th2 cells. Non-CsA treated 5CC7 Th1 or Th2 cells were incubated overnight with Ag-pulsed (Ag+) and bystander (Ag-) B cells in the presence of anti-CD40L blocking antibody at the concentrations listed. ICAM levels were measured on Ag+ B cells after overnight incubation. Results suggest that there is no difference in inhibition of ICAM upregulation by 5CC7 Th1 and Th2 cells at all concentrations of anti-CD40L blocking antibody tested. Grey filled histograms represent ICAM levels on no Ag control B cells in a well with either 5CC7 Th1 (left histogram) or Th2 (right histogram) cells.

Synapse Structure	CsA	Ag-specific	Bystander	Inhibition by anti-CD40L
Th1 Th2 	yes	yes	no	yes
	no	yes	yes, < Ag-specific	Bystander: yes Ag-specific: incomplete

Figure 31. The delivery of help to B cells by T cells is independent of immunological synapse structure. Despite distinct immunological synapse structures, Th1 and Th2 cell delivery of CD40L to B cells is similar. When Th1 and Th2 cells are treated with Cyclosporin A (CsA), they deliver only Ag-specific help. When CD40L synthesis is not inhibited, Th1 and Th2 help both Ag-pulsed and bystander B cells, but the Ag-pulsed cells receive more help as measured by ICAM activation levels on these B cells. Bystander help by non-CsA treated T cells is completely blocked by an anti-CD40L blocking antibody, while Ag-pulsed B cells still receive help.

Chapter 5: Summary and Conclusions:

I. Summary

The experimental results presented in this dissertation provide new insights regarding the delivery of T cell help during antigen-specific reactions. Specifically the experiments presented address the mechanism of CD40L delivery by helper T cells that results in productive signaling and selection of antigen-specific B cells during brief interactions in the germinal center. CD40L does not have to be made *de novo* following helper T cell recognition and engagement of antigen in the context of MHC on an antigen-specific APC, but can be delivered rapidly to the T cell surface from a intracellular storage compartment. This preformed CD40L is sufficient to fulfill many of the functions traditionally thought to require synthesis of *de novo* protein (Chapter 2 and (126)). In this dissertation, I show for the first time that CD40L is deposited on the B cell following antigen-specific interactions. Microscopy experiments revealed that the deposited CD40L exists both intracellularly and on the surface of B cells following overnight incubation of T cells. Since CD40L engages CD40 molecules on B cells, deposition of CD40L on the B cells may provide a means for T cells to initiate sustained productive signaling despite brief interactions in germinal centers. Future experiments will be necessary to determine if the transferred CD40L is functional to induce productive B cell signaling. This could be accomplished by sorting out the cells that have received transferred CD40L and comparing these cells to the antigen specific cells that have not received CD40L. Signaling molecules downstream of CD40-CD40L interaction could then be measured in CD40L transfer positive B cells. Intravital imaging in a mouse with a fluorescently

labeled CD40L may allow for visualization of transfer of CD40L to antigen-specific B cells during an active immune response *in vivo*. Visual proof from intravital microscopy that CD40L is delivered and deposited on B cells during the short interactions in germinal centers would complement the *in vitro* studies presented in Chapter 3 of this dissertation. It was also determined that despite distinct immunological synapse structures, Th1 and Th2 cells deliver help to B cells in an antigen-specific manner, and not to bystander cells in *in vitro* cultures. This directed delivery of T cell help may be a result of the deposition of vesicles containing CD40L on antigen-pulsed B cells at the immunological synapse.

II. Functional significance of preformed CD40L

The second chapter of the dissertation discusses the function of a newly described intracellular secretory compartment containing CD40L. Prior to the discovery of preformed CD40L, it was thought that cytokines had to be made *de novo* upon T cell/APC interaction. The transcription and translation process necessary for *de novo* protein synthesis takes one or more hours to be completed, so how CD40L could possibly be delivered during the brief T/B interactions in the germinal center was not known. Since preformed CD40L is delivered in minutes of TCR stimulation, it offers an explanation as to how this occurs. Experimental results presented in this chapter also show that the small amount of intracellular CD40L is capable of many of the functions which were previously though to require the production of new CD40L protein, including upregulation of costimulatory molecules and production of cytokines by APCs, as well as induction of proliferation of antigen-specific B cells.

Cyclosporine A treatment of T cells *in vitro* allowed for assessment of the functions of

preformed CD40L, but it remains to be determined if preformed CD40L is functional *in vivo*, or if both preformed and newly synthesized CD40L pass through this regulated secretory compartment on their way to the APC. Proof that performed CD40L is functional *in vivo* would require a mouse model in which TCR stimulation does not increase *de novo* CD40L production, and the amount of CD40L synthesized is limited to the amount necessary to fill the intracellular secretory compartments of effector and memory T cells. As part of my thesis work, I tried to generate such a mouse using a tetracycline off system. This mouse was designed to produce CD40L from a transgene with a promoter region dependent on a transactivator that could be inactivated when tetracycline is given to the mouse in its drinking water. On a CD40L knock-out background, the only CD40L produced by the mouse would be from the tetracycline regulated transgene. This would allow for titration of the amount of CD40L that was being produced by the T cells in the mouse, and uncouple CD40L synthesis from TCR signaling. The construct behaved as expected in a transfected cell line, but I obtained only three transgenic mouse lines from the OHSU transgenic facility, and none of them expressed the transgene, whether or not the mothers were treated with tetracycline. This may have been due to silencing of the inserted transgene by epigenetic factors or other types of regulation. I planned experiments to examine this transgenic mouse for deficiencies in germinal center formation and antibody production. The mouse would be immunized with nitrophenyl conjugated ovalbumin, and at day 7, B cells would be assessed with the germinal center markers, CXCR5 and PNA. Antibodies would also be assessed for affinity maturation by looking for known mutations in complementarity determining regions by polymerase chain reaction and for class switching using

antibodies specific for different classes of antibody. If preformed CD40L is sufficient for these changes, then the deficiencies associated with the lack of CD40L during an antibody response would be alleviated. This would indicate that the large amounts of CD40L seen on the surface of T cells *in vitro* upon two hours of TCR stimulation may not be necessary for a germinal center response, and may be an *in vitro* artifact. CD40L expression may be regulated by the availability of antigen during an immune response. Perhaps during the brief antigen-specific interactions *in vivo*, CD40L can be produced *de novo* as a result of summation of all the signals received during multiple antigen-specific interactions. Following this rationale, the CD40L secretory compartment would be continuously replenished when an antigenic challenge is occurring. Alternatively, *de novo* CD40L production following TCR engagement may be necessary only during the long interactions necessary for initial priming of the T cells and/or activation of naïve B cells. I planned to test this possibility by adding tetracycline to the CD40L transgenic animals to turn down CD40L synthesis on days 2 or 3 following antigen exposure or during the secondary response. These suggested experiments would add *in vivo* relevance to the *in vitro* experimental results of Chapter 2.

The knowledge of the function of preformed CD40L that could be gained from the suggested CD40L transgenic line would be complemented with a mouse lacking the secretory compartment that allows preformed CD40L to be delivered to the cell surface following TCR engagement. A Rab27a knock-out mouse (ashen mouse) was tested as a potential candidate in Chapter 2 of the dissertation, and although this mouse lacked surface expression of CD107a after TCR stimulation as reported (212, 213), it was not deficient in rapid surface expression of CD40L. I tested other potential candidates for

regulation of preformed CD40L including a double mutant p38 knock-in mouse (214) that lacks an alternative signaling pathway to p38 downstream of the TCR, SAP knock-out mice (52), and a mouse deficient for transcription factors thought to be associated with transcription of CD40L (TFE3 and TFEB) (215). None of the mice tested were deficient for stimulated surface expression of preformed CD40L. Other candidate genes could be investigated in knock-out mice similar to the ashen mouse (Rab27a knock-out), including beige-J (216) and VAMP8-null (217). These mice have deficiencies in exocytosis and also altered immune responses due to their inability to secrete proteins necessary for T and NK cell killing. If I had been able to find or generate a mouse lacking this function, I would test it in many of the same ways I proposed above for the mouse which only had preformed CD40L.

Regulated exocytosis of effector proteins in cells of the immune system has been a developing field of study in recent years (105, 218-220). These secretory pathways are essential for the function of many immune cells including mast cells (associated with allergic inflammation), macrophages, NK cells, and cytotoxic T lymphocytes. As an alternative to mouse knock-out models, proteins involved in the trafficking and exocytosis of effector proteins have been identified using siRNA technology. I attempted these experiments in primary CD4 lymphocytes with some candidate proteins known to be involved in vesicle trafficking in lymphocytes (syntaxin 11 (221, 222), vamp 8 (223, 224), and SNAP23 (222)), but unfortunately my attempts to knockdown trafficking molecules by electroporation of siRNA into primary lymphocytes were unsuccessful. Knockdown of the control surface molecule CD4 was efficient, but not of the candidate trafficking molecules. New Crispr/Cas9 technology may allow for faster identification of

novel trafficking pathways in immune cells both *in vivo* and *in vitro* (225).

