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# Anti-CD40 Mediated Blockade of the CD40 Costimulatory Pathway

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B.S., University of New Hampshire, 2000

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# Abstract


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## By Christopher Raleigh Gilson

Transplantation is often the sole therapy available for many forms of end-stage organ disease. The field of transplantation owes much of its success to the development of immunosuppressive drugs which predominantly seek to inhibit the T cell-dependent process of rejection. Due to the inherent disadvantages of non-specific immunosuppression, considerable interest has been place in developing more selective strategies. Transient blockade of the CD40 pathway, in conjunction with CD28 blockade, has shown great promise in tolerance induction protocols to inactivate of donor reactive T cells. However, anti-CD154 monoclonal antibodies that have for years been the foundation of these approaches also cause thromboembolism in humans. The central hypothesis of this thesis is that blockade of the CD40 signaling pathway through the use of anti-CD154 or anti-CD40 monoclonal antibodies can equally promote chimerism and graft survival. The functional equivalence between these two approaches highlights the importance of inhibiting the CD40 pathway itself.

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# Chapter 1

# Introduction

*Significance for the Study of Transplantation*

December 23, 1954 marks the date of the first successful human kidney allograft transplant, performed at Boston's Peter Bent Brigham Hospital by Dr. Joseph Murray and colleagues, between Richard Herrick and his twin brother Ronald (1). Over the next half-century, solid organ and hematopoietic transplantation would become an established, while often the only, treatment option for many end stage diseases. However, in the absence of a consenting twin sibling, prevention of immune mediated organ damage is currently dependant upon the continual administration of immunosuppressive drugs. This has been remarkably successful for controlling acute rejection, as exemplified by the current 90% 1-year survival rate following renal transplantation in the United States (2). However, the rate of annual graft attrition after the first post-operative year has virtually remained unchanged (3). An estimated one half to three quarters of the late graft losses are explained by patient death with a functioning graft while chronic rejection accounts for 50 to 80% of graft failures in surviving recipients (4).

Chronic graft injury is not only caused by immune mediated processes, but also by non-immune factors, leading to a progressive diminution of function and ultimately graft failure. This eventuality can be related to two important issues with regards to the use of immunosuppressive drugs. First, most of these drugs act nonspecifically, attenuating the immune system to infectious and cancerous morbidities (5, 6). Second, these drugs must often be taken daily, which continuously exposes the body and the transplanted tissue to direct drug toxicity as well as exacerbating cardiovascular disease, hypertension, hyperlipidemia and new onset diabetes (7).

The U.S. Organ Procurement and Transplantation Network reported that number

of kidney transplants performed in the United States grew from 11,561 in 1997 to 16,646 in 2006 (2). During this same period, the number of candidates on the kidney transplant waiting list at any time during the calendar year increased by 81%, with 66,961 patients still in need of transplant by years-end. This growth in the number of wait-listed patients has been accompanied by a more than doubling in the number of deaths while on the waiting list from 2,184 in 1997 to 4,456 in 2006. The number of kidney donations from both living and deceased donor sources has not kept pace with the growing need. Strategies have been proposed by the transplantation community to increase the efficiency of the current organ allocation and transplantation system, but aggressive changes need to be taken to shift current trends.

The chronic immunosuppression approach has been based on the premise that alloreactivity is a fixed entity, relentlessly at odds with a transplanted organ. However, the expanding understanding of allorecognition and the immune response as a whole has shown that this is not necessarily always the case (8). The interaction is complex and often quite plastic. While the body's goal is normally the complete elimination of an antigen, there are physiologic mechanisms in place to promote tolerance (9-12) and the possibility to harness them for clinical benefit.

*A Brief Historical Context*

Myths have been used by ancient societies to describe seemingly supernatural events in an attempt to make sense of that which cannot be explained or understood by the common man. Stories of the prolongation of life by the replacement of body parts are present in many cultures at a time well before the understanding needed for them to have

actually been performed. The Chinese tell of the physician Pien Ch'iao who reportedly exchanged hearts between two warriors in an attempt to achieve spiritual and physical balance in each (13). Indian scriptures tell of the God Siva, in fits of rage, beheading individuals and then performing xenotransplants as atonement for his outbursts (13). Roman Catholic mythos describe the story of the Saints Cosmas and Damian, who replaced the gangrenous leg of the deacon Justinian with that of a recently deceased Ethiopian (14).

In what is regarded as one of the first credible accounts of transplantation, the second century Indian surgeon Sushruta is credited for performing various types of rotation and pedicle flaps for the treatment of nose amputations, a common form of punishment at the time (15). Centuries later, the Italian surgeon Gasparo Tagliacozzi would also perform successful skin autografts, while noting the perplexingly consistent failure of allografts. He attributed this to the "force and power of individuality" in his 1597 work ‘De Curtorum Chirurgia per Insitionem’ (16).

The exact nature of the ‘…power of individuality’ would take several more centuries to become clear. In the intervening years, a foundation of surgical techniques would be gradually perfected to make performing transplantation practical. The first successful human corneal transplant was performed by Dr. Edward Zirm in Austria in 1905 (17). Without an appreciation of immune privilege, the inexplicable success of corneal transplants and why this could not translate to other tissues confounded physicians of the time. In the early 1900s, the French surgeon Alexis Carrel would pioneer a new technique of artery and vein anastomosis that facilitated the experimental

transplants of animal kidney, heart and spleen. Carrel would receive the Nobel Prize in 1912 and was one of the first to identify rejection as a barrier to transplantation (17).

The origins of modern transplantation immunology are most appropriately attributed to the work of Peter Medawar in the early 1940's. While observing autologous and allogeneic skin grafts used to treat burn victims during World War II, he noted what would become two important tenets of graft rejection. First, allogeneic grafts invariably failed while autografts were accepted. Second, secondary allografts from the same donor were rejected even more promptly than the first. Presenting his findings to the British War Department, he secured funding to follow up these observations with exhaustive rabbit skin graft experiments. Confirming the initial findings from the burn units, he also noted the presence of a lymphocyte infiltrate in the rejecting grafts (18, 19). Medawar concluded that the rejection of allogeneic skin grafts was an immune mediated process with lymphocytes as the likely effector cells. However, the prevailing theories of the time placed a greater importance on antibody mediated responses as an effector mechanism.

A definitive role for lymphocytes in graft rejection would be shown through the work of Nicholas Mitchison during the 1950's. He showed that when lymphocytes from mice immunized against a lymphosarcoma were transferred into naïve mice, that this conferred protective immunity upon tumor re-challenge, while transfer of serum antibodies alone did not (20). Using an allogeneic skin graft model, work by Brent, Billingham and Medawar would further implicate lymphocytes as the central mediators of graft rejection (21).

*T cell Activation*

The development of monoclonal antibodies by Kohler and Millstein in 1975 allowed for the phenotypic characterization of lymphocytes and established their specific roles in graft rejection (22). Both $CD4^+$ (23) and $CD8^+$ T cells (24) were found to be required to mediate graft rejection. Transfer of either subset into severe combined immunodeficiency mice, which lack functional T and B cells, or their depletion from wild type mice have shown that both subsets are involved in graft rejection (25).

Optimal T cell activation requires two signals (26). Signal 1 is initiated by the antigen specific interaction between the T cell receptor (TCR) and the major histocompatibility complex (MHC) presenting peptide. The MHC molecule is divided into two classes: MHC class I and MHC class II. MHC class I molecules bind peptides mainly derived from proteins synthesized inside the cell and are recognized by T cells expressing the CD8 co-receptor. MHC class II molecules mainly bind peptides derived from protein antigens taken up from outside the cell and are recognized by T cells expressing the CD4 co-receptor. Signal 2 takes the form of costimulatory proteins expressed between T cells and APCs that deliver auxiliary stimulation to augment T cell activation. These include, among many others, the CD28, CD40, OX40, 41BB signaling pathways, as well as cytokines such as IL-15, IL-12, IL-10, IL-4 or IL-2.

The specificity and control over the initiation of the immune response is in part provided by the TCR. T cells must be able to both respond to an innumerable array of pathogens while also preserving restraint to self-proteins. Immature T cells undergo a dynamic process in the thymus in which the developing T cells must be able to recognize self MHC within a certain range of affinity to receive signals for continued survival, in

what is termed positive selection (27). Bone marrow derived dendritic cells resident to the thymus contribute to this process by expressing a wide array of self-peptides. During this second interaction, autoreactive T cells with high affinity for these self peptides are deleted through negative selection (28).

Self-reactive T cells do escape these central tolerance mechanisms and can be found in the periphery, but there are additional measures that prevent the emergence of overt autoimmunity. As stated previously, naïve T cells require costimulatory signals to augment antigen recognition by the TCR. Only specialized professional antigen presenting cells are capable of delivering these signals and dendritic cells are regarded as the most potent. They reside throughout most tissues and act as resident sentinels for the immune system. Dendritic cells are highly sensitive to a wide variety of inflammatory stimuli and can be activated by tissue damage, cytokines or pathogenic molecules via Toll-like receptors (29, 30). When dendritic cells sense these 'danger signals', they up-regulate lymph node homing molecules such as CCR7, express costimulatory ligands and enhance antigen presentation all of which allows for efficient T cell priming (31, 32). In the absence of an inflammatory milieu, such as the case when harmless self proteins are taken up, dendritic cells remain in an immature state, reserving their expression of costimulatory molecules such as CD80 and CD86 (33). Under these conditions, autoreactive T cells can be controlled by this suboptimal interaction, leading to anergy, apoptosis, or possibly towards a regulatory T cell like phenotype (34-37).

*The CD28 Signaling Pathway*

CD28 was the first T cell costimulatory pathway identified, is constitutively expressed by most T cells and interacts with its natural ligands CD80 and CD86 expressed by professional APCs (38). In the absence of TCR signaling, the contact between the B7 molecules and CD28 has little impact on T cell activation. When both TCR and CD28 signals are delivered this stimulates proliferation by the transcription and stabilization of IL-2 mRNA (39, 40). CD28 signaling also promotes the secretion of IFNγ, IL-4, IL-8 and RANTES, the expression of CD25 as well as the up-regulation of the survival factor Bcl-xL (41-45).

T cells express a second receptor for CD80 and CD86, known as CTLA4 (CD152). CTLA4 binds to the B7 molecules with a 20-50 fold higher affinity than CD28 (46, 47). It is not normally expressed by naïve T cells, but is rapidly up-regulated following TCR stimulation. It is believed to function by inhibiting the initiation and progression of the immune response (48). CTLA4 deficient mice develop uncontrolled lymphoproliferative disorders and specific blockade of CTLA4 exacerbates allograft rejection (49, 50). Furthermore, some modes of T cell anergy have been shown to require CTLA4 signaling (51).

*The CD40 Signaling Pathway*

The CD40 molecule is an integral membrane protein in the TNF receptor superfamily. It is expressed on a wide array of cells including B cells, mast cells, macrophage, dendritic cells and endothelial cells (52). Its ligand, CD154 (CD40L), is a member of the TNF cytokine superfamily, notably expressed on recently activated $CD4^+$ T cells, but can also be found on other cells such as $CD8^+$ T cell subsets, monocytes,

platelets as well as in a soluble form present in serum (53, 54). The CD40-CD40L interaction was initially characterized for its role during germinal center formation and B cell isotype switching (17). A genetic mutation in CD154 is the cause of a condition in humans known as Hyper-IgM syndrome, distinguished by a failure to form germinal centers and class switch antibody (55).

The CD40 pathway influences a variety of cellular processes including division, survival and differentiation. CD40 ligation can stimulate the secretion of IL-1β, IL-6, IL-8, IL-12, and TNFα, the expression of ICAM-1, LFA-3, CD80 and CD86, the production of matrix metalloproteinases and nitric oxide, as well as inhibiting apoptosis (56-60). The cytoplasmic tail of CD40 contains three TRAF-interacting motifs. Trimerization of CD40 monomers present in the cell membrane lead to the recruitment of tumor necrosis factor receptor-associated factor (TRAF) family members, TRAF2, TRAF3, and TRAF6. These TRAFs activate of several kinase cascades that in turn trigger multiple signal transduction pathways such as NF-κB, JNK, p38, ERK, and PI3K (61). Similar signaling cascades are present in various cell subtypes, however specific functions of the TRAFs during CD40 signaling have been seen in B cells and dendritic cells. The binding to TRAF2 and/or TRAF3 but not TRAF6 is essential for B cell to class switch their immunoglobulin (62). TRAF6 has bee implicated in affinity maturation and plasma cell generation (63). In dendritic cells TRAF6, but not TRAF2/3, play a role in the secretion of IL-12 p40 (64).

CD40 signaling is also critical to many aspects of T cell priming as well. In the absence of CD40L, mice fail to develop experimental autoimmune encephalomyelitis when primed with transgenic $CD4^+$ T cells and MOG peptide. However, the response

can be rescued by the adoptive transfer of CD80 expressing dendritic cells (65). In some models, such as with LCMV infection, CD40L is not required for the primary cytotoxic lymphocyte response or viral clearance (66). However, there is evidence that the CD40 pathway plays a critical role in delivering 'help' during CD4 dependent $CD8^+$ T cell responses. In this situation, CD40 ligation appears to be necessary to fully activate antigen presenting cells which are then responsible for priming an effect $CD8^+$ response (67).

*Clinical Methods of Immunosuppression*

Modern pharmaceutical based immunosuppression had its origins in 1949, when cortisone was shown to alleviate some aspects of rheumatoid arthritis. Corticosteroids have since become an established broad spectrum immunosuppressant to treat other autoimmune disorders and in the prevention of allograft rejection (68). In 1959, cyclophosphamide was demonstrated to suppress antibody formation and began use in bone marrow transplantation (69). In the same year, 6-mercaptopurine was reported to suppress the immune response in rabbits, albeit with significant toxicity. In 1963, a less toxic derivative, azathioprine, was developed which when used in concert with corticosteroids would allowed for advances in experimental heart and liver transplants (70). In 1969, methotrexate was shown to inhibit both antibody formation and the development of delayed hypersensitivity in guinea pigs (71).

It would be the discovery of cyclosporine in 1971 that would begin a new era for transplantation. Cyclosporine was the first immunosuppressive agent that allowed the regulation of T cells with acceptable toxicity. Cyclosporine was originally investigated

as an antibiotic, but its spectrum was found to be too narrow for clinical use. As an immunosuppressant, cyclosporine acts by binding to the cytosolic protein cyclophilin and inhibits the action of calcineurin, which then inhibits the production of IL-2 (70). With its clinical introduction in 1983, patient morbidity fell and it became possible to transplant organs with a one-year success 20% higher than previously possible. Organ transplants which had previously been only experimental, such as heart, liver and lung, were now possible (72).

Several agents would be developed in the ensuing years. In 1982, mycophenolate mofetil, an inosine 5'-monophosphate dehydrogenase inhibitor, was developed and showed very little nephrotoxicity compared to previous drugs (73). In 1987 tacrolimus, another calcineurin inhibitor with a similar mode of action as cyclosporine was also shown to inhibit IL-2 production and lymphocyte proliferation (74). Serendipitously discovered from soil bacteria on the Easter Islands, rapamycin is similar to the calcineurin inhibitors in that both inhibit IL-2 (75). However, rapamycin acts by blocking the mammalian target of rapamycin (mTOR) pathway downstream of IL-2 signaling (76).

Although these drugs have offered physicians a list of agents with which to stave off graft rejection, for the patient they are not without shortcomings. Non-specific broad-spectrum immunosuppression potentates the incidence of both opportunistic infections and malignancies (77, 78). There are also side effects such as hypertension, dyslipidemia, hyperglycemia, peptic ulcers, neurological dysfunction, as well as direct organ toxicity (79, 80). Some of these problems can be managed by careful selection of the drug regime or by decreasing the dosage when appropriate, such as during latent viral

reactivation. In order to maximize patient outcomes and achieve true long-term graft acceptance, novel strategies must be pursued which allow for targeted immune suppression against donor alloantigens, while leaving the remainder of the immune system intact.

*Development of and Prospects for Costimulatory Blockade*

Lafferty and Cunningham put forward the two signal hypothesis for T cell activation in 1974 (81). Since that time, signal 1 has been described as the presentation of the peptide-MHC complex to the TCR and signal 2 as a variety of costimulatory signals. The potential for interrupting costimulation has garnered considerable research interest as appreciation has grown of the vital role it plays in tuning T cell activation.

Initial murine studies demonstrated that long-term graft survival could be achieved by inhibition of the CD28 pathway (82, 83). Blockade of the CD28 pathway is commonly achieved using the recombinant fusion protein CTLA4-Ig, which consists of the extracellular domain of the CTLA4 molecule on an $IgG_1$ backbone (83, 84). It acts as a competitive inhibitor, as CTLA4 binds with a higher affinity to CD80 and CD86 than does CD28 (85). It is believed to primarily effect naïve T cells based on the importance of CD28 in the production of IL-2 and the initiation of T cell activation (86). Studies using the human form of CTLA4-Ig, known as abatacept, showed an encouraging safety profile, but was not significantly efficacious in non-human primate transplant models (87). Abatacept has been applied clinically to treat psoriasis and rheumatoid arthritis (88, 89).

Efforts were undertaken to design a more effective CD28 blocking reagent for use in transplantation (90). It had been shown that CD86 preferentially binds to CD28 while CD80 predominantly interacts with CTLA4 (91, 92). Development thus focused on manipulating abatacept's contact residues to create variants with higher binding affinity with particular emphasis on CD86 (90). Extensive screening resulted in the molecule belatacept, which had a fourfold greater affinity for CD86 and a twofold greater affinity for CD80. Belatacept demonstrated equivalent efficacy and a better side-effects profile when compared to cyclosporine-based treatment in a Phase II clinical study in human recipients of renal allografts (93). Phase III clinical trials are currently underway.

Blockade of the CD40 signaling pathway using anti-CD154 monoclonal antibodies have shown remarkable results in murine models of allograft survival (94). Anti-CD154 has also been shown to prevent acute rejection and promote long-term allograft acceptance in non-human primates (95, 96). Unfortunately, these initial encouraging results in animal models did not predict an easy translation to human application.

A consistent theme emerged when anti-CD154 strategies were applied to clinical testing. hu5C8 was the first of the anti-CD154 agents to be tested in human clinical trials following encouraging animal testing. It allowed for significant prolongation of kidney allografts, but unexpected thromboembolic events emerged (97, 98). The monoclonal antibody IDEC-131 used in combination with rapamycin and donor specific transfusion was successful in prolonging skin graft survival and inducing operational tolerance in a primate renal allotransplant model (99, 100). However thromboembolic events surfaced in patients (101). Another anti-CD154 antibody, ABI793, also prolonged renal allograft

survival in nonhuman primates, however, thromboembolic complications were again present and chronic allograft nephropathy developed following secession of treatment (102). Studies combining the anti-CD154 mAb H106 with belatacept in conjunction with rapamycin and bone marrow transplant significantly improved the length of chimerism in a nonhuman primate models and was notable for the apparent absence of thromboembolism, although no studies of subclinical thrombosis was performed (103). Due to these serious complications, commercial development of anti-CD154 for use in autoimmune disease and transplantation was halted.

It has since been appreciated that CD154 plays an intimate role in coagulation. Platelets serve as a major source of both cell-bound and soluble CD154 (sCD154) in the blood and are involved in thrombi formation and stabilization as well as inflammation. Thrombin-activated platelets rapidly up-regulate CD154 which can then interact with CD40 on endothelial cells, facilitating chemotaxis and the expression of adhesion molecules including E-selectin, ICAM and VCAM (104). Others studies suggest that sCD154 can activate platelets directly through CD40 ligation, although these effects appear to be minimal (105). CD154 can act to stabilize thrombi by an integrin dependant mechanisms, independent of CD40 (53). Furthermore, CD154 deficient mice are characterized as having unstable thrombi, whereas this defect is not seen in CD40 deficient mice (106).

Although complications from clinical use of anti-CD154 have proven unacceptable, recognizing that the thromboembolic side effects occur in a CD40-independent manner suggest a possible alternative. Three anti-CD40 antibodies in particular, 4D11, ch5D12 and Chi220, have been explored in nonhuman primate

transplant models with some measure of success (107-109). 4D11 and Ch5D12 are non-depleting, antagonist antibodies that blocks CD154 binding (110, 111). Both were effective in prolonging renal allograft survival in nonhuman primates, but long-term acceptance and immune tolerance was not absolute (107, 112). Chi220 is a weak agonist and depletes B cells in addition to its ability to block ligand binding (109). The importance of the partial agonist activity in prolonging graft survival has yet to be determined, but it is hypothesized that the weak proliferative signals may promote susceptibility to anergy, death or other immune modulation. When used as monotherapy, Chi220 is capable of moderate prolongation of nonhuman primate kidney engraftment, independent of its B cell depleting effects (109). When paired with belatacept in nonhuman primates, Chi220 was found to facilitate allogeneic islet survival (113). Although anti-CD40 monoclonal antibodies are most often associated with stimulating the immune system, this approach continues to be explored for its use in transplantation.

*Conclusions*

The field of transplantation has progressed considerable from its origins in mythological legend, to the established clinical therapeutic it is today. Significant challenges still remain. Chronic rejection rates have changed little in the past decades, anti-rejection drugs have created a population of immunosuppressed individuals, and the very agents that prolong graft acceptance also contribute to graft failure. Investigations into the utility of costimulation blockade have established an enormous potential for success. Although the clinical translation of anti-CD154 has not proceeded as productively as seen with CTLA4-Ig, it is clear that modulation of the CD40/CD154

pathway can play a crucial role in the induction of transplant tolerance. The current challenge is to understand the mechanisms by which these strategies both succeed and fail so that long-term tolerance may become a reality.

# Chapter 2

## Equivalent Efficacies Between Anti-CD40 and Anti-CD154 Monoclonal Antibodies for use in Costimulation Blockade

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## Abstract

Although inhibiting the CD40/CD154 T cell costimulatory signaling pathway through the use of anti-CD154 blocking antibodies is effective in murine models, thromboembolic side effects observed in human clinical trials have raised significant questions regarding the safety of this approach. Nevertheless, blockade of the CD40 signaling pathway has shown compelling utility, thus, appropriately designed anti-CD40 antibodies may provide a suitable alternative. We investigated a novel monoclonal rat anti-murine CD40 antibody, 7E1-G2b, for characteristics mirroring anti-CD154. 7E1-G2b was found to be as effective as anti-CD154 when used in concert with CTLA4-Ig in promoting both allogeneic skin graft survival and hematopoietic bone marrow chimerism. These effects were not due to the depletion of CD40 bearing antigen presenting cells or inhibitory signals delivered by Fc receptors. These data show that an anti-CD40 antibody can promote graft survival as well as anti-CD154 treatment, making 7E1-G2b an attractive substitute in murine models of costimulation blockade-based tolerance regimens.

## Introduction

According to the two signal hypothesis, generating an effective T cell response requires signals delivered via T cell receptor engagement, known as signal 1, as well as accessory signals in the form of costimulatory molecules, known as signal 2 (114). When signal 1 is delivered in the absence of signal 2, T cell activation can be severely altered, resulting in anergy, apoptosis, abortive proliferation, or immunoregulation (10, 115, 116). The potential of harnessing this phenomenon through the use of agents that interrupt these signaling events has captivated the interest of the transplant community since its inception. Several signaling pathways have been targeted for blockade with varying success including ICOS, OX40, 4-1BB and CD70 (117-120). In particular, inhibition of the CD28 and CD40 costimulatory pathways have shown impressive synergy in murine models for the prevention of allograft rejection (121). Clinical translation of CTLA4-Ig led to the development of LEA29Y (belatacept), a second generation CD28 blocker which has proved efficacious in non-human primate models (90), paving the way for its use in human clinical trials of kidney transplantation (93).

Blockade of the CD40 signaling pathway initially showed great promise in non-human primates (96, 122). However, these early animal studies did not envisage the difficulties that would later arise. Clinical trials using anti-CD154 monoclonal antibodies would be halted following alarming evidence of unanticipated thromboembolic side effects (102, 123, 124). It is now appreciated that CD154 expressed by platelets, as well as in soluble form, is intimately involved in the formation and stabilization of thrombi (53, 105). Although these side effects have not been observed in all anti-CD154 reagents

and dosing strategies, (101, 123, 124), the potential for serious complications must nonetheless be of consideration. To continue exploring the inhibition of the CD40 pathway as a therapeutic modality, alternative means must be sought which avoid these complications.

Investigations outside of the field of transplantation using anti-CD40 mAb have generally focused on their agonistic properties in order to augment immune responses to thymus-independent antigens or to boost anti-tumor and anti-virus activity (125-129). There are reports of successful immune attenuation achieved in non-human primate models employing anti-CD40 monoclonal antibodies (107, 110, 112). In particular, the chimeric anti-human CD40 mAb Chi220 displayed impressive synergy when combined with LEA29Y for promoting islet allograft survival in non-human primates (113).

Herein, we describe a novel murine analogue of Chi220, the anti-CD40 monoclonal antibody 7E1-G2b. When used in concert with CTLA4-Ig, 7E1-G2b was as effective as anti-CD154 in promoting skin graft survival and bone marrow chimerism. The mechanisms behind anti-CD40 mediated suppression in comparison to anti-CD154 mediated suppression remains unclear, although depletion of CD40 expressing cells and inhibitory Fc receptors were not found to be prominently involved.

## Methods

*Ab generation*

A natural isotype switch variant of the rat anti-mouse CD40 antibody clone 7E1-G1 was generated by the sib-selection technique (130). Briefly, anti-CD40 monoclonal antibodies of the $IgG_{2b}$ isotype were identified by ELISA among supernatants of 96 well plates that had been seeded at 1000 cells/well with the original 7E1-G1 hybridoma. Subsequent rounds of plating and identification of $IgG_{2b}$ positive wells at seeding densities of 200 and then 20 cells/well followed by two rounds of cloning by limiting dilution led to the isolation of the clonal $IgG_{2b}$ switch variant, 7E1-G2b (Bristol-Myers Squibb, New York, NY).

*Mice*

Adult male 6- to 8-wk-old C57BL/6, BALB/c, and C3H/HeJ were obtained from The Jackson Laboratory (Bar Harbor, ME). Act-mOVA mice were generously provided by M. Jenkins (University of Minnesota, Minneapolis, MN). B6.129S4-*Fcgr2b*$^{tm1TtK}$ N12 mice were obtained from Taconic Farms (Germantown, NY). Animals received humane care in pathogen-free housing conditions in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

*Skin grafting*

Full thickness ear and tail skin grafts (~1$cm^2$) were transplanted onto the dorsal thorax of recipient mice and secured with a plastic adhesive bandage for 6 days. Recipients

received 500μg i.p. of CTLA4-Ig (Bristol-Myers Squibb, New York, NY) and either 7E1-G2b, rat $IgG_{2b}$ isotype control, or anti-CD154 (MR1: BioXCell, West Lebanon, NH) on days 0, 2, 4, and 6. Graft survival was monitored by daily visual inspection and rejection was defined as the complete loss of viable epidermal tissue.

*Bone marrow chimerism*

Donor bone marrow was flushed from tibiae and femora then resuspended at $2x10^7$cells/500μL in sterile saline. Recipient mice were transfused on days 0 and 6 with donor marrow i.v, along with a single 500mg dose of busulfan on day 5. On days 0, 2, 4, and 6, 500μg i.p. of CTLA4-Ig and either 7E1-G2b, rat IgG isotype control or hamster anti-mouse CD154 mAb.

*Flow cytometry for phenotype and absolute number*

At the indicated time points, animals were sacrificed, spleens harvested and single cell suspension prepared in phosphate buffered saline supplemented with 0.5% bovine serum albumin and 1 mM EDTA. Unless previously stated, antibodies obtained from BD Pharmingen (San Jose, CA) include the following: anti- $V_\beta$5-PE, $V_\beta$8-PE, $V_\beta$11-PE, CD3-APC, -Pacific Blue, CD4-APC, -Pacific Blue, CD8-FITC, -PerCP, CD11b-PerCP-Cy5.5, CD11c-APC, CD19-APC, -FITC, CD40-PE, and CD45R-PerCP-Cy5.5. Antibodies obtained from Invitrogen (Carlsbad, CA) include: Streptavidin-Pacific Orange, anti- CD8-Pacific Orange. Antibodies obtained from eBioscience (San Diego, CA) include anti-CD19-Pacific Blue. When noted, absolute numbers of splenocyte subsets were determined by TruCount Bead Analysis according to the manufacturer's

instructions (BD Biosciences). Flow cytometric data was analyzed using FlowJo Software (Treestar, Ashland, OR).