III. CD40L is transferred to antigen-presenting B cells by helper T cells

In Chapter 3 of my dissertation, I identified a new mechanism of delivery of CD4 T cell help to antigen-presenting B cells. To my knowledge, this is the first time that the transfer of CD40L to antigen-specific B cells or other APCs has been shown. Prior to this discovery, it was assumed that T cell CD40L engages with CD40 on APCs and then remained on the T cell surface for a period long enough to initiate productive downstream signaling in the APC at which point it was downmodulated from the T cell surface (137). However, transfer of CD40L suggests a different way in which delivery of CD40L can induce signaling in antigen-specific B cells. Instead of a short engagement of CD40L on the surface of the T cell with CD40 on the surface of the B cell, CD40L may engage with CD40 after it is deposited on the B cell. This would allow for continuing signaling from CD40 after the end of a short T cell-B cell engagement (Figure 32). As stated in Chapter 3, this idea has recently been proposed by Michael Dustin in a review (125), though it has not yet been evaluated experimentally. Deposition of CD40L onto antigen-specific B cells could provide a means by which B cells can quantify the amount of help they received during multiple short interactions with T cells in the germinal center. The CD40L deposited on the B cell may be essential for effective signaling necessary for survival, proliferation, and differentiation to antibody secretion by the B cell.

The initial discovery of transfer of CD40L from helper T cells to antigen-bearing B cells resulted from the method used to measure CD40L expression on T cells in the *in vitro* overnight assay as discussed in Chapter 3 of the dissertation. The fluorescently labeled

antibody was included during the incubation of the B and T cells allowing the existence of transfer to be revealed. This may be due to the fact that the addition of the anti-CD40L antibody to the T/B cell cultures allowed for accumulation of CD40L on the surface of T cells without downregulation by CD40+ cells in the cultures (137). Therefore large amounts of labeled CD40L may accumulate on the T cell surface and also be labeled during delivery to antigen-specific B cells, allowing the process of antigen-specific CD40L transfer to be revealed. Despite the perhaps artificially high amounts of CD40L labeled with a fluorescent antibody in these cell cultures, bystander transfer was still not detected, indicating that the process of transfer is solely directed towards antigen-presenting cells. The fluorescently labeled anti-CD40L antibody may also allow for detection of antigen-specific transfer due to longer periods of stabilized expression on the B cells following interaction with T cells. CD40L transfer to antigen-specific B cells was also verified experimentally without the overnight inclusion of the fluorescently labeled anti-CD40L antibody by fixing and permeabilizing CD40L knock-out B cells following overnight incubation with helper T cells before staining with anti-CD40L (Figure 18). Since the CD40L knock-out B cells were incapable of producing CD40L, this internal CD40L found on antigen-presenting B cells had to be of T cell origin, further supporting the existence of antigen-specific transfer. This also indicates that transfer is not just an artifact of addition of anti-CD40L to the system.

Transfer of molecules and exosomes between activated T cells and APCs has been shown previously and linked to T cell activation (226, 227). *In vitro* overnight incubation of T cells with DCs results in the accumulation of APC derived MHC molecules on the T cells (228-231). These transferred molecules can stay at high levels for up to two hours (232).

In addition adoptive transfer of T cells into a mouse results in the surface of T cells being coated with Ig molecules and non-T cell derived membrane fragments in a process called trogocytosis (228-230). Therefore transfer does not just occur in environments created *in vitro*. It will have to be determined if transfer of CD40L also occurs *in vivo* and if this is necessary for selection of antigen specific B cells. As suggested in chapter 3, this transfer may be a way for B cells to sustain CD40 signaling despite short interactions. However productive signaling downstream of CD40 will need to be assessed in future proposed experiments. This has been shown for other factors including TGFβ (233) and NGF (234), which are internalized and continue to signal. In addition MHC transfer to T cells has resulted in increased signaling in T cells after APCs have been removed from the culture (235). Through microscopy and fixing and permeabilizing the antigen-bearing B cells, I was able to determine that CD40L accumulates intracellularly following interaction with helper T cells. However whether transferred CD40L can engage CD40 and lead to productive downstream signaling and whether this occurs on the B cell surface or needs to be internalized in an endocytic compartment is not known. The form of CD40L also has to be further characterized. Although transferred CD40L appears in punctate spots on and inside B cells, whether this is in the form of an exosome, microvesicle, or patches of transferred membrane that have fused with the B cell surface has not yet been determined. Electron microscopy and markers which specifically defined these different extracellular vesicles could be used in this characterization (236).

It has recently been recognized that extracellular vesicles provide a means of communication among different cell types, and this subject has only recently been investigated in terms of immune cells (237). Exosomes delivered between two different

cell types contain proteins, mRNA, and microRNAs that can influence the protein expression and function in the receiving cells. Recently it has been shown that another means of suppression by regulatory T cells may be the delivery of microRNAs that can downregulate inflammatory genes in interacting cells (238, 239). It seems like the immunological synapse, which is contact zone between T cell and APCs upon recognition of antigen may allow for communication and signals to be sent between these two cell types, and therefore may offer a place for such short-range communication events to occur during an immune response. Many more advances will probably be made following this relatively new line of investigation in immunology in the areas of cellular communication via extracellular vesicles, including both microvesicles and exosomes. Delivery of extracellular vesicles within the immunological synapse would allow T cells to deliver short-range antigen-specific signals capable of inducing sustained signaling in target cells despite brief interactions. The transferred extracellular vesicles may be the way in which highly motile cells, like T and B cells within the germinal centers, deliver functional signals and communicate with their target cells. In fact, FasL and CTLA-4, which are known to be stored preformed in intracellular compartments like CD40L have both been suggested to exist in functional membrane bound vesicles (240, 241). Therefore, functional vesicles may be a common mechanism of delivery of membrane bound effector proteins, like CD40L.

IV. Functional significance of Immunological Synapse

The function of the immunological synapse in terms of delivery of help to APCs was investigated in Chapter 4 of this dissertation. It had previously been discovered in the

Parker lab that different helper T cell subsets have different synapse structures, however the function of these differences is not known. I hypothesized that the Th1 ring structure synapse is required for specific delivery of CD40L to antigen-bearing B cells, while Th2 cells, with a multifocal synapse, would be more likely to activate bystander B cells. However, I was unable to uncover differences in delivery of CD40L to antigen-specific and bystander B cells between Th1 and Th2 cells as assessed by the methods described in Chapter 2. Th1 and Th2 cells form stable synapses upon interaction with antigen-presenting cells (94) as opposed to Tregs which form motile synapses as they move rapidly over APCs. At high concentrations of CD80 on the APC, Tregs stop moving, form a ring synapse, and modulate expression of surface molecules (25) or deliver cytokines like IL-10, which is known to be necessary for some of the inhibitory properties of Tregs and delivered directionally to APCs (105). Th1 cells (172) and Tregs (242) can behave like cytotoxic T cells under certain circumstances, so it may be that the bull's eye synapse seen is solely necessary for the release of lytic granules. T follicular helper cells are the main helper T subset involved in the selection processes necessary for development of a memory B cell population and affinity maturation of antibodies. The immunological synapse structure of T follicular cells has yet to be determined due to the lack of knowledge of the signals required for consistent *in vitro* generation of Tfh (202). I would predict that if the immunological synapse is necessary solely for deliver cytolytic granules, which are not produced by Tfh cells, then Tfh cells would have a multifocal synapse structure similar to that seen with Th2 cells.

Future experiments will be necessary to assess the delivery of CD40L in the context of the immunological synapse. My hypothesis is that CD40L is delivered at the points of

TCR-peptide-MHC interaction within the synapse but not within the areas of adhesion molecule interactions in the synapse. I generated a lentiviral pHlourin-CD40L construct to approach this question. Phlourin is derived from GFP but has mutations which make it non-fluorescent with GFP filters at low pH and fluorescent like GFP at neutral pH at the cell surface (243). CD40L is stored intracellularly within a secretory lysosome, which is an acidic compartment. Therefore pHlourin-CD40L which traffics to the appropriate intracellular location should be non-fluorescent, and upon TCR stimulation when pHlourin-CD40L is brought to the cell surface, it should become fluorescent similar to GFP. I have transduced activated CD40L knock-out T cells with this construct and upon PMA/Ionomycin stimulation I detect an increase in fluorescence in the GFP channel as well as measurable expression of CD40L by flow cytometry. Since these T cells are CD40L knock-out, the only CD40L measureable in these cells would be of lentiviral origin. In future experiments, I will add the transduced T cells to lipid bilayers to visualize where within the immunological synapse CD40L is delivered allowing me to address my proposed hypothesis that CD40L is delivered to the points of TCR-pMHC interaction and not where adhesion molecules are interacting in the pSMAC. In addition to the wide-field microscopy experiments proposed, using the pHlourin construct, I can also measure delivery of CD40L at the T cell surface upon antigen recognition using TIRF microscopy. TIRF microscopy allows for visualization of proteins at and just below (200 nm) the cell surface (244). Therefore it should be possible to see the GFP fluorescence as the CD40L is delivered upon interaction with antigen displayed in the context of MHC on the lipid bilayer. Alternatively, this construct can also be imaged via spinning disk microscopy which will allow all delivery of CD40L, even that opposite the

source of stimulation to be measured and compared. I would hypothesize that CD40L is delivered mostly towards the APC, but using this technique I could assess the opposite pole of the cell as well.