*Statistical analysis*

Skin graft rejection was represented by Kaplan-Meier survival curves. Statistical comparisons of mean survival times were performed using nonparametric one-way ANOVA (Kruskal-Wallis test), followed by Dunn's multiple comparisons posttest of the relevant datasets. Additional statistical analysis was performed using two-tailed unpaired Students t-test. (GraphPad Software, La Jolla, CA)

## Results

*Anti-CD40 treatment combined with CTLA4-Ig promotes allogeneic skin graft survival.*

Previous studies from our laboratory and others have demonstrated the efficacy of costimulatory blockade-based protocols for promoting murine skin graft survival (121, 131-133). Historically, these have involved the use of an anti-CD154 binding antibody, paired with the CD28 pathway-blocking reagent CTLA4-Ig. Here we sought to determine whether an anti-CD40 antibody could function as an effective substitute for anti-CD154 in a costimulation blockade-based tolerance induction regimen. For the first experimental setup, groups of C57BL/6 mice were grafted with allogeneic BALB/c skin and treated with CTLA4-Ig and either 7E1-G2b, anti-CD154, or rat $IgG_{2b}$ isotype control i.p. on days 0, 2, 4, and 6. Recipients treated with either 7E1-G2b or anti-CD154 show a modest prolongation in skin graft survival (MST = 27 and 23 days, respectively), compared to isotype control and untreated animals (MST = 13 and 11 days, respectively), indicative of the 'costimulation blockade resistant' phenotype (134) seen with this particular strain combination of donor and recipient (Figure 2.1a). In the more permissive BALB/c to C3H to model (135), graft survival was also prolonged, for a greater duration than the previous strain combination, with 7E1-G2b or anti-CD154 treatment (MST > 75 days), as compared to isotype control treatment (MST = 19 days) (Figure 2.1b).

*Anti-CD40 treatment combined with CTLA4-Ig promotes bone marrow chimerism.*

In the second experimental setup, C57BL/6 mice received allogeneic BALB/c

bone marrow ($2x10^7$cells i.v. on days 0 and 6) and BALB/c skin grafts on day 0, as well as busulfan, an alkylating agent toxic to hematopoietic stem cells, on day 5. Recipient mice were given CTLA4-Ig and either 7E1-G2b, anti-CD154, or rat IgG isotype control i.p. on days 0, 2, 4, and 6. Peripheral blood was then sampled for the presence of donor-derived B cells and T cells. The percentage of donor $B220^+$ B cells (Figure 2.2a) and $CD4^+$ T cells (Figure 2.2b) reached similar levels in 7E1-G2b and anti-CD154 treated groups, while isotype treated animals failed to sustain donor chimerism.

As a surrogate marker of central tolerance, we tracked the frequency of donor reactive $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells in these chimeric recipients. T cells possessing these Vβ segments are normally deleted in the thymus of BALB/c mice due to their high affinity for endogenous retroviral superantigens (mouse mammary tumor viruses, MMTVs) presented by I-E MHC molecules. Wild-type C57BL/6 mice do not express I-E, thus $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells constitute roughly 4-5% and 2-3% of the peripheral $CD4^+$ T cell compartment, respectively. The thymic deletion of these specific Vβ expressing T cells in our chimeric mice can serve as a measure of successful donor tolerance (136). As shown on day 58, the percentage of $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells in the isotype (black bar) treated group was consistent with wild-type C57BL/6 levels (Figure 2.2c). In contrast, 7E1-G2b (striped bar) and anti-CD154 (white bar) treated groups show a significant loss of $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells from the periphery. Vβ deletion was shown to be specific as the use of $V\beta8.1/8.2^+$ by $CD4^+$ T cells remained comparable in all groups. The successful induction of central tolerance directly related to peripheral tolerance, as both 7E1-G2b and anti-CD154 treated recipients had prolonged graft survival (MST > 75 days) not seen with isotype treatment

(MST = 16 days) (Figure 2.2d).

*The mechanism of anti-CD40's efficacy does not depend on the depletion of CD40 expressing cells.*

We hypothesized that 7E1-G2b may act by depleting CD40-expressing cells, resulting in a dearth remaining with which to mediate graft rejection. This has been proposed as a possible mechanism for anti-CD154 (137) that would potentially selectively impact recently activated T cells. However, the CD40 molecule has a much broader expression range. The majority of professional antigen presenting cells such as macrophage, dendritic cells and B cells are all positive for CD40 (Figure 2.3a). We found that less than ten percent of $CD4^+$ and $CD8^+$ T cells express CD40, fifteen percent of granulocytes and forty percent of natural killer cells. Although a small percentage of T cells express CD40, the density present on the cell surface is greater than that seen for B cells (Figure 2.3b). The highest density can be found by natural killer cells.

To determine if these CD40 expressing cells were depleted by anti-CD40 treatment, groups of mice were injected i.p. on days 0, 2, 4, and 6 with either 7E1-G2b, isotype matched control antibody, FGK4.5 or left untreated. On day 7, mice were sacrificed and the absolute numbers of each cell subset were enumerated from the spleen by TruCount analysis. We found that treatment with 7E1-G2b did not lead to a significant change in the numbers of T cells, granulocytes, natural killer cells, macrophage, dendritic cells or B cells as compared to isotype control or untreated animals (Figure 2.4). Mice treated with FGK4.5 showed a two-fold increase in $CD4^+$ T cells, three-fold increase in $CD8^+$ T cells, granulocytes and macrophage, a four-fold

increase in dendritic cells and a six-fold increase in B cells. Interestingly, there was no significant difference in the numbers of natural killer cells, in spite of the fact that these cells showed the highest surface density of CD40.

*The involvement of inhibitory Fc receptors cannot fully account for the beneficial effects of 7E1-G2b treatment on skin graft survival.*

Another possible mechanisms for the efficacy of 7E1-G2b may involve Fc receptors. The ligation of 7E1-G2b by inhibitory Fc receptors could be necessary for the antibody's prolongation of graft survival. To test hypothesis, we made use of a different model of skin graft rejection than previously presented herein. As we have previously shown, untreated C57BL/6 mice reject a BALB/c skin graft aggressively, with a mean survival time of around 11 days, while costimulation blockade modestly extends survival to around 25 days (Figure 2.1a). To investigate potentially subtle influences by Fc receptors, we used a single minor mismatched skin graft rejection model. The mOVA-C57BL/6 mouse constitutively expresses full length, membrane-bound chicken ovalbumin driven by the β-actin promoter (138). When used as skin graft donors for untreated C57BL/6 recipients, these single mismatched grafts are rejected with an MST of 18 days (Figure 2.5a). Costimulation blockade consisting of 7E1-G2b or anti-CD154 along with CTLA4-Ig extends graft survival indefinitely (MST > 200 days). Isotype matched control antibody and CTLA4-Ig are able to extend graft survival for > 100 days in about half of the recipients tested, highlighting the significant effect of blocking the CD28 pathway in this model.

To test the hypothesis that inhibitory Fc receptors are necessary for the

immunosuppressive effects seen for 7E1-G2b, we obtained FcγRIIb deficient mice, which lack any expression of inhibitory Fc receptors (139). Following the same protocol outline above, we observed no difference in long-term graft survival between FcγRIIb deficient 7E1-G2b treated animals as compared to similarly treated wild type recipients (MST > 75 days) (Figure 2.5b). Interestingly, the 50% long-term graft survival that was observed in wild-type recipients of mOVA skin grafts treated with CTLA4-Ig alone was abrogated in the FcγRIIb deficient hosts, as all grafts were promptly rejected (MST = 20 days).

## Discussion

Although effective in murine models, anti-CD154 antibodies tested in human clinical trials lead to thromboembolic side effects, due to the expression of CD154 on platelets and its involvement in the formation and stabilization of thrombi (53, 102, 105, 123, 124). As a result, enthusiasm for targeting this molecule has cooled considerably. However, interrupting the CD40/CD154 signaling pathway remains a potent means for inhibiting rejection and an attractive, albeit onerous, therapeutic target. Herein, we investigated a novel murine anti-CD40 antibody, 7E1-G2b, for characteristics mirroring anti-CD154 blockade. When used in concert with CTLA4-Ig, 7E1-G2b effectively promoted skin graft survival and allogeneic bone marrow chimerism, similar to what was seen for anti-CD154. Although a wide range of immune cells expresses CD40, treatment with the anti-CD40 antibody did not lead to the depletion of CD40 bearing cells. Furthermore, the involvement of inhibitory Fc receptors was not found to play a dominant role in the antibody's efficacy.

These results further demonstrate the therapeutic potential of an anti-CD40 blocking agent. Although the exact mechanism by which the therapeutic effect is achieved through blocking either CD40 or CD154 is most likely quite different, we have shown that the outcome can be very similar. It is currently debatable how anti-CD154 exerts its immunosuppressive effects. It has been postulated that antibodies bound to CD154 serve to deplete activated T cells (137) or may induce reciprocal negative signaling upon the T cell itself (140). Interrupting CD40 signals to antigen presenting cells during T cell priming has been shown to have profound impacts on the dynamics of

T cell-APC interactions. Among these include limiting the up-regulation of activation markers such as CD80 and CD86 (141), decreasing the production of proinflammatory cytokines such as IL-8, IL-12, MIP-1α and TNFα (142, 143) as well as shortening the duration of the cognate T cell-APC interaction (144). These effects divert the T cell from an effector phenotype and towards an alternative program of anergy (145), abortive proliferation (146), apoptosis (147) or a regulatory cell phenotype (148). Whether these same mechanisms are at play when 7E1-G2b is used as a blocking agent have yet to be determine.

Among the murine IgG subclasses, which share a high degree of homology with rats (149), $IgG_{2b}$ is generally considered to be a potent mediator of immune responses based on its ability to fix complement and capacity to promote antibody dependent cellular cytotoxicity (150, 151). One might predict that an anti-CD40 antibody would be an efficient depleting agent, however, we have no evidence to support the depletion of CD40 expressing cell subsets. Studies investigating the efficacy of Chi220, a primate analog of 7E1-G2b, do report a significant depletion of B cells in non-human primates (109). However, in agreement with the conclusion for Chi220, our findings suggest that immune suppression can be achieved without a necessity for depletion. An important caveat to the current study is that many of the characterizations were examined in the absence of an ongoing alloresponse and inflammation. A multitude of factors are modulated during ongoing immune responses, so it is possible that the activity of 7E1-G2b may vary depending on the inflammatory milieu.

Antibodies can cross-link Fc receptors present on antigen presenting cells, providing signals to alert the presence of opsonized bacteria or antibody coated cells in

need of clearance, as well as to down-regulate B cell responses (139). However, in our system Fc receptors were not closely involved. Inhibitory signals through FcγRIIb were not required for the immunosuppressive effects seen for 7E1-G2b, as graft survival was unchanged between FcγRIIb deficient and wild type animals.

We have demonstrated that CD28 blockade can effectively synergize with CD40 blockade using either anti-CD154 or anti-CD40 monoclonal antibody. Although clinical translation of anti-CD154 has encountered difficulties, this should not belie the clear utility that can be gained by further studying the inhibition of the CD40 pathway in transplantation. Interrupting the CD40/CD154 costimulatory pathway alters a myriad of signal transduction cascades. Understanding which of these events are critical for the immunosuppressive effects would provide even greater specificity for the development of novel therapeutics.

## Figure Legends

**Figure 2.1. Anti-CD40 blocking antibody treatment promotes skin graft survival equivalent to anti-CD154 when used in concert with CTLA4-Ig.** C57BL/6 mice were treated with 500μg of CTLA4-Ig and either 7E1-G2b, anti-CD154, or rat isotype IgG control, i.p. on days 0, 2, 4, and 6. Recipients treated with CTLA4-Ig and either 7E1-G2b or anti-CD154 show a modest prolongation of skin graft survival in (A) the robust BALB/c to C57BL/6 model, and long-term survival in (B) the more permissive BALB/c to C3H model.

**Figure 2.2. Anti-CD40 blocking antibody treatment promotes allogeneic bone marrow chimerism equivalent to anti-CD154 when used in concert with CTLA4-Ig.** C57BL/6 mice were treated with 500μg of CTLA4-Ig and either 7E1-G2b, anti-CD154, or rat isotype IgG control, i.p. on days 0, 2, 4, and 6. Busulfan was given i.v. on day 5, 600mg per mouse, along with allogeneic BALB/c bone marrow, $2x10^7$ cells i.v. on days 0 and 6, and skin graft on day 0. The percentage of donor (A) $B220^+$ B cells and (B) $CD4^+$ T cells reached similar levels in 7E1-G2b and anti-CD154 treated groups, while isotype treated animals failed to sustain chimerism. (C) The percentage of $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells in the isotype (black bar) treated group was consistent with wild-type B6 levels. In contrast, $CD4^+$ T cells from 7E1-G2b (striped bar) and anti-CD154 (white bar) treated groups show significant deletion of $V\beta11^+$ and $V\beta5.1/5.2^+$. Vβ deletion is shown to be specific as the use of $V\beta8.1/8.2^+$ by $CD4^+$ T cells remained comparable in all groups. Data is shown from day 58-post transplant. (D) Chimeric mice are tolerant to

donor skin grafts, as both 7E1-G2b and anti-CD154 treated recipients had prolonged graft survival not seen for the isotype treated group. (∗ p value <0.001)

**Figure 2.3. CD40 expression is not restricted to professional antigen presenting cells.** Single cell suspensions of splenocytes from naïve C57BL/6 mice were stained for each noted cell subset and gated for expression of CD40. (A) Representative flow cytometry plots showing the percentage of CD40 positive cells. (B) Summary of the percentage of CD40 positive cells and the corresponding mean fluorescent intensity. The data represent three mice per group.