It has recently been determined that the same signaling pathways involved in hedgehog signaling and intraflagellar transport exist in T cells and function in the immunological synapse (102, 103, 245). Hedgehog signaling is a primary mechanism of communication and diversification between cells during the early developmental stages of an organism. However the molecular details of delivery of this essential cellular signal to its target cells have not been clearly defined. The centrosome reorganization and polarization of proteins involved in the immunological synapse is similar to events that occurs in cilia and flagella suggesting that these structures may be functional homologs for cellular communication (246). Activation of the hedgehog signaling pathway has been shown to be important in activation of Rac1 which is necessary for the actin remodeling needed for efficient killing of target cells (247). This supports the idea that the immunological synapse functions in communication between T cells and target APCs. The signaling pathways involved in developmental biology may actually impact broader scientific fields. Therefore a more generalized knowledge of the early developmental pathways in biology may inform scientific discoveries in immunology.

Figure:

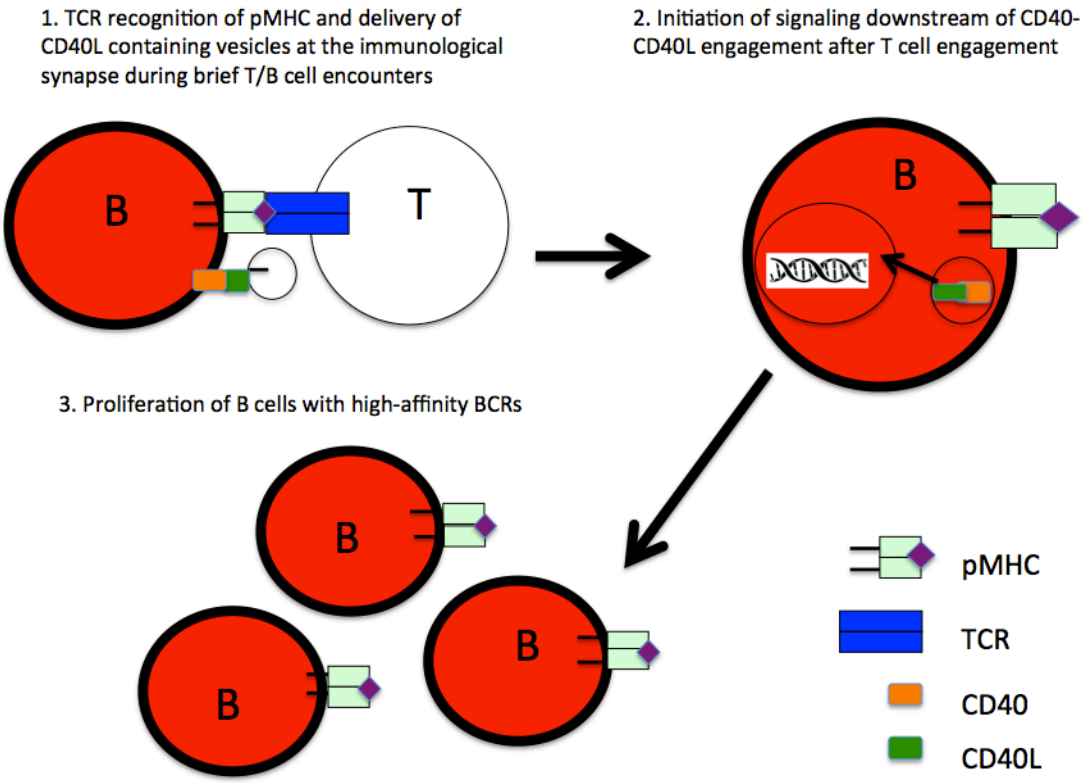


Figure 32. CD40L is transferred to antigen-bearing B cells resulting in selection and proliferation of B cells with high-affinity BCRs. T cells recognize peptide in the context of MHC molecules on B cells through their TCR. This results in the formation of an immunological synapse. Upon formation of the synapse, T cells release CD40L-containing vesicles that engage with CD40 on B cells. The CD40L vesicle engaged with CD40 is internalize within the B cells and downstream signaling results in proliferation of these high-affinity B cells.

References:

1. Paus, D., T. G. Phan, T. D. Chan, S. Gardam, A. Basten, and R. Brink. 2006. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* 203: 1081-1091.
2. Cyster, J. G., S. B. Hartley, and C. C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature* 371: 389-395.
3. Lee, S. K., R. J. Rigby, D. Zotos, L. M. Tsai, S. Kawamoto, J. L. Marshall, R. R. Ramiscal, T. D. Chan, D. Gatto, R. Brink, D. Yu, S. Fagarasan, D. M. Tarlinton, A. F. Cunningham, and C. G. Vinuesa. 2011. B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J Exp Med* 208: 1377-1388.
4. Miller, J. F., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J Exp Med* 128: 801-820.
5. Mitchell, G. F., and J. F. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J Exp Med* 128: 821-837.
6. Rajewsky, K., V. Schirrmacher, S. Nase, and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J Exp Med* 129: 1131-1143.
7. Mitchison, N. A. 1971. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. *Eur J Immunol* 1: 18-27.
8. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the

activity of I region-specific Ir genes in macrophages and B lymphocytes. *J Immunol* 120: 1809-1812.

9. Lanzavecchia, A. 1985. Antigen-specific interaction between T and B cells. *Nature* 314: 537-539.

10. Tony, H. P., and D. C. Parker. 1985. Major histocompatibility complex-restricted, polyclonal B cell responses resulting from helper T cell recognition of antiimmunoglobulin presented by small B lymphocytes. *J Exp Med* 161: 223-241.

11. Adiko, A. C., J. Babdor, E. Gutierrez-Martinez, P. Guermonprez, and L. Saveanu. 2015. Intracellular Transport Routes for MHC I and Their Relevance for Antigen Cross-Presentation. *Front Immunol* 6: 335.

12. Hulpke, S., and R. Tampe. 2013. The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem Sci* 38: 412-420.

13. Bevan, M. J. 2010. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. 1976. *J Immunol* 185: 1361-1366.

14. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J Exp Med* 158: 303-316.

15. Babbitt, B. P., P. M. Allen, G. Matsueda, E. Haber, and E. R. Unanue. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 317: 359-361.

16. Marshall, L. S., A. Aruffo, J. A. Ledbetter, and R. J. Noelle. 1993. The molecular basis for T cell help in humoral immunity: CD40 and its ligand, gp39. *J Clin Immunol* 13: 165-174.

17. Tsytsykova, A. V., E. N. Tsitsikov, and R. S. Geha. 1996. The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. *J Biol Chem*271: 3763-3770.

18. Lindgren, H., K. Axcrona, and T. Leanderson. 2001. Regulation of transcriptional activity of the murine CD40 ligand promoter in response to signals through TCR and the costimulatory molecules CD28 and CD2. *J Immunol* 166: 4578-4585.

19. Sun, S. C. 2012. The noncanonical NF-kappaB pathway. *Immunol Rev* 246: 125-140.

20. Elgueta, R., M. J. Benson, V. C. de Vries, A. Wasiuk, Y. Guo, and R. J. Noelle. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 229: 152-172.

21. Fife, B. T., and J. A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* 224: 166-182.

22. Parry, R. V., J. M. Chemnitz, K. A. Frauwirth, A. R. Lanfranco, I. Braunstein, S. V. Kobayashi, P. S. Linsley, C. B. Thompson, and J. L. Riley. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 25: 9543-9553.

23. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30: 531-564.

24. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-787.

25. Thauland, T. J., Y. Koguchi, M. L. Dustin, and D. C. Parker. 2014. CD28-CD80 interactions control regulatory T cell motility and immunological synapse formation.

J Immunol 193: 5894-5903.

26. Qureshi, O. S., Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E. M. Schmidt, J. Baker, L. E. Jeffery, S. Kaur, Z. Briggs, T. Z. Hou, C. E. Futter, G. Anderson, L. S. Walker, and D. M. Sansom. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332: 600-603.
27. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol* 8: 523-532.
28. Wolchok, J. D., H. Kluger, M. K. Callahan, M. A. Postow, N. A. Rizvi, A. M. Lesokhin, N. H. Segal, C. E. Ariyan, R. A. Gordon, K. Reed, M. M. Burke, A. Caldwell, S. A. Kronenberg, B. U. Agunwamba, X. Zhang, I. Lowy, H. D. Inzunza, W. Feely, C. E. Horak, Q. Hong, A. J. Korman, J. M. Wigginton, A. Gupta, and M. Sznol. 2013. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* 369: 122-133.
29. Sharma, P., and J. P. Allison. 2015. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* 161: 205-214.
30. Burnet, F. M. 1962. The immunological significance of the thymus: an extension of the clonal selection theory of immunity. *Australas Ann Med* 11: 79-91.
31. Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 410: 101-105.
32. Crotty, S. 2012. The 1-1-1 fallacy. *Immunol Rev* 247: 133-142.
33. Gitlin, A. D., Z. Shulman, and M. C. Nussenzweig. 2014. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* 509: 637-640.