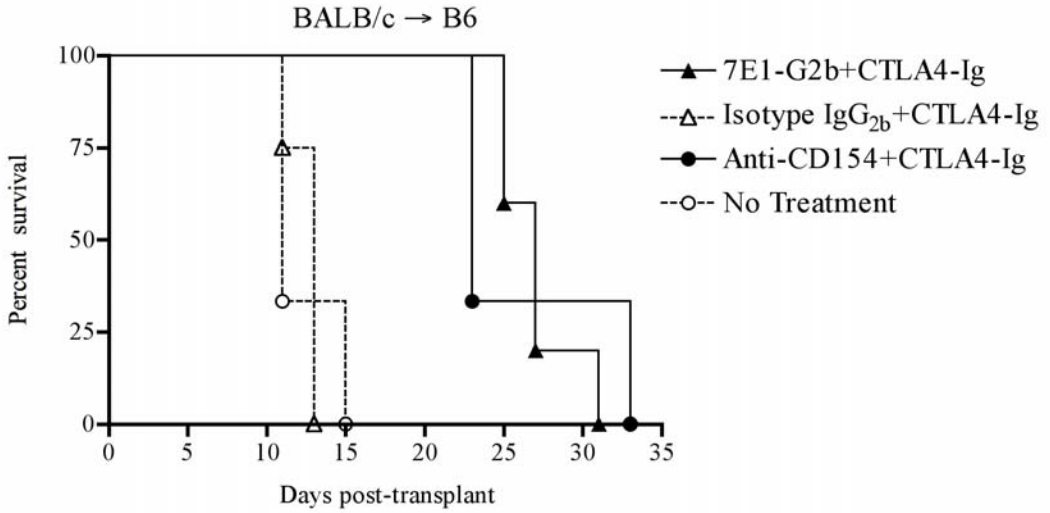
**Figure 2.4. 7E1-G2b treatment does not lead to depletion of CD40 expressing cell subsets *in vivo*.** C57BL/6 mice were injected on days 0, 2, 4, and 6 with 500μg of either 7E1-G2b, isotype $IgG_{2b}$ control, FGK4.5, or left untreated. The absolute number of splenic cell subsets was enumerated by TruCount analysis on day 7. No significant differences were noted for any cell subsets between 7E1-G2b and isotype control. FGK4.5 treatment leads to a significant increase in the total number of all subsets except for natural killer cells. (∗ p value <0.05, ∗∗ p value <0.001)

**Figure 2.5. The inhibitory Fc-receptors cannot fully account for the immunosuppressive effects of 7E1-G2b.** (A) Wild-type C57BL/6 mice were transplanted with minor antigen mismatched mOVA x B6 skin grafts and treated with 500μg of CTLA4-Ig and either 7E1-G2b, isotype $IgG_{2b}$ control, or anti-CD154 i.p on days 0, 2, 4, and 6. (B) FcγRIIb deficient recipient mice, lacking the inhibitory Fc

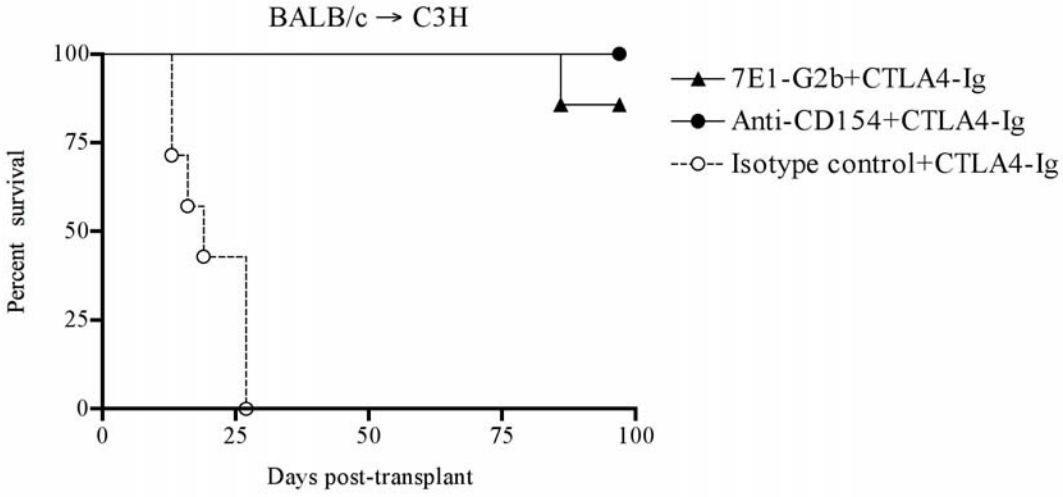
receptor, have no diminution of long-term graft survival for 7E1-G2b treated mice, while all other treatment groups promptly reject their grafts.

# Figure 2.1

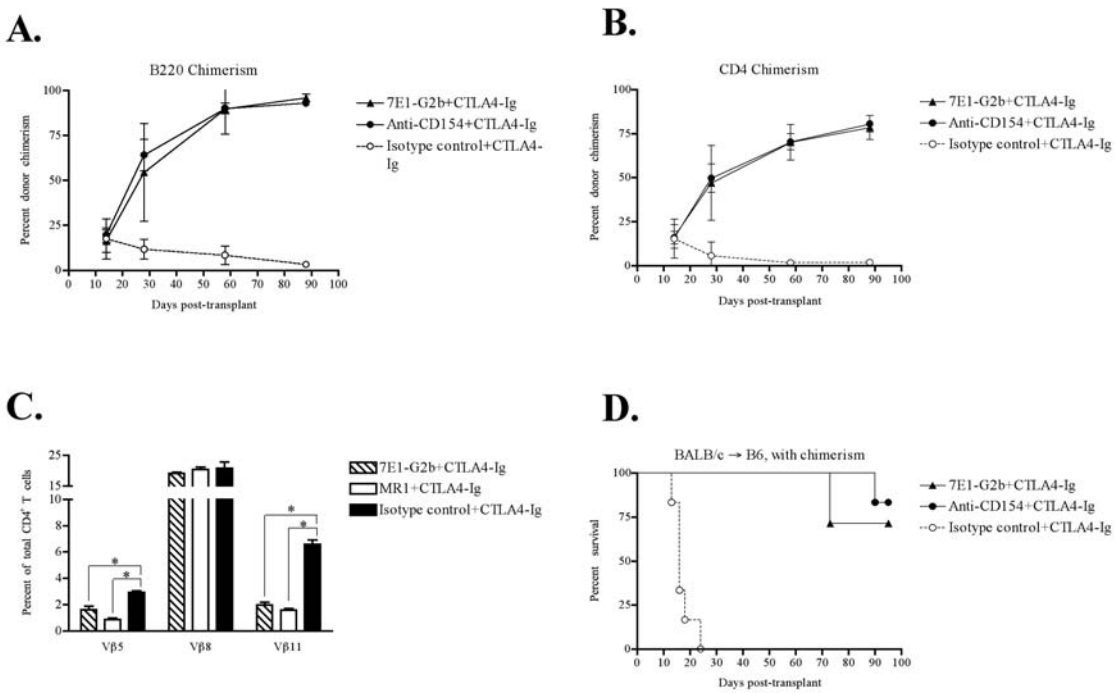
**A.**


BALB/c → B6
Percent survival
100
75
50
25
0
0
5
10
15
20
25
30
35
Days post-transplant
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
No Treatment

**B.**

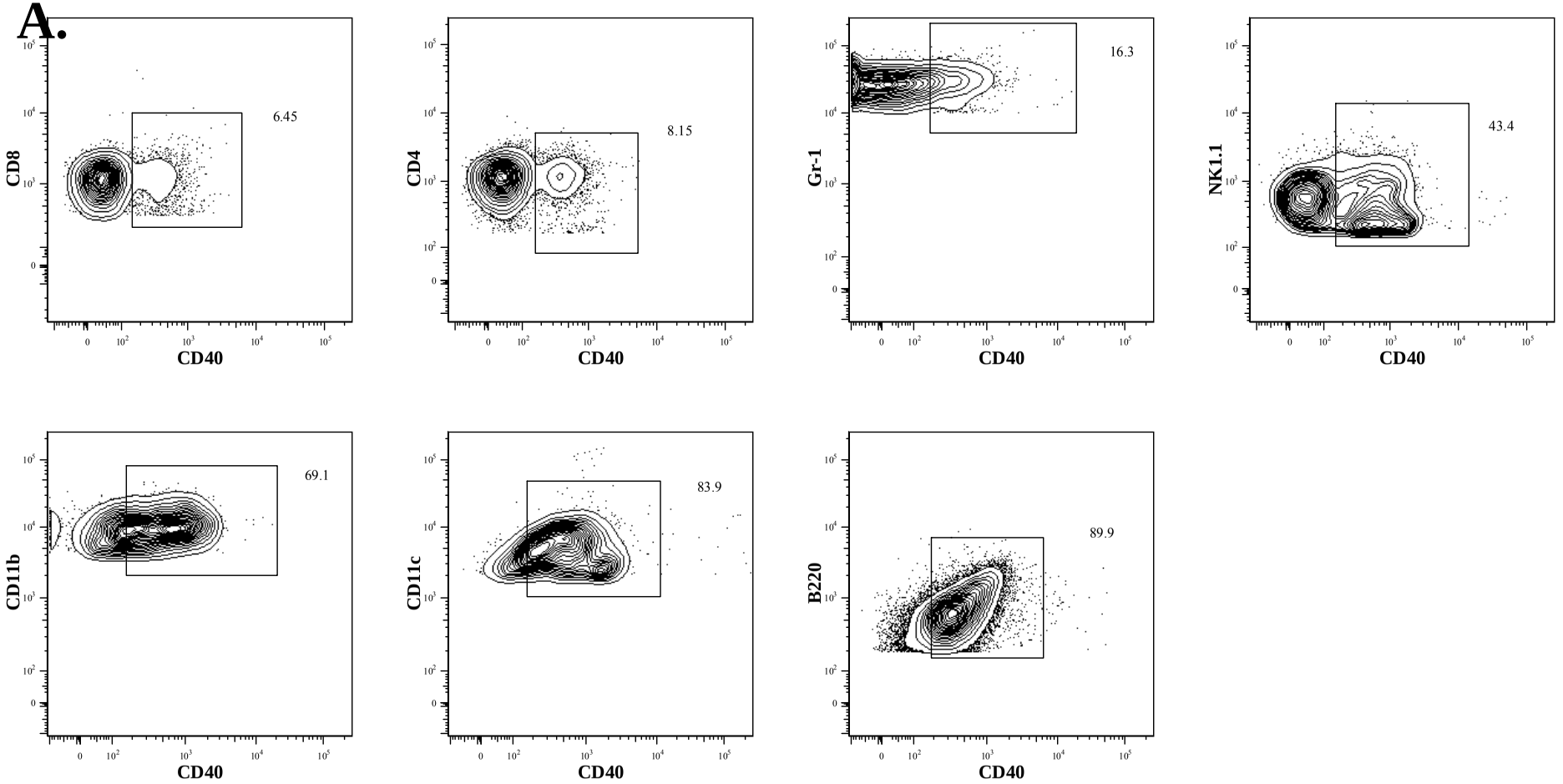

BALB/c → C3H
Percent survival
100
75
50
25
0
0
25
50
75
100
Days post-transplant
7E1-G2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
Isotype control+CTLA4-Ig

# Figure 2.2


A.
B220 Chimerism
Percent donor chimerism
Days post-transplant
7E1-G2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
Isotype control+CTLA4-Ig
B.
CD4 Chimerism
Percent donor chimerism
Days post-transplant
7E1-G2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
Isotype control+CTLA4-Ig
C.
Percent of total CD4+ T cells
Vβ5
Vβ8
Vβ11
7E1-G2b+CTLA4-Ig
MR1+CTLA4-Ig
Isotype control+CTLA4-Ig
D.
BALB/c → B6, with chimerism
Percent survival
Days post-transplant
7E1-G2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
Isotype control+CTLA4-Ig

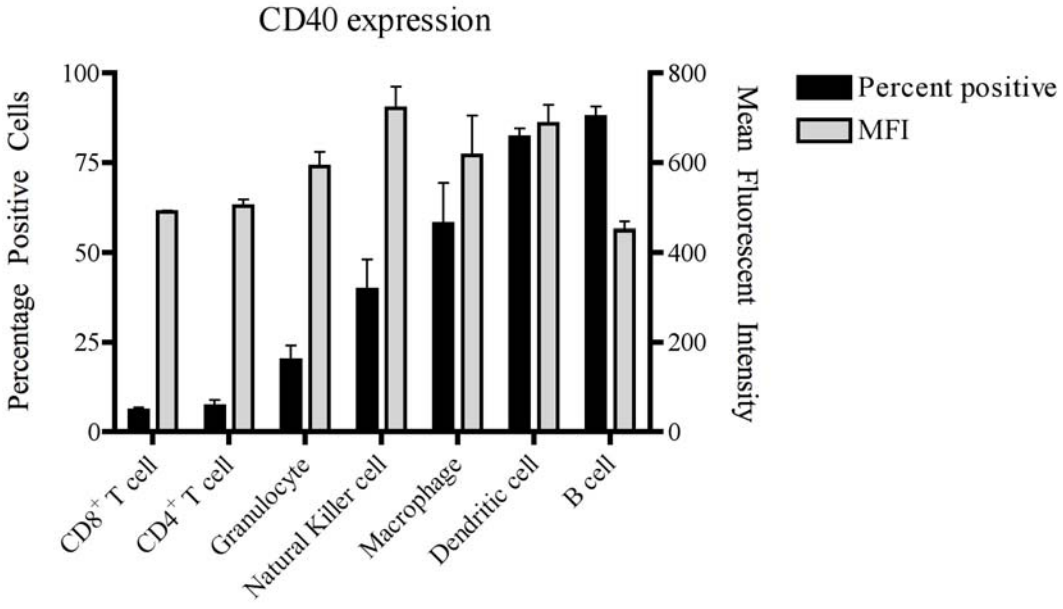
# Figure 2.3

A.