34. Cattoretti, G., M. Buttner, R. Shaknovich, E. Kremmer, B. Alobeid, and G. Niedobitek. 2006. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. *Blood* 107: 3967-3975.

35. Moldenhauer, G., S. W. Popov, B. Wotschke, S. Bruderlein, P. Riedl, N. Fissolo, R. Schirmbeck, O. Ritz, P. Moller, and F. Leithauser. 2006. AID expression identifies interfollicular large B cells as putative precursors of mature B-cell malignancies. *Blood* 107: 2470-2473.

36. Zhang, J., I. C. MacLennan, Y. J. Liu, and P. J. Lane. 1988. Is rapid proliferation in B centroblasts linked to somatic mutation in memory B cell clones? *Immunol Lett* 18: 297-299.

37. Allen, C. D., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* 27: 190-202.

38. Cyster, J. G. 2010. Shining a light on germinal center B cells. *Cell* 143: 503-505.

39. De Silva, N. S., and U. Klein. 2015. Dynamics of B cells in germinal centres. *Nat Rev Immunol* 15: 137-148.

40. Mueller, J., M. Matloubian, and J. Zikherman. 2015. Cutting edge: An in vivo reporter reveals active B cell receptor signaling in the germinal center. *J Immunol* 194: 2993-2997.

41. Shlomchik, M. J., and F. Weisel. 2012. Germinal center selection and the development of memory B and plasma cells. *Immunol Rev* 247: 52-63.

42. Sprent, J., and H. von Boehmer. 1976. Helper function of T cells depleted of alloantigen-reactive lymphocytes by filtration through irradiated F1 hybrid recipients. I. Failure to collaborate with allogeneic B cells in a secondary response

to sheep erythrocytes measured in vivo. *J Exp Med* 144: 617-626.

43. Sprent, J. 1978. Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the opposite parental strain not associated with active suppression. *J Exp Med* 147: 1142-1158.

44. Sprent, J. 1978. Role of the H-2 complex in induction of T helper cells in vivo. I. Antigen-specific selection of donor T cells to sheep erythrocytes in irradiated mice dependent upon sharing of H-2 determinants between donor and host. *J Exp Med* 148: 478-489.

45. Miller, J. F., and J. Sprent. 1971. Cell-to-cell interaction in the immune response. VI. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. *J Exp Med* 134: 66-82.

46. Allen, C. D., T. Okada, H. L. Tang, and J. G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. *Science* 315: 528-531.

47. Liu, D., H. Xu, C. Shih, Z. Wan, X. Ma, W. Ma, D. Luo, and H. Qi. 2014. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* 517: 214-8.

48. Shulman, Z., A. D. Gitlin, J. S. Weinstein, B. Lainez, E. Esplugues, R. A. Flavell, J. E. Craft, and M. C. Nussenzweig. 2014. Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* 345: 1058-1062.

49. Victora, G. D., T. A. Schwickert, D. R. Fooksman, A. O. Kamphorst, M. Meyer-Hermann, M. L. Dustin, and M. C. Nussenzweig. 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter.

Cell 143: 592-605.

50. Qi, H. 2012. From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. *Immunol Rev* 247: 24-35.

51. Qi, H., J. L. Cannons, F. Klauschen, P. L. Schwartzberg, and R. N. Germain. 2008. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* 455: 764-769.

52. Kamperschroer, C., D. M. Roberts, Y. Zhang, N. P. Weng, and S. L. Swain. 2008. SAP enables T cells to help B cells by a mechanism distinct from Th cell programming or CD40 ligand regulation. *J Immunol* 181: 3994-4003.

53. Zhao, F., J. L. Cannons, M. Dutta, G. M. Griffiths, and P. L. Schwartzberg. 2012. Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis. *Immunity* 36: 1003-1016.

54. Chesnut, R. W., and H. M. Grey. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J Immunol* 126: 1075-1079.

55. Croft, M., and S. L. Swain. 1992. Analysis of CD4+ T cells that provide contact-dependent bystander help to B cells. *J Immunol* 149: 3157-3165.

56. Whalen, B. J., H. P. Tony, and D. C. Parker. 1988. Characterization of the effector mechanism of help for antigen-presenting and bystander resting B cell growth mediated by Ia-restricted Th2 helper T cell lines. *J Immunol* 141: 2230-2239.

57. Marrack, P., and J. W. Kappler. 1980. The role of H-2-linked genes in helper T cell function. VII. Expression of I region and immune response genes by B cells in bystander help assays. *J Exp Med* 152: 1274-1288.

58. Benson, M. J., R. Elgueta, W. Schpero, M. Molloy, W. Zhang, E. Usherwood, and R. J. Noelle. 2009. Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals. *J Exp Med* 206: 2013-2025.

59. Quah, B. J., V. P. Barlow, V. McPhun, K. I. Matthaei, M. D. Hulett, and C. R. Parish. 2008. Bystander B cells rapidly acquire antigen receptors from activated B cells by membrane transfer. *Proc Natl Acad Sci U S A* 105: 4259-4264.

60. Pollok, K. E., V. O'Brien, L. Marshall, J. W. Olson, R. J. Noelle, and E. C. Snow. 1991. The development of competence in resting B cells. The induction of cyclic AMP and ornithine decarboxylase activity after direct contact between B and T helper cells. *J Immunol* 146: 1633-1641.

61. Noelle, R. J., M. Roy, D. M. Shepherd, I. Stamenkovic, J. A. Ledbetter, and A. Aruffo. 1992. 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 U S A* 89: 6550-6554.

62. Armitage, R. J., W. C. Fanslow, L. Strockbine, T. A. Sato, K. N. Clifford, B. M. Macduff, D. M. Anderson, S. D. Gimpel, T. Davis-Smith, C. R. Maliszewski, and et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature* 357: 80-82.

63. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity* 1: 167-178.

64. Xu, J., T. M. Foy, J. D. Laman, E. A. Elliott, J. J. Dunn, T. J. Waldschmidt, J.

Elsemore, R. J. Noelle, and R. A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity* 1: 423-431.

65. Renshaw, B. R., W. C. Fanslow, 3rd, R. J. Armitage, K. A. Campbell, D. Liggitt, B. Wright, B. L. Davison, and C. R. Maliszewski. 1994. Humoral immune responses in CD40 ligand-deficient mice. *J Exp Med* 180: 1889-1900.

66. Grewal, I. S., J. Xu, and R. A. Flavell. 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* 378: 617-620.

67. Han, S., K. Hathcock, B. Zheng, T. B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 155: 556-567.

68. Allen, R. C., R. J. Armitage, M. E. Conley, H. Rosenblatt, N. A. Jenkins, N. G. Copeland, M. A. Bedell, S. Edelhoff, C. M. Disteche, D. K. Simoneaux, and et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science* 259: 990-993.

69. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L. S. Grosmaire, R. Stenkamp, M. Neubauer, and et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell* 72: 291-300.

70. Seyama, K., S. Nonoyama, I. Gangsaas, D. Hollenbaugh, H. F. Pabst, A. Aruffo, and H. D. Ochs. 1998. Mutations of the CD40 ligand gene and its effect on CD40 ligand expression in patients with X-linked hyper IgM syndrome. *Blood* 92: 2421-2434.

71. Kroczek, R. A., D. Graf, D. Brugnoni, S. Giliani, U. Korthuer, A. Ugazio, G.

Senger, H. W. Mages, A. Villa, and L. D. Notarangelo. 1994. Defective expression of CD40 ligand on T cells causes “X-linked immunodeficiency with hyper-IgM (HIGM1)”. *Immunol Rev* 138: 39-59.

72. Vavassori, S., and L. R. Covey. 2009. Post-transcriptional regulation in lymphocytes: the case of CD154. *RNA Biol* 6: 259-265.

73. Matus-Nicodemos, R., S. Vavassori, M. Castro-Faix, A. Valentin-Acevedo, K. Singh, V. Marcelli, and L. R. Covey. 2011. Polypyrimidine tract-binding protein is critical for the turnover and subcellular distribution of CD40 ligand mRNA in CD4+ T cells. *J Immunol* 186: 2164-2171.

74. Vavassori, S., Y. Shi, C. C. Chen, Y. Ron, and L. R. Covey. 2009. In vivo post-transcriptional regulation of CD154 in mouse CD4+ T cells. *Eur J Immunol* 39: 2224-2232.

75. Casamayor-Palleja, M., M. Khan, and I. C. MacLennan. 1995. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J Exp Med* 181: 1293-1301.

76. Koguchi, Y., T. J. Thauland, M. K. Slifka, and D. C. Parker. 2007. Preformed CD40 ligand exists in secretory lysosomes in effector and memory CD4+ T cells and is quickly expressed on the cell surface in an antigen-specific manner. *Blood* 110: 2520-2527.

77. Bossi, G., and G. M. Griffiths. 1999. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med* 5: 90-96.

78. Ebsen, H., M. Lettau, D. Kabelitz, and O. Janssen. 2015. Subcellular localization and activation of ADAM proteases in the context of FasL shedding in T lymphocytes. *Mol Immunol* 65: 416-428.