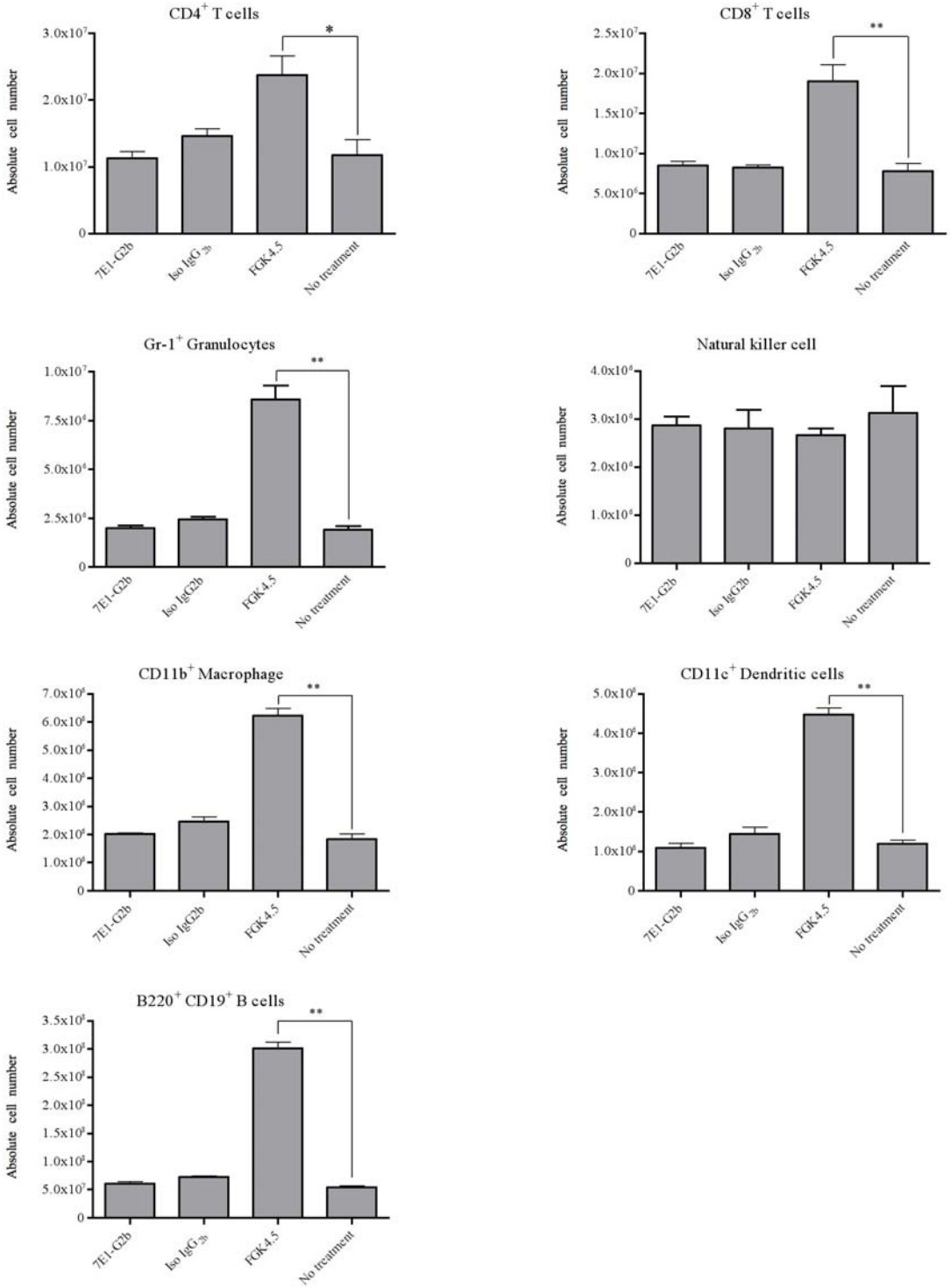

CD8
CD40
6.45
CD4
CD40
8.15
Gr-1
CD40
16.3
NK1.1
CD40
43.4
CD11b
CD40
69.1
CD11c
CD40
83.9
B220
CD40
89.9

# Figure 2.3

## B.

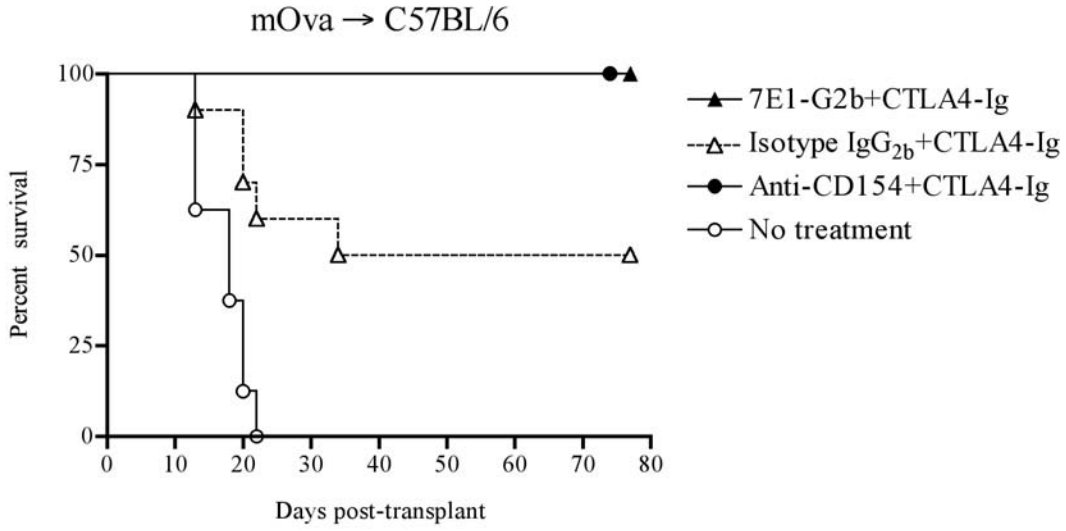

CD40 expression
Percentage Positive Cells
Mean Fluorescent Intensity
100
75
50
25
0
800
600
400
200
0
Percent positive
MFI
CD8+ T cell
CD4+ T cell
Granulocyte
Natural Killer cell
Macrophage
Dendritic cell
B cell

# Figure 2.4

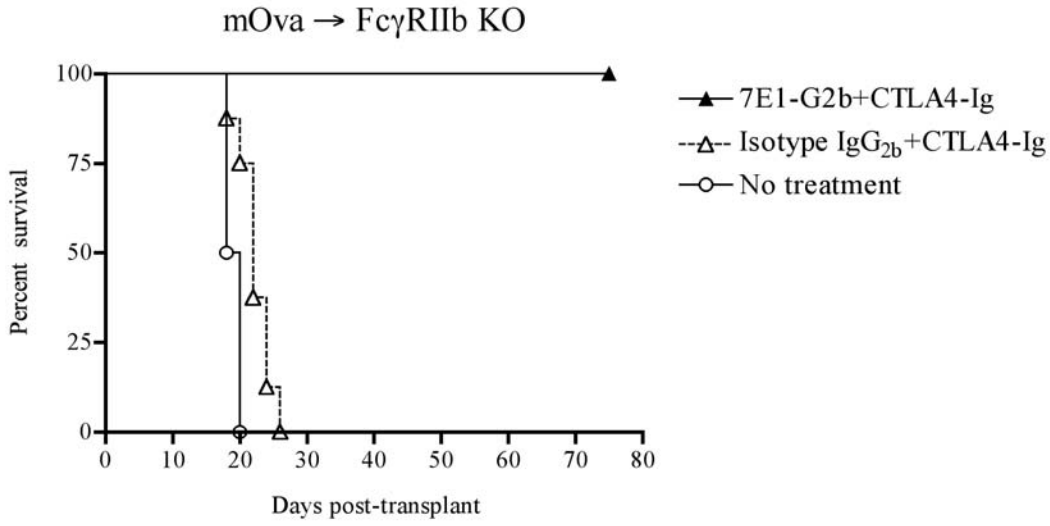

CD4+ T cells
CD8+ T cells
Gr-1+ Granulocytes
Natural killer cell
CD11b+ Macrophage
CD11c+ Dendritic cells
B220+ CD19+ B cells
Absolute cell number
7E1-G2b
Iso IgG2b
FGK4.5
No treatment
*
**

# Figure 2.5

## A.


mOva → C57BL/6
Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
70
80
Days post-transplant
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
Anti-CD154+CTLA4-Ig
No treatment

## B.


mOva → FcγRIIb KO
Percent survival
100
75
50
25
0
0
10
20
30
40
50
60
70
80
Days post-transplant
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
No treatment

# Chapter 3

# Divergent Functionality Between Anti-CD40 Monoclonal Antibody of $IgG_1$ and $IgG_{2b}$ Isotype in Blockade of the CD40 Costimulatory Pathway

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## Abstract

The utility of blocking anti-CD154 antibodies in humans has been hampered by the emergence of thromboembolic side effects during clinical trials. However, anti-CD40 blocking antibodies can provide an effective alternative method, while avoiding the involvement of coagulation. We compared two isoforms of a novel monoclonal rat anti-murine CD40 antibody, 7E1-G1, an $IgG_1$ isotype, and 7E1-G2b, an $IgG_{2b}$ isotype. 7E1-G2b was as effective when used in concert with CTLA4-Ig in promoting both allogeneic bone marrow chimerism and skin graft survival, while 7E1-G1 was not. The protection was not due to depletion of CD40 bearing antigen-presenting cells. Naïve mice injected with 7E1-G1, but not 7E1-G2b, show maturation of B cells and dendritic cells along with marked proliferation of B cells and T cells. Although B cells showed significant activation in 7E1-G1 treated mice and may have contributed to the overall stimulated immune environment, this may not have been sufficient for graft rejection as both wild-type and B cell deficient mice promptly rejected allogeneic skin grafts when treated with 7E1-G1 and CTLA4-Ig. *In vitro* proliferation assays to measure the agonist properties of the two 7E1 variants revealed similar weak responses when plate-bound. However, when added as a soluble stimulus, 7E1-G1, but not 7E1-G2b, led to strong proliferation, suggesting interactions mediated by the Fc region. Mice deficient in either the activating FcγRIII receptor, or the common Fc signaling chain, FcεRγ1, showed no difference in skin graft survival compared to wild type mice when treated with 7E1-G1 or 7E1-G2b and CTLA4-Ig. These data suggest that an appropriately designed anti-CD40 antibody, with specific attention paid to the isotype, can act as an antagonist CD40 blocking agent.

## Introduction

Long-term outcomes in transplantation have changed little despite dramatic reductions in acute rejection rates. This fact potentates the need to develop novel tolerance induction strategies and safer immunosuppressive modalities (3, 152). Given the central role of T cells in transplant rejection, a unifying goal of clinical tolerance induction is to selectively inactivate or delete donor-reactive T cells. Costimulation blockade strategies have the potential to meet both goals. In particular, blockade of the CD40 signaling pathway has shown great promise in murine and non-human primate models (121, 122). However, thromboembolic side effects observed when using anti-CD154 monoclonal antibodies in humans preclude its clinical use (53, 105).

To continue exploring the inhibition of the CD40 pathway as a therapeutic, anti-CD40 monoclonal antibodies have been considered as a possible alternative. Since its discovery in 1985, the CD40 molecule has been recognized for its central role in the control of T-dependant B cell responses (153). Cognate interactions between CD40 and CD154 are crucial to isotype switching and germinal center formation (154).

The first murine anti-CD40 monoclonal antibodies were described in 1994 (155). It was found that while some of these antibodies led to proliferation and activation of B cells, others did not. It was hypothesized that the position of antibody binding on CD40 relative to the natural ligand CD154 determined whether the antibody would function as an agonist or antagonist (155-157). In contrast, other studies have argued that antibody mediated CD40 signaling is epitope-independent (158). These data argue that receptor aggregation is the primary mechanism by which CD40 is activated by antibody ligation,

regardless of where the antibody binds.

The importance of the monoclonal antibody's isotype to its biological activity and how this influences its immunotherapeutic properties has long been appreciated (159-162). Extensive study of the various immunoglobulin isotypes has defined a hierarchy with regards to compliment fixation and Fc receptor binding. Murine and rat $IgG_{2b}$ isotypes, which share a high degree of both sequence and functional homology (151), are often described as being the potent mediators of cellular depletion due to their ability to fix compliment and high affinity for activating Fc receptors (163-165). In contrast, $IgG_1$ isotypes are characterized by a poor affinity for complement activation and a weak ability to bind Fc receptors (150, 151, 165).

Predicting how an antibody will function *in vivo* is confounded by more factors than simply complement or Fc receptor interactions. The flexibility of the hinge region, which differs across the various isotypes, can affect the functional valency of an antibody, due to steric hindrance (166, 167). Inflammation and tissue injury, such as incurred during organ transplant, can effect the regulation of complement control factors, the expression of Fc receptors or the sialylation of polysaccharides on circulating antibodies, all of which influence how antibodies are perceived by the immune system (168-171). Furthermore, antibodies binding to various target cells may have different outcomes depending on the cell's particular stage of development and activation status, or due to cell-type specific signaling of the bound molecule (63, 64, 172).

Herein, we describe two murine anti-CD40 binding antibodies, 7E1-G1, a rat $IgG_1$ isotype, and its natural isotype switch variant 7E1-G2b, a rat $IgG_{2b}$ isotype. 7E1-G1 was developed by standard hybridoma selection and purification techniques, and selected

based on its specificity for murine CD40, ability to inhibit ligand binding, and paucity of agonism (173). It was hypothesized that an antibody that strongly induced both complement fixation and Fc receptor interactions would be the most favorable for promoting graft survival. Such an antibody would possibly deplete CD40 expressing antigen-presenting cells before the priming of an effective response could occur. As rat $IgG_1$ antibody isotypes have been shown in various models to poorly fix complement and interact weakly with Fc receptors, an antibody having the same specificity but of the $IgG_{2b}$ isotype was sought. To this end, the natural isotype switch variant 7E1-G2b was generated by the sib-selection technique (130, 173).

When used in concert with CTLA4-Ig, the anti-CD40 antibody 7E1-G2b, but not 7E1-G1, was found to be effective in promoting bone marrow chimerism and skin graft survival. Although neither antibody was depleting, 7E1-G1, unlike 7E1-G2b, functioned as a potent CD40 agonist suggesting the involvement of Fc receptors. However, Fc receptors or the presence of CD40 activated B cells could not account for the difference in efficacy between these two 7E1 variants.

## Methods

*Ab generation*

A recombinant murine CD40 immunoglobulin fusion protein consisting of the extracellular region of mouse CD40 fused to the hinge, CH2 and CH3 domains of a mouse $IgG_{2a}$ antibody was used to immunize Lewis rats. Three days following the last immunization, leukocytes were fused with X63-Ag8.653 mouse myeloma cells to create rat/mouse heterohybridomas. Supernatants were tested for reactivity with mouse CD40 and for the ability to inhibit the binding of soluble murine CD40 to its ligand CD154. Stimulatory properties were measured by proliferation of splenic B cells in the presence of anti-IgM. Suppression of antibody production was measured by immunization of mice with sheep red blood cells. The clone 7E1-G1 was selected as the lead anti-CD40 mAb candidate for murine studies (173).