79. He, J. S., D. E. Gong, and H. L. Ostergaard. 2010. Stored Fas ligand, a mediator of rapid CTL-mediated killing, has a lower threshold for response than degranulation or newly synthesized Fas ligand. *J Immunol* 184: 555-563.

80. Zuccato, E., E. J. Blott, O. Holt, S. Sigismund, M. Shaw, G. Bossi, and G. M. Griffiths. 2007. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J Cell Sci* 120: 191-199.

81. Alegre, M. L., P. J. Noel, B. J. Eisfelder, E. Chuang, M. R. Clark, S. L. Reiner, and C. B. Thompson. 1996. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J Immunol* 157: 4762-4770.

82. Iida, T., H. Ohno, C. Nakaseko, M. Sakuma, M. Takeda-Ezaki, H. Arase, E. Kominami, T. Fujisawa, and T. Saito. 2000. Regulation of cell surface expression of CTLA-4 by secretion of CTLA-4-containing lysosomes upon activation of CD4+ T cells. *J Immunol* 165: 5062-5068.

83. Linsley, P. S., J. Bradshaw, J. Greene, R. Peach, K. L. Bennett, and R. S. Mittler. 1996. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* 4: 535-543.

84. Dustin, M. L., T. Starr, R. Varma, and V. K. Thomas. 2007. Supported planar bilayers for study of the immunological synapse. *Curr Protoc Immunol* Chapter 18:Unit 18.13.

85. Vardhana, S., and M. Dustin. 2008. Supported Planar Bilayers for the Formation of Study of Immunological Synapses and Kinapse. *J Vis Exp* pii: 947. doi:

10.3791/947.

86. Monks, C. R., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395: 82-86.

87. Crites, T. J., K. Padhan, J. Muller, M. Krogsgaard, P. R. Gudla, S. J. Lockett, and R. Varma. 2014. TCR Microclusters pre-exist and contain molecules necessary for TCR signal transduction. *J Immunol* 193: 56-67.

88. Yokosuka, T., K. Sakata-Sogawa, W. Kobayashi, M. Hiroshima, A. Hashimoto-Tane, M. Tokunaga, M. L. Dustin, and T. Saito. 2005. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol* 6: 1253-1262.

89. Varma, R., G. Campi, T. Yokosuka, T. Saito, and M. L. Dustin. 2006. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity* 25: 117-127.

90. Bromley, S. K., W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 2001. The immunological synapse. *Annu Rev Immunol* 19: 375-396.

91. Beal, A. M., N. Anikeeva, R. Varma, T. O. Cameron, G. Vasiliver-Shamis, P. J. Norris, M. L. Dustin, and Y. Sykulev. 2009. Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. *Immunity* 31: 632-642.

92. Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221-227.

93. Davis, D. M., and M. L. Dustin. 2004. What is the importance of the immunological synapse? *Trends Immunol* 25: 323-327.

94. Thauland, T. J., Y. Koguchi, S. A. Wetzel, M. L. Dustin, and D. C. Parker. 2008. Th1 and Th2 cells form morphologically distinct immunological synapses. *J Immunol* 181: 393-399.

95. Clayton, K. L., M. S. Haaland, M. B. Douglas-Vail, S. Mujib, G. M. Chew, L. C. Ndhlovu, and M. A. Ostrowski. 2014. T cell Ig and mucin domain-containing protein 3 is recruited to the immune synapse, disrupts stable synapse formation, and associates with receptor phosphatases. *J Immunol* 192: 782-791.

96. Lee, K. H., A. R. Dinner, C. Tu, G. Campi, S. Raychaudhuri, R. Varma, T. N. Sims, W. R. Burack, H. Wu, J. Wang, O. Kanagawa, M. Markiewicz, P. M. Allen, M. L. Dustin, A. K. Chakraborty, and A. S. Shaw. 2003. The immunological synapse balances T cell receptor signaling and degradation. *Science* 302: 1218-1222.

97. Chauveau, A., A. Le Floc'h, N. S. Bantilan, G. A. Koretzky, and M. Huse. 2014. Diacylglycerol kinase alpha establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci Signal* 7: ra82. doi: 10.1126/scisignal.2005287

98. Bossi, G., C. Trambas, S. Booth, R. Clark, J. Stinchcombe, and G. M. Griffiths. 2002. The secretory synapse: the secrets of a serial killer. *Immunol Rev* 189: 152-160.

99. Stinchcombe, J. C., and G. M. Griffiths. 2003. The role of the secretory immunological synapse in killing by CD8+ CTL. *Semin Immunol* 15: 301-305.

100. Stinchcombe, J. C., and G. M. Griffiths. 2007. Secretory mechanisms in cell-mediated cytotoxicity. *Annu Rev Cell Dev Biol* 23: 495-517.

101. Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* 315: 1687-1691.

102. Finetti, F., S. R. Paccani, J. Rosenbaum, and C. T. Baldari. 2011. Intraflagellar transport: a new player at the immune synapse. *Trends Immunol* 32: 139-145.

103. Finetti, F., A. Onnis, and C. T. Baldari. 2015. Regulation of vesicular traffic at the T cell immune synapse: lessons from the primary cilium. *Traffic* 16: 241-249.

104. Finetti, F., S. R. Paccani, M. G. Riparbelli, E. Giacomello, G. Perinetti, G. J. Pazour, J. L. Rosenbaum, and C. T. Baldari. 2009. Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse. *Nat Cell Biol* 11: 1332-1339.

105. Huse, M., B. F. Lillemeier, M. S. Kuhns, D. S. Chen, and M. M. Davis. 2006. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* 7: 247-255.

106. Chemin, K., A. Bohineust, S. Dogniaux, M. Tourret, S. Guegan, F. Miro, and C. Hivroz. 2012. Cytokine secretion by CD4+ T cells at the immunological synapse requires Cdc42-dependent local actin remodeling but not microtubule organizing center polarity. *J Immunol* 189: 2159-2168.

107. Le Floc'h, A., Y. Tanaka, N. S. Bantilan, G. Voisinne, G. Altan-Bonnet, Y. Fukui, and M. Huse. 2013. Annular PIP3 accumulation controls actin architecture and modulates cytotoxicity at the immunological synapse. *Journal Exp Med* 210: 2721-

2737.

108. Pulecio, J., J. Petrovic, F. Prete, G. Chiaruttini, A. M. Lennon-Dumenil, C. Desdouets, S. Gasman, O. R. Burrone, and F. Benvenuti. 2010. Cdc42-mediated MTOC polarization in dendritic cells controls targeted delivery of cytokines at the immune synapse. *J Exp Med* 207: 2719-2732.

109. Bouma, G., A. Mendoza-Naranjo, M. P. Blundell, E. de Falco, K. L. Parsley, S. O. Burns, and A. J. Thrasher. 2011. Cytoskeletal remodeling mediated by WASp in dendritic cells is necessary for normal immune synapse formation and T-cell priming. *Blood* 118: 2492-2501.

110. Rak, G. D., E. M. Mace, P. P. Banerjee, T. Svitkina, and J. S. Orange. 2011. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* 9: e1001151.

111. Ritter, A. T., Y. Asano, J. C. Stinchcombe, N. M. Dieckmann, B. C. Chen, C. Gawden-Bone, S. van Engelenburg, W. Legant, L. Gao, M. W. Davidson, E. Betzig, J. Lippincott-Schwartz, and G. M. Griffiths. 2015. Actin depletion initiates events leading to granule secretion at the immunological synapse. *Immunity* 42: 864-876.

112. Tourret, M., S. Guegan, K. Chemin, S. Dogniaux, F. Miro, A. Bohineust, and C. Hivroz. 2010. T cell polarity at the immunological synapse is required for CD154-dependent IL-12 secretion by dendritic cells. *J Immunol* 185: 6809-6818.

113. Soares, H., R. Henriques, M. Sachse, L. Ventimiglia, M. A. Alonso, C. Zimmer, M. I. Thoulouze, and A. Alcover. 2013. Regulated vesicle fusion generates signaling nanoterritories that control T cell activation at the immunological synapse. *J Exp Med* 210: 2415-2433.

114. Thauland, T. J., and D. C. Parker. 2010. Diversity in immunological synapse structure. *Immunol* 131: 466-472.

115. Moens, L., and S. G. Tangye. 2014. Cytokine-Mediated Regulation of Plasma Cell Generation: IL-21 Takes Center Stage. *Front Immunol* 5: 65.

116. Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 5: 853-865.

117. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J. I. Ellyard, I. A. Parish, C. S. Ma, Q. J. Li, C. R. Parish, C. R. Mackay, and C. G. Vinuesa. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31: 457-468.

118. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325: 1001-1005.

119. Luthje, K., A. Kallies, Y. Shimohakamada, T. B. GT, A. Light, D. M. Tarlinton, and S. L. Nutt. 2012. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* 13: 491-498.

120. Spolski, R., and W. J. Leonard. 2010. IL-21 and T follicular helper cells. *Int Immunol* 22: 7-12.

121. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, S. Fagarasan, A. Liston, K. G. Smith, and C. G. Vinuesa. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* 17: 975-982.

122. Lu, K. T., Y. Kanno, J. L. Cannons, R. Handon, P. Bible, A. G. Elkahloun, S. M. Anderson, L. Wei, H. Sun, J. J. O'Shea, and P. L. Schwartzberg. 2011. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity* 35: 622-632.

123. Chung, Y., S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal, Y. H. Wang, H. Lim, J. M. Reynolds, X. H. Zhou, H. M. Fan, Z. M. Liu, S. S. Neelapu, and C. Dong. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 17: 983-988.

124. Choudhuri, K., J. Llodra, E. W. Roth, J. Tsai, S. Gordo, K. W. Wucherpfennig, L. C. Kam, D. L. Stokes, and M. L. Dustin. 2014. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature* 507: 118-123.

125. Dustin, M. L. 2014. What Counts in the Immunological Synapse? *Mol Cell* 54: 255-262.

126. Koguchi, Y., J. L. Gardell, T. J. Thauland, and D. C. Parker. 2011. Cyclosporine-resistant, Rab27a-independent mobilization of intracellular preformed CD40 ligand mediates antigen-specific T cell help in vitro. *J Immunol* 187: 626-634.

127. Parker, D. C. 1993. T cell-dependent B cell activation. *Annu Rev Immunol* 11: 331-360.

128. van Kooten, C., and J. Banchereau. 2000. CD40-CD40 ligand. *J Leukoc Biol* 67: 2-17.

129. McHeyzer-Williams, L. J., and M. G. McHeyzer-Williams. 2005. Antigen-specific memory B cell development. *Annu Rev Immunol* 23: 487-513.

130. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation.

Annu Rev Immunol 25: 171-192.

131. Okada, T., M. J. Miller, I. Parker, M. F. Krummel, M. Neighbors, S. B. Hartley, A. O'Garra, M. D. Cahalan, and J. G. Cyster. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol* 3: e150.
132. Matheu, M. P., C. Beeton, A. Garcia, V. Chi, S. Rangaraju, O. Safrina, K. Monaghan, M. I. Uemura, D. Li, S. Pal, L. M. de la Maza, E. Monuki, A. Flugel, M. W. Pennington, I. Parker, K. G. Chandy, and M. D. Cahalan. 2008. Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity* 29: 602-614.
133. Cannons, J. L., H. Qi, K. T. Lu, M. Dutta, J. Gomez-Rodriguez, J. Cheng, E. K. Wakeland, R. N. Germain, and P. L. Schwartzberg. 2010. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* 32: 253-265.
134. Blott, E. J., and G. M. Griffiths. 2002. Secretory lysosomes. *Nat Rev Mol Cell Biol* 3: 122-131.
135. Stinchcombe, J. C., G. Bossi, S. Booth, and G. M. Griffiths. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* 15: 751-761.
136. Jenkins, M. R., and G. M. Griffiths. 2010. The synapse and cytolytic machinery of cytotoxic T cells. *Cur Opin Immunol* 22: 308-313.
137. Yellin, M. J., K. Sippel, G. Inghirami, L. R. Covey, J. J. Lee, J. Sinning, E. A. Clark, L. Chess, and S. Lederman. 1994. CD40 molecules induce down-modulation and

endocytosis of T cell surface T cell-B cell activating molecule/CD40-L. Potential role in regulating helper effector function. *J Immunol* 152: 598-608.

138. Stinchcombe, J. C., D. C. Barral, E. H. Mules, S. Booth, A. N. Hume, L. M. Machesky, M. C. Seabra, and G. M. Griffiths. 2001. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol* 152: 825-834.
139. Oxenius, A., M. F. Bachmann, R. M. Zinkernagel, and H. Hengartner. 1998. Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. *Eur J Immunol* 28: 390-400.
140. Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 9: 310-318.
141. Huddleston, C. A., A. D. Weinberg, and D. C. Parker. 2006. OX40 (CD134) engagement drives differentiation of CD4+ T cells to effector cells. *Eur J Immunol* 36: 1093-1103.
142. Son, Y. I., S. Egawa, T. Tatsumi, R. E. Redlinger, Jr., P. Kalinski, and T. Kanto. 2002. A novel bulk-culture method for generating mature dendritic cells from mouse bone marrow cells. *J Immunol Methods* 262: 145-157.
143. Fuleihan, R., N. Ramesh, A. Horner, D. Ahern, P. J. Belshaw, D. G. Alberg, I. Stamenkovic, W. Harmon, and R. S. Geha. 1994. Cyclosporin A inhibits CD40 ligand expression in T lymphocytes. *J Clin Invest* 93: 1315-1320.
144. Kawakami, K., and D. C. Parker. 1993. Antigen and helper T lymphocytes activate B lymphocytes by distinct signaling pathways. *Eur J Immunol* 23: 77-84.
145. Hermann-Kleiter, N., and G. Baier. 2010. NFAT pulls the strings during CD4+ T

helper cell effector functions. *Blood* 115: 2989-2997.

146. Borst, J., J. Hendriks, and Y. Xiao. 2005. CD27 and CD70 in T cell and B cell activation. *Curr Opin Immunol* 17: 275-281.

147. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 281: 65-78.

148. Van den Eertwegh, A. J., R. J. Noelle, M. Roy, D. M. Shepherd, A. Aruffo, J. A. Ledbetter, W. J. Boersma, and E. Claassen. 1993. 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* 178: 1555-1565.

149. Han, S., K. Hathcock, B. Zheng, T. B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J Immunol* 155: 556-567.

150. Takahashi, Y., P. R. Dutta, D. M. Cerasoli, and G. Kelsoe. 1998. 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* 187: 885-895.

151. O'Connor, B. P., L. A. Vogel, W. Zhang, W. Loo, D. Shnider, E. F. Lind, M. Ratliff, R. J. Noelle, and L. D. Erickson. 2006. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. *J Immunol* 177: 7723-7732.

152. Chan, T. D., D. Gatto, K. Wood, T. Camidge, A. Basten, and R. Brink. 2009. Antigen affinity controls rapid T-dependent antibody production by driving the

expansion rather than the differentiation or extrafollicular migration of early plasmablasts. *J Immunol* 183: 3139-3149.

153. Harwood, N. E., and F. D. Batista. 2008. New insights into the early molecular events underlying B cell activation. *Immunity* 28: 609-619.

154. Depoil, D., R. Zaru, M. Guiraud, A. Chauveau, J. Harriague, G. Bismuth, C. Utzny, S. Muller, and S. Valitutti. 2005. Immunological synapses are versatile structures enabling selective T cell polarization. *Immunity* 22: 185-194.

155. Reinhardt, R. L., H. E. Liang, and R. M. Locksley. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 10: 385-393.

156. Ozaki, K., R. Spolski, R. Ettinger, H. P. Kim, G. Wang, C. F. Qi, P. Hwu, D. J. Shaffer, S. Akilesh, D. C. Roopenian, H. C. Morse, 3rd, P. E. Lipsky, and W. J. Leonard. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* 173: 5361-5371.

157. Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 207: 353-363.

158. Zotos, D., J. M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L. M. Corcoran, D. I. Godfrey, K. M. Toellner, M. J. Smyth, S. L. Nutt, and D. M. Tarlinton. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* 207: 365-378.

159. Calame, K. L., K. I. Lin, and C. Tunyaplin. 2003. Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol* 21:

205-230.

160. Saito, M., J. Gao, K. Basso, Y. Kitagawa, P. M. Smith, G. Bhagat, A. Pernis, L. Pasqualucci, and R. Dalla-Favera. 2007. A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell* 12: 280-292.

161. Erickson, L. D., B. G. Durell, L. A. Vogel, B. P. O'Connor, M. Cascalho, T. Yasui, H. Kikutani, and R. J. Noelle. 2002. Short-circuiting long-lived humoral immunity by the heightened engagement of CD40. *J Clin Invest* 109: 613-620.

162. Bolduc, A., E. Long, D. Stapler, M. Cascalho, T. Tsubata, P. A. Koni, and M. Shimoda. 2010. Constitutive CD40L expression on B cells prematurely terminates germinal center response and leads to augmented plasma cell production in T cell areas. *J Immunol* 185: 220-230.

163. Kishi, Y., Y. Aiba, T. Higuchi, K. Furukawa, T. Tokuhisa, T. Takemori, and T. Tsubata. 2010. Augmented antibody response with premature germinal center regression in CD40L transgenic mice. *J Immunol* 185: 211-219.

164. Polo, J. M., W. Ci, J. D. Licht, and A. Melnick. 2008. Reversible disruption of BCL6 repression complexes by CD40 signaling in normal and malignant B cells. *Blood* 112: 644-651.

165. Ci, W., J. M. Polo, and A. Melnick. 2008. B-cell lymphoma 6 and the molecular pathogenesis of diffuse large B-cell lymphoma. *Curr Opin Hematol* 15: 381-390.

166. Damdinsuren, B., Y. Zhang, A. Khalil, W. H. Wood, 3rd, K. G. Becker, M. J. Shlomchik, and R. Sen. 2010. Single round of antigen receptor signaling programs naive B cells to receive T cell help. *Immunity* 32: 355-366.

167. MacDonald, K. P., Y. Nishioka, P. E. Lipsky, and R. Thomas. 1997. Functional CD40 ligand is expressed by T cells in rheumatoid arthritis. *J Clin Invest* 100: 2404-2414.