A natural isotype switch variant of 7E1-G1, from an $IgG_1$ to an $IgG_{2b}$, was generated by the sib-selection technique (130). Briefly, ELISA identified an anti-CD40 mAb of the IgG2b isotype among supernatants of 96 well plates that had been seeded at 1000 cells/well with the original 7E1-G1 hybridoma. Subsequent rounds of plating and identification of $IgG_{2b}$ positive wells at seeding densities of 200 and then 20 cells/well followed by two rounds of cloning by limiting dilution led to the isolation of the clonal $IgG_{2b}$ switch variant, 7E1-G2b.

*Mice*

Adult male 6- to 8-wk-old C57BL/6, BALB/c, C3H/HeJ, B6.129S2-*Igh*-$6^{tm1Cgn}$/J, and B6.129P2-*Fcgr3*$^{tm1Sjv}$/J, were obtained from The Jackson Laboratory (Bar Harbor, ME). Act-mOVA mice were generously provided by M. Jenkins (University of Minnesota, Minneapolis, MN). B6.129P2-*Fcer1g*$^{tm1Rav}$ N12, and B6.129S4-*Fcgr2b*$^{tm1TtK}$ N12, were obtained from Taconic Farms (Germantown, NY). Animals received humane care in pathogen-free housing conditions in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

*Competitive binding and affinity assays*

The M12 B cell lymphoma cell line was generously provided by N. Iwakoshi (Emory University, Atlanta, GA). To compare the binding affinity, M12 B cells were incubated with 1mg/mL to 8pg/mL of 7E1-G1, 7E1-G2b, rat $IgG_1$ isotype control, rat $IgG_{2b}$ isotype control, or FGK4.5. Isotype control antibodies and FGK4.5 were purchased from BioXCell (West Lebanon, NH). Bound antibody was then detected with R-phycoerythrin-conjugated $F(ab')_2$ goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). For the competitive binding assay, cells were incubated with the same titrations as noted above, along with 10ug/mL of FLAG-tagged soluble recombinant murine CD154 (Axxora, San Diego, CA). Bound CD154 was detected with FITC-conjugated anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO).

*In vitro proliferation assay*

To test the 7E1 variants as an immobilized stimulus, Immulon 4 HBX plates (Daigger, Vernon Hills, IL) were incubated overnight with 1mg/mL to 1ηg/mL of either 7E1-G1,

7E1-G2b, rat $IgG_1$ isotype control, rat $IgG_{2b}$ isotype control, or FGK4.5. For a soluble stimulus, Costar cell culture plates (Sigma-Aldrich, St. Louis, MO) were blocked with 10% bovine serum albumin in phosphate buffered saline prior to the addition of the same titrated concentrations of the antibodies as noted above. B cell responders from C57BL/6 mice were enriched with Lymphocyte Separation Media (Mediatech, Herndon, VA) then purified using the Mouse B cell Isolation Kit and VarioMACS Separator (Miltenyi Biotec, Auburn, CA). Purity was confirmed to be >98% $B220^+$ B cells. Purified B Cells were added to both immobilized and soluble stimulus plates in parallel and following 72 hours in culture were pulsed for an additional 12 hours with 0.5μCi/well of [methyl-$^3$H]thymidine. Cells were harvested and [methyl-$^3$H]thymidine incorporation was measured on a standard β counter microplate reader (Biotek, Winooski, VT).

*Bone marrow chimerism*

Donor bone marrow was flushed from tibiae and femora then resuspended at $2x10^7$cells/500μL in sterile saline. Recipient mice were transfused on days 0 and 6 with donor marrow i.v, along with a single 500mg dose of busulfan on day 5. On days 0, 2, 4, and 6, 500μg i.p. of CTLA4-Ig (Bristol-Myers Squibb, New York, NY) and either 7E1-G1, 7E1-G2b or rat IgG isotype control.

*Skin grafting*

Full thickness ear and tail skin grafts (~1$cm^2$) were transplanted onto the dorsal thorax of recipient mice and secured with a plastic adhesive bandage for 6 days. Recipients received 500μg i.p. of CTLA4-Ig and either 7E1-G1, 7E1-G2b, rat $IgG_1$ isotype control

or rat $IgG_{2b}$ isotype control on days 0, 2, 4, and 6. Graft survival was monitored by daily visual inspection and rejection was defined as the complete loss of viable epidermal tissue.

*BrdU labeling for in vivo proliferation*

Mice were treated on days 0, 2, 4 and 6 with 500μg/dose i.p. of either 7E1-G1, 7E1-G2b, rat $IgG_1$ isotype control, rat $IgG_{2b}$ isotype control or FGK4.5. On day 7, mice were pulsed i.p. with 2 mg of 5-bromo-2-deoxyuridine (BrdU) then sacrificed 4 hrs later. BrdU incorporation was detected using the BrdU Flow Kit according to the manufactures protocol (BD Biosciences, San Jose, CA).

*Flow cytometry for phenotype and absolute number*

At the indicated time points, animals were sacrificed, spleens harvested and single cell suspension prepared in phosphate buffered saline supplemented with 0.5% bovine serum albumin and 1 mM EDTA. Unless previously stated, antibodies obtained from BD Pharmingen (San Jose, CA) include the following: anti- 41BBL-PE, H-$2K^d$-FITC, I-$A^b$-FITC, -biotin, $V_\beta$5-PE, $V_\beta$8-PE, $V_\beta$11-PE, CD3-APC, -Pacific Blue, CD4-APC, -Pacific Blue, -PE, CD8-FITC, -PerCP, CD11b-FITC, -PerCP-Cy5.5, CD11c-APC, CD19-APC, -FITC, -Pacific Blue, -PE, CD25-APC, CD43-FITC, -PE, CD44-APC, -FITC, CD45R-PE, -PerCP-Cy5.5, CD69-PerCP-Cy5.5, CD70-PE, CD80-APC, -FITC, and CD86-FITC, -PE. Antibodies obtained from Invitrogen (Carlsbad, CA) include: Streptavidin-Pacific Orange, anti- CD8-Pacific Orange, and CD45R-Pacific Orange. Antibodies obtained from eBioscience (San Diego, CA) include anti-CD19-Pacific Blue. When noted,

absolute numbers of splenocyte subsets were determined by TruCount Bead Analysis according to the manufacturer's instructions (BD Biosciences). Flow cytometric data was analyzed using FlowJo Software (Treestar, Ashland, OR).

*Statistical analysis*

Skin graft rejection was represented by Kaplan-Meier survival curves. Statistical comparisons of mean survival times were performed using nonparametric one-way ANOVA (Kruskal-Wallis test), followed by Dunn's multiple comparisons posttest of the relevant datasets. Titration curves were compared by nonlinear regression best curve fit using a sigmoidal dose-response (variable slope) equation over the exponential portion of each data set. Additional statistical analysis was performed using two-tailed unpaired Students t-test. (GraphPad Software, La Jolla, CA)

## Results

*7E1-G1 and 7E1-G2b possess equivalent affinities for murine CD40 and both effectively inhibit the interaction of CD154 to CD40.*

Preliminary characterization during the development of the 7E1 variants proposed that 7E1-G2b was an isotype switch variant of 7E1-G1 and thus should possess the same specificity and affinity for CD40 (173). In order to confirm this finding, we sought to compare their binding affinities to their natural ligand, murine CD40. We utilized the B cell line M12, which constitutively expresses a high level of CD40 on its surface as an *in vitro* target (Figure 3.1). Cultured M12 cells were incubated with increasing concentrations of 7E1-G1, 7E1-G2b, or the strong murine CD40 agonist FGK4.5 (174), and bound antibody was subsequently detected with a fluorescently labeled polyclonal anti-rat IgG secondary antibody. The resulting titration curves for the 7E1 variants were not significantly different, as the null hypothesis that one curve best fit both data sets could not be rejected (P value = 0.207, $R^2 = 0.994$, $EC_{50} = 81.63$ng/mL). The titration curve for the unrelated antibody FGK4.5 was distinct from either of the 7E1 variants (P value < 0.0001, $R^2 = 0.749$, $EC_{50} = 95.00$ng/mL) (Figure 3.2a). These results suggested that any potential alterations in the idiotypic region of the 7E1 variants did not cause significant deviations in their affinity for murine CD40.

We next sought to assess the ability of the 7E1 variants to compete with soluble CD154 for binding to cell-bound CD40. We hypothesized that both 7E1-G1 and 7E1-G2b would inhibit this interaction to a similar degree. To test this, M12 B cells were again incubated with increasing concentrations of 7E1-G1, 7E1-G2b, or FGK4.5, along

with a constant concentration of Flag-tagged soluble CD154 fusion protein (sCD154). Bound sCD154 then was detected with a fluorescently labeled anti-FLAG secondary antibody. The mean fluorescence intensity (MFI) observed at each concentration of competitor antibody was divided by the MFI of M12 cells cultured with Flag-tagged sCD154 alone. The competitive inhibition curves generated for the 7E1 variants were statistically identical, as the null hypothesis that one curve best fit both data sets could not be rejected (P value = 0.071, $R^2 = 0.992$, $EC_{50} = 0.238\mu g/mL$). FGK4.5 was unique in its ability to block sCD154 from binding and best fit its own curve (P value < 0.0001, $R^2 = 0.6992$, $EC_{50} = 1.362\mu g/mL$) (Figure 3.2b). These results further confirm that the idiotypic region of 7E1-G2b has not undergone significant functional changes, as well as showing that both 7E1-G1 and 7E1-G2b have the same ability to block the interaction between CD40 and CD154.

*Anti-CD40 treatment combined with CTLA4-Ig promotes allogeneic chimerism and skin graft survival.*

We have previous shown that the anti-CD40 antibody 7E1-G2b can effectively substitute for anti-CD154 in costimulatory blockade-based protocols for promoting murine hematopoietic chimerism and skin graft survival. Here we sought to determine whether the specific isotype of the anti-CD40 antibody used would affect this functional outcome. Groups of C57BL/6 mice were grafted with allogeneic BALB/c skin and treated with CTLA4-Ig and either 7E1-G1, 7E1-G2b, or rat IgG isotype control i.p. on days 0, 2, 4, and 6. Recipients treated with 7E1-G2b show a modest prolongation of skin graft survival (MST = 27 and 23 days, respectively), compared to 7E1-G1 (MST = 18

days), in the robust BALB/c to C57BL/6 'costimulation blockade resistant' model (134) (Figure 3.3a). In the more permissive BALB/c to C3H to model (135), graft survival was further prolonged with 7E1-G2b (MST > 75 days), as compared to 7E1-G1 treatment (MST = 29 days) (Figure 3.3b).

To test these agents in a chimerism model, C57BL/6 mice were transfused with allogeneic BALB/c bone marrow ($2x10^7$cells i.v. on days 0 and 6) and BALB/c skin grafts on day 0, as well as busulfan, an alkylating agent toxic to hematopoietic stem cells, on day 5. Groups of mice also received CTLA4-Ig and either 7E1-G1, 7E1-G2b or rat IgG isotype control i.p. on days 0, 2, 4, and 6. Peripheral blood was sampled for the presence of donor-derived B cells and T cells. The percentage of donor $B220^+$ B cells (Figure 3.3c) and $CD4^+$ T cells (Figure 3.3d) were greater than 75% with 7E1-G2b treatment, while 7E1-G1 and isotype treated animals failed to sustain significant donor chimerism. The successful induction of central tolerance directly related to peripheral tolerance, as 7E1-G2b recipients had prolonged graft survival (MST > 75 days) not seen for 7E1-G1 treatment (MST = 16 days) (Figure 3.3e).

We also tracked the frequency of donor reactive $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells in these chimeric recipients. T cells possessing these Vβ segments are normally deleted in the thymus of BALB/c mice due to their high affinity for endogenous retroviral superantigens (mouse mammary tumor viruses, MMTVs) presented by I-E MHC molecules. Wild-type C57BL/6 mice do not express I-E, thus $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells constitute roughly 4-5% and 2-3% of the peripheral $CD4^+$ T cell compartment, respectively. The thymic deletion of these specific Vβ expressing T cells in our chimeric mice can serve as a surrogate measure of successful donor tolerance

(136). As shown on day 58, the percentage of Vβ11$^+$ and Vβ5.1/5.2$^+$ CD4$^+$ T cells in the isotype (black bar) and 7E1-G1 (checkered bar) treated groups were consistent with wild-type C57BL/6 levels (Figure 3.3f). In contrast, CD4$^+$ T cells from 7E1-G2b (striped bar) treated groups show a significant loss of Vβ11$^+$ and Vβ5.1/5.2$^+$ from the periphery. Vβ deletion was shown to be specific as the use of Vβ8.1/8.2$^+$ by CD4$^+$ T cells remained comparable in all groups.

*The agonist potential of the 7E1 variants are dependent on both the isotype and the mode of stimulation in vitro.*

The data presented above demonstrated that although the 7E1 variants have an equivalent ability to block CD154 binding to CD40, they show dramatically different synergy with CTLA4-Ig for promoting allogeneic graft survival. We hypothesized that this observation may be due in part to differences in their agonist properties. To test this, we used the induction of B cell proliferation *in vitro* as an indirect measure of CD40 agonism.

Spleens were taken from C57BL/6 mice and either used as whole splenocytes or B cells were purified by magnetic bead separation. Each was then cultured with either soluble or plate-bound antibodies. For an immobilized stimulus high binding affinity culture plates were pre-coated with increasing concentrations of 7E1-G1, 7E1-G2b, isotype matched control antibody or FGK4.5 at concentrations ranging from 1ng/mL to 100ug/mL. Alternatively, the ability of soluble antibody to induce proliferation was measured by incubations in conventional culture plates that were pre-blocked prior to the addition of the antibodies as described in the Materials and Methods. We found that

when 7E1-G1 or 7E1-G2b was presented as a plate bound stimulus, similar levels of B cell proliferation as with FGK4.5 were observed (Figure 3.4a). However, when the antibodies were free in solution, the ability of 7E1-G2b to induce proliferation was significantly reduced. In contrast, soluble 7E1-G1, although not as robust as FGK4.5, continued to promote B cell expansion (Figure 3.4b). The results were similar when using whole splenocyte responders, treated with 50μg of the indicated antibodies (Figure 3.4c,d). This demonstrated that although both 7E1-G1 and 7E1-G2b exhibited the same affinities for CD40 and could induce proliferation when immobilized, only 7E1-G1 retained its agonistic properties when soluble.

*Anti-CD40 treatment does not cause depletion of CD40 expressing cells, although depending on the isotype, can induce in vivo proliferation.*

Because we observed that soluble 7E1-G1 could function as a CD40 agonist *in vitro*, whereas 7E1-G2b could not, we hypothesized that these differences may explain their different *in vivo* efficacy for preventing graft rejection. As such, we explored the *in vivo* effects of treatment with the 7E1 variants. We speculated that the CD40-binding antibodies might act by depleting CD40-expressing cells. Mice were injected i.p. on days 0, 2, 4, and 6 with either 7E1-G1, 7E1-G2b, isotype matched control antibody, FGK4.5 or left them untreated. On day 7, mice were sacrificed and the absolute numbers of various cell subsets were enumerated from the spleen by TruCount analysis. We found that treatment with 7E1-G2b did not lead to the depletion of any cell subset enumerated, as the absolute numbers of each did not significantly differ from isotype control or untreated animals (Figure 3.5a). However, mice treated with 7E1-G1 showed a three-

fold increase in $CD8^+$ T cells, B cells and granulocytes and an approximately two-fold increase in dendritic cells and macrophage, at levels close to those seen with FGK4.5. Interestingly, no change in either CD4+ T cells or natural killer cells occurred with 7E1-G1 treatment, despite both of these cells expressing significant levels of CD40 on their surface (Figure 2.3).

To determine if these increases in cell numbers were due to increased trafficking to the spleen or *de novo* cell division, groups of mice were given the same treatment as above and pulsed on day 7 with 2mg of BrdU for an additional 4 hours. BrdU, when paired with the DNA stain 7AAD, can reveal both the presence of dividing cells and their specific state in the cell cycle. B cells, $CD8^+$ T cells and to a lesser extent $CD4^+$ T cells show a clear population of cells progressing through the cell cycle in 7E1-G1 and FGK4.5 treated animals, but not in 7E1-G2b or isotype control treated animals (Figure 3.5b). This brief snapshot of division represents a 5-fold increase in proliferating B cells, a 3-fold increase in proliferating $CD4^+$ T cells and a 11-fold increase in proliferating $CD8^+$ T cells with 7E1-G1 as compared to isotype control treatment (Figure 3.5c). Thus, the ability of 7E1-G2b to promote graft survival is not due to the depletion of CD40-expressing cells. In contrast, 7E1-G1 treatment leads to the significant proliferation of T cells and B cells, as well as the accumulation of macrophage, dendritic cells and granulocytes in the spleen.

We also looked for the presence of bound antibody on circulating B cells. Mice were injected i.p. on days 0, 2, 4, and 6 with CTLA4-Ig and either 7E1-G1 or 7E1-G2b, then sacrificed at the indicated time points. Bound antibody was detected with a polyclonal anti-rat IgG secondary antibody. When viewed as an absolute number of cells

with bound anti-CD40 antibody, the increased number of B cells in the 7E1-G1 treated group is most likely reflective of the agonist properties characterized for this antibody (Figure 3.6a). 7E1-G2b displays a relatively consistent number of B cells with bound antibody out to at least 50 days, while 7E1-G1 is undetectable after 30 days. The data presented as a percentage of total B cells shows that 7E1-G1 is absent from almost all B cells after 30 days (Figure 3.6b). The percentage of total B cells bound with 7E1-G2b does not begin to drop until at least 40 days.

*7E1-G1, but not 7E1-G2b, induces the expression of activation markers on antigen presenting cells.*

Due to the findings that 7E1-G1 can induce B cell proliferation *in vitro* and *in vivo*, we hypothesized that administration of 7E1-G1, but not 7E1-G2b, would also result in the up-regulation of activation markers on antigen presenting cells. To examine this, C57BL/6 mice were given a single injection of either 7E1-G1, 7E1-G2b, isotype matched control antibody, FGK4.5, or left untreated. Mice were sacrificed at various time points and stained for the presence of activation markers on $CD11c^+$ dendritic cells. 7E1-G1 treatment led to three-fold increase in MHC class II expression, a four-fold increase in CD80 expression and a seven-fold increase in the expression of CD86 on dendritic cells, closely mirroring the level of expression observed for FGK4.5 treated animals (Figure 3.7a). The percentage of splenic dendritic cells expressing CD70 increased by over one hundred-fold, 41BBL increased by 12-fold and OX40L increased by fifteen-fold, again only for 7E1-G1 and FGK4.5 but not 7E1-G2b treated animals.

$B220^+$ $CD19^+$ B cells were also measured for activation markers in these same

treated animals. B cells in 7E1-G1 treated animals showed a three-fold increase in the expression of MHC class II, and an almost two-fold increase in both CD80 and CD86 (Figure 3.7b). The percentage of CD69 expressing B cells increased almost two-fold in the spleen immediately following 7E1-G1 treatments. This was in contrast to what was observed in 7E1-G2b treated animals, which did not differ from isotype and untreated animals.

*Increased expression of activation markers on T cells following treatment with 7E1-G1, but not 7E1-G2b.*

We hypothesized that the effects of the anti-CD40 antibodies on antigen presenting cells would subsequently impact T cell activation as well. To test this, C57BL/6 mice were again given a single injection of either 7E1-G1, 7E1-G2b, isotype matched control antibody, FGK4.5 or left untreated. Mice were sacrificed at various time points and stained for the expression of activation markers on $CD3^+$ T cell subsets. $CD4^+$ T cells from 7E1-G1 treated mice showed an almost two-fold increase in the percentage of cells expressing CD25 and CD43, which peak by day 7 (Figure 3.8a). The percentage of cells expressing CD44 and CD69 increased by two-fold, which can be seen as early as day 3. In contrast, the percentage of $CD4^+$ T cells expressing each of these activation markers for 7E1-G2b treated animals was at or below what was observed in isotype control treated animals. The percentage of $CD8^+$ T cells from 7E1-G1 treated animals expressing CD25 increased by four-fold, CD43 by almost twenty-fold, CD44 by three-fold and CD69 by five-fold, all of which peak by day 7 (Figure 3.8b). 7E1-G2b did not induce any notable activation marker expression on $CD8^+$ T cells beyond that seen with

isotype and untreated animals.

*Morphological changes in the spleen following 7E1-G1 treatment.*

We consistently observed a prominent splenomegaly, beginning at day 3, in 7E1-G1 treated animals, similar to FGK4.5, but not for 7E1-G2b (data not shown). To investigate this further, mice were injected i.p. on days 0, 2, 4, and 6 with either 7E1-G1, 7E1-G2b or isotype matched control antibody, and spleen section were prepared on day 7. Under H&E staining, the overall spleen morphology between treatments did not appear overtly dissimilar, however the white pulp in 7E1-G1 treated animals was more prominent and irregularly shaped in comparison to 7E1-G2b and control treatments (Figure 3.9a). Using immunohistochemistry, we found the distribution of $B220^+$ B cells was unique in the 7E1-G1 treated animals, as the cells were diffuse throughout the entire white pulp, including the T cell zones. In the 7E1-G2b and control treated sections, B cells were restricted to the marginal zones along the outer rim of the white pulp (Figure 3.9b).

*The presence of activated B cells alone cannot explain the differences observed between the two isotypes of anti-CD40 antibodies.*

Resting B cells make up a large percentage of the total cells present in the spleen and express a high basal level of CD40 (Figure 2.3) (52). As we have shown, B cells aggressively responded to 7E1-G1, up-regulating activation markers, robustly proliferate and migrate into the T cell zones of the spleen. We hypothesized that the failure of 7E1-G1 to act as an effective CD40 blocking antibody is due to its activation of B cells. To

test this, we again made use of the mOVA-C57BL/6 single minor mismatched skin graft rejection model. When used as skin graft donors for untreated C57BL/6 recipients, these single mismatched grafts are rejected with an MST of 18 days (Fig. 3.10a). Costimulation blockade consisting of 7E1-G2b and CTLA4-Ig extends graft survival indefinitely (MST > 200 days). Isotype matched control antibody and CTLA4-Ig are able to extend graft survival for > 100 days in about half of the recipients tested, highlighting the significant effects of blocking the CD28 pathway alone in this model. Interestingly, when 7E1-G1 is used, the beneficial effects seen with CTLA4-Ig alone are significantly reduced and the MST drops to 22 days and all grafts are rejected.

Given this observation, we postulated that the prominent activation of B cells by 7E1-G1 may have created an overall stimulated environment that supercedes the effects of CTLA4-Ig, thus directly prompting graft rejection. To test this, we obtained B cell deficient mice for use as recipients of mOVA skin grafts. B cell deficient recipients were treated with 500μg of both CTLA4-Ig and either 7E1-G1 or isotype matched control antibody i.p. on days 0, 2, 4, and 6, along with a mOVA skin graft on day 0. In the absence of B cells, the MST of 7E1-G1 treated animals was 21 days, not significantly different from that seen for wild type recipients (Figure 3.10b). Untreated recipients promptly reject grafts with an MST of 13 days, while a third of isotype control recipients show long term survival.

*The involvement of individual Fc receptors cannot fully account for the differences observed between the two isotypes of anti-CD40 antibodies.*

We next sought to explore the possibility that Fc receptors could account for the

difference in efficacy observed between the 7E1 variants. To test the hypothesis that activating signals through the Fc receptor are responsible for the graft rejection seen with 7E1-G1, we obtained FcγRIII deficient mice for use as recipients of mOVA skin grafts. FcγRIII is the main activating Fc receptor for murine $IgG_1$ antibody isotype (139). Recipients were treated with 500 μg of both CTLA4-Ig and either 7E1-G1, 7E1-G2b or isotype matched control antibody i.p. on days 0, 2, 4, and 6, along with a mOVA skin graft on day 0. The MST of 7E1-G1 treated FcγRIII deficient animals was 20 days, which is not significantly different when compared to isotype matched control antibody or similarly treated wild type recipients (MST = 17 days) (Figure 3.10c). 7E1-G2b treated FcγRIII deficient animals show no diminution in their long-term graft survival.

Several other activating Fc receptors have been identified, for which $IgG_1$ isotype antibodies have varying affinities. In order to determine if interactions with other activating Fc receptors, besides FcγRIII, contributed to the graft rejection seen for 7E1-G1 treated animals, we obtained FcRγ deficient mice. FcRγ, also referred to as FcεRIγ, is the common signaling chain that associates with and is responsible for the surface expression of all of the currently reported murine activating Fc-receptors (175). In the absence of all activating Fc receptors, 7E1-G1 treated animals again did not exhibit prolongation of graft survival and have an MST of only 20 days, compared to the long-term survival seen for 7E1-G2b treated animals (Figure 3.10d, MST > 75 days). Approximately 50% of the isotype treated animals experienced long term graft survival, while untreated animals rejected their grafts with an MST of 11 days.

## Discussion

We compared the efficacy of two isoforms of a murine anti-CD40 antibody, 7E1-G1 and 7E1-G2b. We demonstrated that both antibodies have an equivalent affinity for their ligand CD154, and are able to inhibit the interaction between CD40 and CD154 to the same extent. When used in concert with CTLA4-Ig, 7E1-G2b effectively promotes allogeneic bone marrow chimerism and skin graft survival, while 7E1-G1 does not. When plate bound, both antibodies can function as CD40 agonists. However, only 7E1-G1 was found to act as a CD40 agonist when soluble *in vitro* and *in vivo*. Activated B cells or Fc receptor signaling were not found to play a dominant role in these differential effects.

Affinity and epitope profiling of murine anti-CD40 antibodies have described a correlation between the location where the antibody binds, with respect to the binding site of CD154, and the resultant activation signals delivered (156, 157). Based on the distinct agonist profiles described for the 7E1 variants, it could be concluded that the 7E1-G1 binds proximal to the CD154 binding site while 7E1-G2b binds distally, thus allowing the former to mimic receptor ligation and the latter to be an antagonist blocking agent. However, N-terminal peptide sequencing of the heavy chain confirm that both antibodies are identical for the first 35 amino acids and PCR using primers specific for the variable heavy chain CDRs of 7E1-G1 yield a band of appropriate size from cDNA obtained from either antibody (173). This is supported by our findings that both antibodies have an essentially identical binding affinity for cell bound CD40 and an equivalent capacity to inhibit the interaction between CD40 and CD154. If the possibility remains that there are

differences in the variable region, it does not appear to significantly impact the binding characteristics and is therefore unlikely to alter the binding epitope. We therefore conclude that the different biological function of these two antibodies is likely due to other mechanisms.

Fc receptors can deliver activating signals to antigen presenting cells when cross linked by antibody aggregates (139). Murine $IgG_{2b}$ isotype antibodies have been characterized as having a greater affinity for activating verses inhibitory Fc receptors than $IgG_1$ isotypes (165). However, the delivery of proinflammatory signals through activating Fc receptors were not found to be responsible for the failure of 7E1-G1 to promote graft survival, as FcεRγI deficient mice have the same kinetics of graft survival as wild-type mice. The contributions by Fc receptors cannot be completely excluded without blocking all Fc receptor interactions such as by generating $F(ab')_2$ fragments of the 7E1 variants or using FcεRγI x FcγRIIb deficient mice, which lack surface expression of all murine Fc receptors.

Simultaneous blockade of the CD40 and CD28 pathways has been aggressively explored as a clinically applicable approach to induce transplantation tolerance. However, the use of anti-CD154 blocking antibodies has been hampered by the occurrence of thromboembolism. Antibodies targeting CD40 rather than anti-CD154 show promise as an alternative in non-human primate transplant models (109, 113). We have provided evidence that an anti-CD40 monoclonal antibody can substitute for anti-CD154 in protocols designed to promote the induction of murine hematopoietic chimerism and allogeneic skin graft survival. The data shown here describes that the antibody isotype plays an important a role in the biological function and further studies

are merited to define the specific mechanisms responsible for these effects.

## Figure Legends

**Figure 3.1. The M12 B cell lymphoma cell line expresses a uniformly high level of surface CD40.** M12 B cells were grown for at least 1 week in at 37°C in RPMI media supplemented with 10% fetal bovine serum. Cells were stained with B220-FITC and CD40-PE.

**Figure 3.2. 7E1-G1 and 7E1-G2b have an equivalent affinity for murine CD40 and effectively inhibit the binding of CD154.** (A) M12 B cells were incubated with the indicated concentrations of 7E1-G1, 7E1-G2b or FGK4.5. Bound antibody was detected with a PE-labeled polyclonal anti-rat IgG secondary antibody by flow cytometry. The values are presented as the percentage of maximum fluorescence observed for each antibody. (B) M12 B cells were incubated with the indicated concentrations of 7E1-G1, 7E1-G2b or FGK4.5 along with 10μg/mL of Flag-tagged soluble CD154 fusion protein. A FITC-labeled anti-Flag secondary antibody was used to detect bound CD154. The values represent a percentage of the maximum fluorescence observed with sCD154 alone. Data points represent triplicate samples, and experiments were repeated four times.