168. Yi, Y., M. McNerney, and S. K. Datta. 2000. Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J Immunol* 165: 6627-6634.

169. Katsiari, C. G., S. N. Liossis, A. M. Dimopoulos, D. V. Charalambopoulo, M. Mavrikakis, and P. P. Sfikakis. 2002. CD40L overexpression on T cells and monocytes from patients with systemic lupus erythematosus is resistant to calcineurin inhibition. *Lupus* 11: 370-378.

170. Mathur, R. K., A. Awasthi, P. Wadhone, B. Ramanamurthy, and B. Saha. 2004. Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. *Nat Med* 10: 540-544.

171. Guarda, G., C. Dostert, F. Staehli, K. Cabalzar, R. Castillo, A. Tardivel, P. Schneider, and J. Tschopp. 2009. T cells dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes. *Nature* 460: 269-273.

172. Brown, D. M., C. Kamperschroer, A. M. Dilzer, D. M. Roberts, and S. L. Swain. 2009. IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4+ T cells. *Cell Immunol* 257: 69-79.

173. Keller, A. M., Y. Xiao, V. Peperzak, S. H. Naik, and J. Borst. 2009. Costimulatory ligand CD70 allows induction of CD8+ T-cell immunity by immature dendritic cells in a vaccination setting. *Blood* 113: 5167-5175.

174. Johnson, S., Y. Zhan, R. M. Sutherland, A. M. Mount, S. Bedoui, J. L. Brady, E. M. Carrington, L. E. Brown, G. T. Belz, W. R. Heath, and A. M. Lew. 2009. Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. *Immunity* 30: 218-227.

175. Henn, V., J. R. Slupsky, M. Grafe, I. Anagnostopoulos, R. Forster, G. Muller-Berghaus, and R. A. Kroczek. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-594.

176. Otterdal, K., C. Smith, E. Oie, T. M. Pedersen, A. Yndestad, E. Stang, K. Endresen, N. O. Solum, P. Aukrust, and J. K. Damas. 2006. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes. *Blood* 108: 928-935.

177. Koguchi, Y., A. C. Buenafe, T. J. Thauland, J. L. Gardell, E. R. Bivins-Smith, D. B. Jacoby, M. K. Slifka, and D. C. Parker. 2012. Preformed CD40L is stored in Th1, Th2, Th17, and T follicular helper cells as well as CD4+ 8- thymocytes and invariant NKT cells but not in Treg cells. *PloS One* 7: e31296.

178. Victora, G. D., and M. C. Nussenzweig. 2012. Germinal centers. *Annu Rev Immunol* 30: 429-457.

179. Crotty, S. 2015. A brief history of T cell help to B cells. *Nat Rev Immunol* 15: 185-9.

180. Kupfer, A., and S. J. Singer. 1989. The specific interaction of helper T cells and antigen-presenting B cells. IV. Membrane and cytoskeletal reorganizations in the bound T cell as a function of antigen dose. *J Exp Med* 170: 1697-1713.

181. Boisvert, J., S. Edmondson, and M. F. Krummel. 2004. Immunological synapse formation licenses CD40-CD40L accumulations at T-APC contact sites. *J Immunol* 173: 3647-3652.

182. Bertrand, F., M. Esquerre, A. E. Petit, M. Rodrigues, S. Duchez, J. Delon, and S. Valitutti. 2010. Activation of the ancestral polarity regulator protein kinase C zeta at the immunological synapse drives polarization of Th cell secretory machinery toward APCs. *J Immunol* 185: 2887-2894.

183. Lineberry, N. B., L. L. Su, J. T. Lin, G. P. Coffey, C. M. Seroogy, and C. G. Fathman. 2008. Cutting edge: The transmembrane E3 ligase GRAIL ubiquitinates the costimulatory molecule CD40 ligand during the induction of T cell anergy. *J Immunol* 181: 1622-1626.

184. Yacoub, D., N. Benslimane, L. Al-Zoobi, G. Hassan, A. Nadiri, and W. Mourad. 2013. CD154 is released from T-cells by ADAM10 and ADAM17 in a CD40-dependent manner. *J Biol Chem* 288: 36083-93.

185. Ludewig, B., V. Henn, J. M. Schroder, D. Graf, and R. A. Kroczek. 1996. Induction, regulation, and function of soluble TRAP (CD40 ligand) during interaction of primary CD4(+) CD45RA(+) T cells with dendritic cells. *Eur J Immunol* 26: 3137-3143.

186. Chen, Y., J. Chen, Y. Xiong, Q. Da, Y. Xu, X. Jiang, and H. Tang. 2006. Internalization of CD40 regulates its signal transduction in vascular endothelial cells. *Biochem Biophys Res Commun* 345: 106-117.

187. Lang, P., J. C. Stolpa, B. A. Freiberg, F. Crawford, J. Kappler, A. Kupfer, and J. C. Cambier. 2001. TCR-induced transmembrane signaling by peptide/MHC class II via associated Ig-alpha/beta dimers. *Science* 291: 1537-1540.

188. Seder, R. A., W. E. Paul, M. M. Davis, and B. Fazekas de St Groth. 1992. The presence of interleukin 4 during in vitro priming determines the lymphokine-

producing potential of CD4+ T cells from T cell receptor transgenic mice. *J Exp Med* 176: 1091-1098.

189. Lee, B. O., L. Haynes, S. M. Eaton, S. L. Swain, and T. D. Randall. 2002. The biological outcome of CD40 signaling is dependent on the duration of CD40 ligand expression: reciprocal regulation by interleukin (IL)-4 and IL-12. *J Exp Med* 196: 693-704.
190. Wykes, M. 2003. Why do B cells produce CD40 ligand? *Immunol Cell Biol* 81: 328-331.
191. Roy, M., T. Waldschmidt, A. Aruffo, J. A. Ledbetter, and R. J. Noelle. 1993. The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4+ T cells. *J Immunol* 151: 2497-2510.
192. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat Med* 11: 1118-1124.
193. Chattopadhyay, P. K., J. Yu, and M. Roederer. 2005. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. *Nat Med* 11: 1113-1117.
194. Hawkins, E. D., M. L. Turner, C. J. Wellard, J. H. Zhou, M. R. Dowling, and P. D. Hodgkin. 2013. Quantal and graded stimulation of B lymphocytes as alternative strategies for regulating adaptive immune responses. *Nat Commun* 4: 2406.
195. Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S. C. Sun, and M. Karin. 2001. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* 293: 1495-1499.

196. Xiao, G., E. W. Harhaj, and S. C. Sun. 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 7: 401-409.

197. Rathmell, J. C., S. E. Townsend, J. C. Xu, R. A. Flavell, and C. C. Goodnow. 1996. Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. *Cell* 87: 319-329.

198. Kupfer, A., and S. J. Singer. 1989. Cell biology of cytotoxic and helper T cell functions: immunofluorescence microscopic studies of single cells and cell couples. *Annu Rev Immunol* 7: 309-337.

199. McHeyzer-Williams, M., S. Okitsu, N. Wang, and L. McHeyzer-Williams. 2012. Molecular programming of B cell memory. *Nat Rev Immunol* 12: 24-34.

200. Schulten, V., J. A. Greenbaum, M. Hauser, D. M. McKinney, J. Sidney, R. Kolla, C. S. Lindestam Arlehamn, C. Oseroff, R. Alam, D. H. Broide, F. Ferreira, H. M. Grey, A. Sette, and B. Peters. 2013. Previously undescribed grass pollen antigens are the major inducers of T helper 2 cytokine-producing T cells in allergic individuals. *Proc Natl Acad Sci U S A* 110: 3459-3464.

201. Brown, A. C., S. Oddos, I. M. Dobbie, J. M. Alakoskela, R. M. Parton, P. Eissmann, M. A. Neil, C. Dunsby, P. M. French, I. Davis, and D. M. Davis. 2011. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol* 9: e1001152.

202. Crotty, S. 2014. T follicular helper cell differentiation, function, and roles in disease. *Immunity* 41: 529-542.

203. Schwickert, T. A., G. D. Victora, D. R. Fooksman, A. O. Kamphorst, M. R. Mugnier, A. D. Gitlin, M. L. Dustin, and M. C. Nussenzweig. 2011. A dynamic T

cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J Exp Med* 208: 1243-1252.

204. Crotty, S., R. J. Johnston, and S. P. Schoenberger. 2010. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 11: 114-120.

205. Fazilleau, N., L. Mark, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells: lineage and location. *Immunity* 30: 324-335.

206. Gu-Trantien, C., S. Loi, S. Garaud, C. Equeter, M. Libin, A. de Wind, M. Ravoet, H. Le Buanec, C. Sibille, G. Manfouo-Foutsop, I. Veys, B. Haibe-Kains, S. K. Singhal, S. Michiels, F. Rothe, R. Salgado, H. Duvillier, M. Ignatiadis, C. Desmedt, D. Bron, D. Larsimont, M. Piccart, C. Sotiriou, and K. Willard-Gallo. 2013. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* 123: 2873-2892.

207. Bindea, G., B. Mlecnik, M. Tosolini, A. Kirilovsky, M. Waldner, A. C. Obenauf, H. Angell, T. Fredriksen, L. Lafontaine, A. Berger, P. Bruneval, W. H. Fridman, C. Becker, F. Pages, M. R. Speicher, Z. Trajanoski, and J. Galon. 2013. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 39: 782-795.

208. Clegg, C. H., J. T. Rulffes, H. S. Haugen, I. H. Hoggatt, A. Aruffo, S. K. Durham, A. G. Farr, and D. Hollenbaugh. 1997. Thymus dysfunction and chronic inflammatory disease in gp39 transgenic mice. *Int Immunol* 9: 1111-1122.

209. Dunn, R. J., C. J. Luedecker, H. S. Haugen, C. H. Clegg, and A. G. Farr. 1997. Thymic overexpression of CD40 ligand disrupts normal thymic epithelial

organization. *J Histochem Cytochem* 45: 129-141.

210. Brown, M. P., D. J. Topham, M. Y. Sangster, J. Zhao, K. J. Flynn, S. L. Surman, D. L. Woodland, P. C. Doherty, A. G. Farr, P. K. Pattengale, and M. K. Brenner. 1998. Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in mice. *Nat Med* 4: 1253-1260.

211. Crow, M. K., and K. A. Kirou. 2001. Regulation of CD40 ligand expression in systemic lupus erythematosus. *Curr Opin Rheumatol* 13: 361-369.

212. Wilson, S. M., R. Yip, D. A. Swing, T. N. O'Sullivan, Y. Zhang, E. K. Novak, R. T. Swank, L. B. Russell, N. G. Copeland, and N. A. Jenkins. 2000. A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proc Natl Acad Sci U S A* 97: 7933-7938.

213. Haddad, E. K., X. Wu, J. A. Hammer, 3rd, and P. A. Henkart. 2001. Defective granule exocytosis in Rab27a-deficient lymphocytes from Ashen mice. *J Cell Biol* 152: 835-842.

214. Jirmanova, L., M. L. Giardino Torchia, N. D. Sarma, P. R. Mittelstadt, and J. D. Ashwell. 2011. Lack of the T cell-specific alternative p38 activation pathway reduces autoimmunity and inflammation. *Blood* 118: 3280-3289.

215. Huan, C., M. L. Kelly, R. Steele, I. Shapira, S. R. Gottesman, and C. A. Roman. 2006. Transcription factors TFE3 and TFEB are critical for CD40 ligand expression and thymus-dependent humoral immunity. *Nat Immunol* 7: 1082-1091.

216. Huynh, C., D. Roth, D. M. Ward, J. Kaplan, and N. W. Andrews. 2004. Defective lysosomal exocytosis and plasma membrane repair in Chediak-Higashi/beige cells. *Proc Natl Acad Sci U S A* 101: 16795-16800.

217. Zhu, D., Y. Zhang, P. P. Lam, S. Dolai, Y. Liu, E. P. Cai, D. Choi, S. A. Schroer, Y. Kang, E. M. Allister, T. Qin, M. B. Wheeler, C. C. Wang, W. J. Hong, M. Woo, and H. Y. Gaisano. 2012. Dual role of VAMP8 in regulating insulin exocytosis and islet beta cell growth. *Cell Metab* 16: 238-249.

218. Stow, J. L., and R. Z. Murray. 2013. Intracellular trafficking and secretion of inflammatory cytokines. *Cytokine Growth Factor Rev* 24: 227-239.

219. Benado, A., Y. Nasagi-Atiya, and R. Sagi-Eisenberg. 2009. Protein trafficking in immune cells. *Immunobiol* 214: 507-525.

220. Huse, M., E. J. Quann, and M. M. Davis. 2008. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nat Immunol* 9: 1105-1111.

221. Arneson, L. N., A. Brickshawana, C. M. Segovis, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2007. Cutting edge: syntaxin 11 regulates lymphocyte-mediated secretion and cytotoxicity. *J Immunol* 179: 3397-3401.

222. Valdez, A. C., J. P. Cabaniols, M. J. Brown, and P. A. Roche. 1999. Syntaxin 11 is associated with SNAP-23 on late endosomes and the trans-Golgi network. *J Cell Sci* 112: 845-854.

223. Dressel, R., L. Elsner, P. Novota, N. Kanwar, and G. Fischer von Mollard. 2010. The exocytosis of lytic granules is impaired in Vti1b- or Vamp8-deficient CTL leading to a reduced cytotoxic activity following antigen-specific activation. *J Immunol* 185: 1005-1014.

224. Loo, L. S., L. A. Hwang, Y. M. Ong, H. S. Tay, C. C. Wang, and W. Hong. 2009. A role for endobrevin/VAMP8 in CTL lytic granule exocytosis. *Eur J Immunol* 39: 3520-3528.

225. Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, and F. Zhang. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819-823.

226. Sprent, J. 2005. Swapping molecules during cell-cell interactions. *Sci STKE* 2005: pe8.

227. Hudrisier, D., and P. Bongrand. 2002. Intercellular transfer of antigen-presenting cell determinants onto T cells: molecular mechanisms and biological significance. *FASEB J* 16: 477-486.

228. Hudson, L., and J. Sprent. 1976. Specific adsorption of IgM antibody onto H-2-activated mouse T lymphocytes. *J Exp Med* 143: 444-449.

229. Nagy, Z., B. E. Elliott, M. Nabholz, P. H. Krammer, and B. Pernis. 1976. Specific binding of alloantigens to T cells activated in the mixed lymphocyte reaction. *J Exp Med* 143: 648-659.

230. Hudson, L., J. Sprent, J. F. Miller, and J. H. Playfair. 1974. B cell-derived immunoglobulin on activated mouse T lymphocytes. *Nature* 251: 60-62.

231. Cone, R. E., J. Sprent, and J. J. Marchalonis. 1972. Antigen-binding specificity of isolated cell-surface immunoglobulin from thymus cells activated to histocompatibility antigens. *Proc Natl Acad Sci U S A* 69: 2556-2560.

232. Huang, J. F., Y. Yang, H. Sepulveda, W. Shi, I. Hwang, P. A. Peterson, M. R. Jackson, J. Sprent, and Z. Cai. 1999. TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. *Science* 286: 952-954.

233. Di Guglielmo, G. M., C. Le Roy, A. F. Goodfellow, and J. L. Wrana. 2003. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell*

Biol 5: 410-421.

234. Grimes, M. L., J. Zhou, E. C. Beattie, E. C. Yuen, D. E. Hall, J. S. Valletta, K. S. Topp, J. H. LaVail, N. W. Bunnett, and W. C. Mobley. 1996. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. *J Neurosci* 16: 7950-7964.

235. Hudrisier, D., J. Riond, H. Mazarguil, J. E. Gairin, and E. Joly. 2001. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. *J Immunol* 166: 3645-3649.

236. Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, and C. Hivroz. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* 168: 3235-3241.

237. Raposo, G., and W. Stoorvogel. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 200: 373-383.

238. Okoye, I. S., S. M. Coomes, V. S. Pelly, S. Czieso, V. Papayannopoulos, T. Tolmachova, M. C. Seabra, and M. S. Wilson. 2014. MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. *Immunity* 41: 89-103.

239. Chatila, T. A., and C. B. Williams. 2014. Regulatory T cells: exosomes deliver tolerance. *Immunity* 41: 3-5.

240. Esposito, L., K. M. Hunter, J. Clark, D. B. Rainbow, H. Stevens, J. Denesha, S. Duley, S. Dawson, G. Coleman, S. Nutland, G. L. Bell, C. Moran, M. Pekalski, J. A. Todd, and L. S. Wicker. 2014. Investigation of soluble and transmembrane CTLA-4 isoforms in serum and microvesicles. *J Immunol* 193: 889-900.

241. Alonso, R., M. C. Rodriguez, J. Pindado, E. Merino, I. Merida, and M. Izquierdo. 2005. Diacylglycerol kinase alpha regulates the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes. *J Biol Chem* 280: 28439-28450.

242. Zhao, D. M., A. M. Thornton, R. J. DiPaolo, and E. M. Shevach. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* 107: 3925-3932.

243. Ashby, M. C., K. Ibaraki, and J. M. Henley. 2004. It's green outside: tracking cell surface proteins with pH-sensitive GFP. *Trends Neurosci* 27: 257-261.

244. Burchfield, J. G., J. A. Lopez, K. Mele, P. Vallotton, and W. E. Hughes. 2010. Exocytotic vesicle behaviour assessed by total internal reflection fluorescence microscopy. *Traffic* 11: 429-439.

245. Dustin, M. L. 2014. T-cells play the classics with a different spin. *Mol Biol Cell* 25: 1699-1703.

246. Stinchcombe, J. C., and G. M. Griffiths. 2014. Communication, the centrosome and the immunological synapse. *Philos Trans R Soc Lond B Biol Sci* 369:

247. de la Roche, M., A. T. Ritter, K. L. Angus, C. Dinsmore, C. H. Earnshaw, J. F. Reiter, and G. M. Griffiths. 2013. Hedgehog signaling controls T cell killing at the immunological synapse. *Science* 342: 1247-1250.