**Figure 3.3. 7E1-G2b promotes allogeneic bone marrow chimerism and skin graft survival when used in concert with CTLA4-Ig, not seen with 7E1-G1.**
C57BL/6 mice were treated with 500μg of CTLA4-Ig and either 7E1-G1, 7E1-G2b or rat isotype IgG control, i.p. on days 0, 2, 4, and 6. Recipients treated with CTLA4-Ig and 7E1-G2b show a modest prolongation of skin graft survival in (A) the robust BALB/c to

C57BL/6 model, and long-term survival in (B) the more permissive BALB/c to C3H model. (C) C57BL/6 mice were treated with 500μg of CTLA4-Ig and either 7E1-G1, 7E1-G2b or rat isotype IgG control, i.p. on days 0, 2, 4, and 6. Busulfan was given i.v. on day 5, 600mg per mouse, along with allogeneic BALB/c bone marrow, $2x10^7$ cells i.v. on days 0 and 6, and skin graft on day 0. The percentage of donor (C) $B220^+$ B cells and (D) $CD4^+$ T cells reached similar levels in 7E1-G2b, while 7E1-G1 and isotype treated animals failed to sustain chimerism. (E) Chimeric mice are tolerant to donor skin grafts 7E1-G2b treated recipients had prolonged graft survival not seen in the 7E1-G1 and isotype treated groups. (F) The percentage of $V\beta11^+$ and $V\beta5.1/5.2^+$ $CD4^+$ T cells in the isotype (black bar) and 7E1-G1 (crossed bar) treated groups were consistent with wild-type B6 levels. In contrast, $CD4^+$ T cells from 7E1-G2b (striped bar) treated groups show significant deletion of $V\beta11^+$ and $V\beta5.1/5.2^+$. Vβ deletion is shown to be specific as the use of $V\beta8.1/8.2^+$ by $CD4^+$ T cells remained comparable in all groups. Data is shown from day 58-post transplant. (∗ p value <0.001)

**Figure 3.4. 7E1-G2b induces B cell proliferation *in vitro* only when present as an immobilized stimulus.** (A) Purified B cells were added to high binding affinity 96 well plates that had been coated with titrated concentrations of either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b, isotype $IgG_{2b}$ control, or FGK4.5. In parallel, (B) purified B cells were added to conventional 96 well plates, pre-blocked with bovine serum albumen, to which the same antibody concentrations were added. Following 72-hour incubation at 37°C, all plates were pulsed for an additional 18 hours with 0.5μCi/well of [methyl-$^3$H]thymidine. Actively dividing cells which had taken up the radioactive probe were

quantified by scintillation counter. Data points represent triplicate samples, and experiments were repeated three times. Whole splenocytes were incubated using the same (C) plate bound stimulus protocol or the (D) soluble stimulus protocol as outline above. 50μg/mL of each of the indicated antibodies were used and cells were incubated and assayed as noted above. Data points represent triplicate samples, and experiments were repeated twice.

**Figure 3.5. *In vivo* proliferation induced through CD40 ligation is dependent upon the specific isotype of the anti-CD40 antibody.** C57BL/6 mice were injected on days 0, 2, 4, and 6 with 500μg of either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b, isotype $IgG_{2b}$ control, FGK4.5, or left untreated. (A) The absolute number of splenic cell subsets was enumerated by TruCount analysis. No significant differences were noted for any cell subsets between 7E1-G2b and isotype control. 7E1-G1 treated animals show a significant increase in the total number of $CD8^+$ T cells, $B220^+$ B cells, $CD11c^+$ Dendritic cells, Gr-$1^+$ granulocytes and $CD11b^+$ macrophage in the spleen, to a slightly lesser extent as seen with FGK4.5. (B) Similarly treated animals were pulsed on day 7 by i.v. injection with 2mg of BrdU for 4 hours. The cell cycle positions and total DNA synthetic activities of spleen B cells, $CD4^+$ and $CD8^+$ T cells can be determined by the expression of total DNA by 7-AAD and detection of BrdU incorporation into newly formed DNA. Cells in G0/G1 are present in the lower left gate, S phase in the upper gate, and G2 + M in the lower right gate, with the corresponding percentages noted in each plot's legend. These data are representative examples of three independent experiments with three mice per group. (C) A summary of the percentage of BrdU positive dividing cells from the

three independent experiments described above. The percentage of $B220^+$ $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells were statistically significant between 7E1-G1 and isotype $IgG_1$ control treated groups, as well as between FGK4.5 and no treatment groups. No significant difference was noted between 7E1-G2b and isotype $IgG_{2b}$ control treatment. (* p value <0.05, ** p value <0.001)

**Figure 3.6. Both 7E1 variants can be detected on the surface of circulating B cells *in vivo*.** Groups of C57BL/6 mice were injected with 500μg of CTLA4-Ig and either 7E1-G1 or 7E1-G2b. Blood was collected from mice at the indicated time points. $B220^+$ B cells were stained for the presence of bound 7E1-G1 or 7E1-G2b using a PE-labeled polyclonal anti-rat IgG secondary antibody. The data are presented as an (A) absolute number, determined by TrueCount analysis, or (B) as a percentage of total B cells. These data are represent three mice per group.

**Figure 3.7. 7E1-G1, but not 7E1-G2b, increases expression of co-stimulatory molecules and matures antigen presenting cells.** C57BL/6 mice were given a single injection with 500μg of either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b, isotype $IgG_{2b}$ control, FGK4.5 or left untreated. For analysis of (A) $CD11c^+$ Dendritic cells, animals were sacrificed after 6, 24, 36, 60 hours, and 7 days. Splenocytes were stained for CD11c and the indicated markers. For analysis of (B) $B220^+CD19^+$ B cells, animals were sacrificed after 3, 5, 7 and 14 days. The expression is displayed as a fold change compared to untreated animals of the mean fluorescence intensity or of the percentage of

positive cells, as noted on axes. These data are representative examples of three independent experiments with three mice per group.

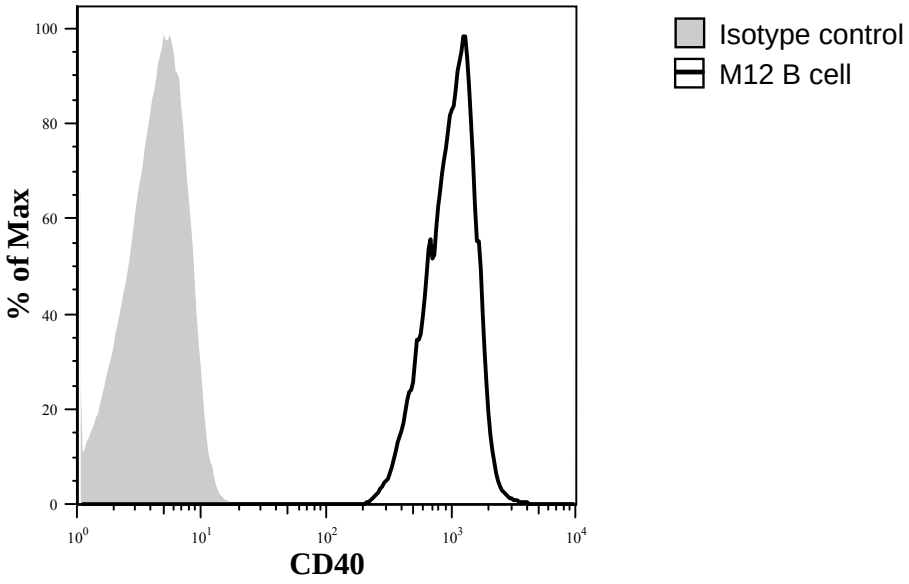
**Figure 3.8. Isotype specific CD40 agonism translates to T cell activation.** C57BL/6 mice were given a single injection with 500μg of either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b, isotype $IgG_{2b}$ control, FGK4.5 or left untreated. For analysis of (A) $CD4^+$ T cells and (B) $CD8^+$ T cells, animals were sacrificed after 3, 5, 7 and 14 days and stained for the indicated markers. The expression is displayed as a fold change of the percentage of positive cells compared to untreated animals. These data are representative examples of three independent experiments with three mice per group.

**Figure 3.9. Histological analysis of the spleen following treatment with the 7E1 variants.** C57BL/6 mice were injected with 500μg of CTLA4-Ig and either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b or isotype $IgG_{2b}$ control i.p on days 0, 2, 4, and 6. Animals were sacrificed on day 14, and spleens were collected for (A) H&E staining (4x magnification) and for (B) immunohistochemistry staining for $B220^+$ B cells (20x magnification). Images are representative of three mice per group.

**Figure 3.10. Fc-receptor and B cell involvement cannot fully account for the differences seen between 7E1-G1 and 7E1-G2b.** (A) Wild-type C57BL/6 mice were transplanted with minor antigen mismatched mOVA x B6 skin grafts and treated with 500μg of CTLA4-Ig and either 7E1-G1, isotype $IgG_1$ control, 7E1-G2b, isotype $IgG_{2b}$ control, or anti-CD154 i.p on days 0, 2, 4, and 6. (B) muMT B cell deficient mice were

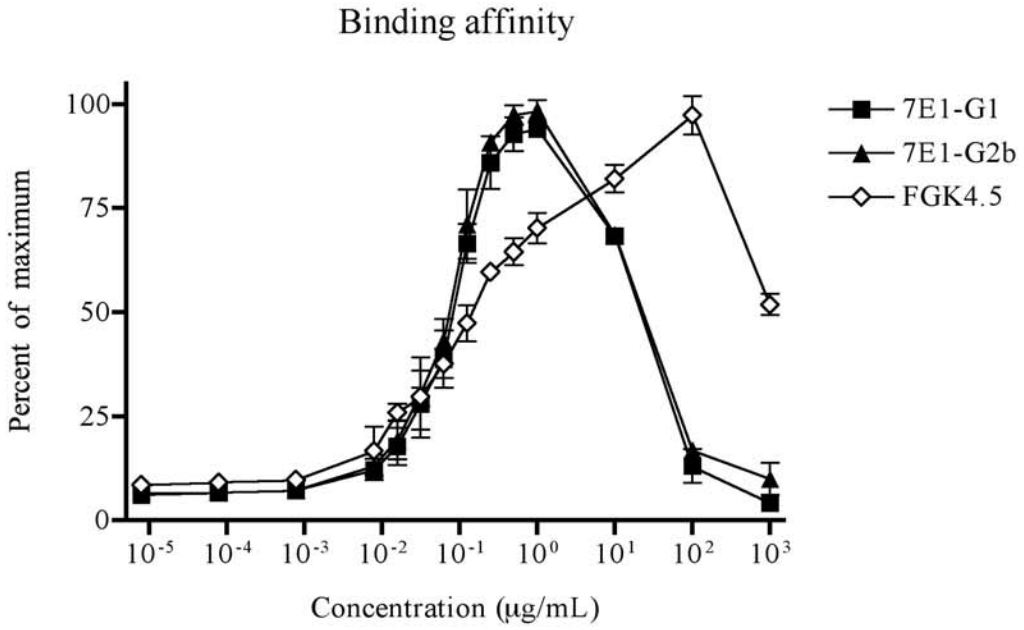
used as recipients of mOVA x B6 skin graft with the same treatment regime, and show a survival pattern very similar to that seen for wild-type recipients. (C) FcγRIII deficient recipients do not show significant prolongation of mOVA x B6 skin graft survival when treated with CTLA4-Ig and 7E1-G1. (D) FcεRγI deficient recipients also do not show significant differences in the prolongation of mOVA x B6 skin graft survival as compared to wild-type recipients.

# Figure 3.1


100
80
60
40
20
0
% of Max
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
CD40
Isotype control
M12 B cell

# Figure 3.2

**A.**


Binding affinity
Percent of maximum
100
75
50
25
0
$10^{-5}$ $10^{-4}$ $10^{-3}$ $10^{-2}$ $10^{-1}$ $10^{0}$ $10^{1}$ $10^{2}$ $10^{3}$
Concentration (μg/mL)
7E1-G1
7E1-G2b
FGK4.5

**B.**

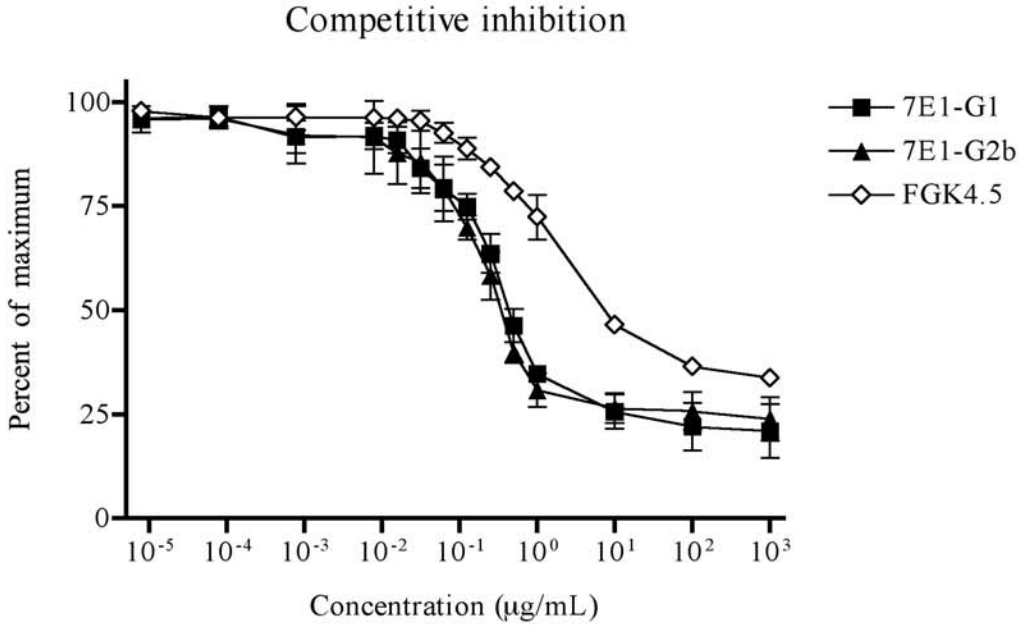
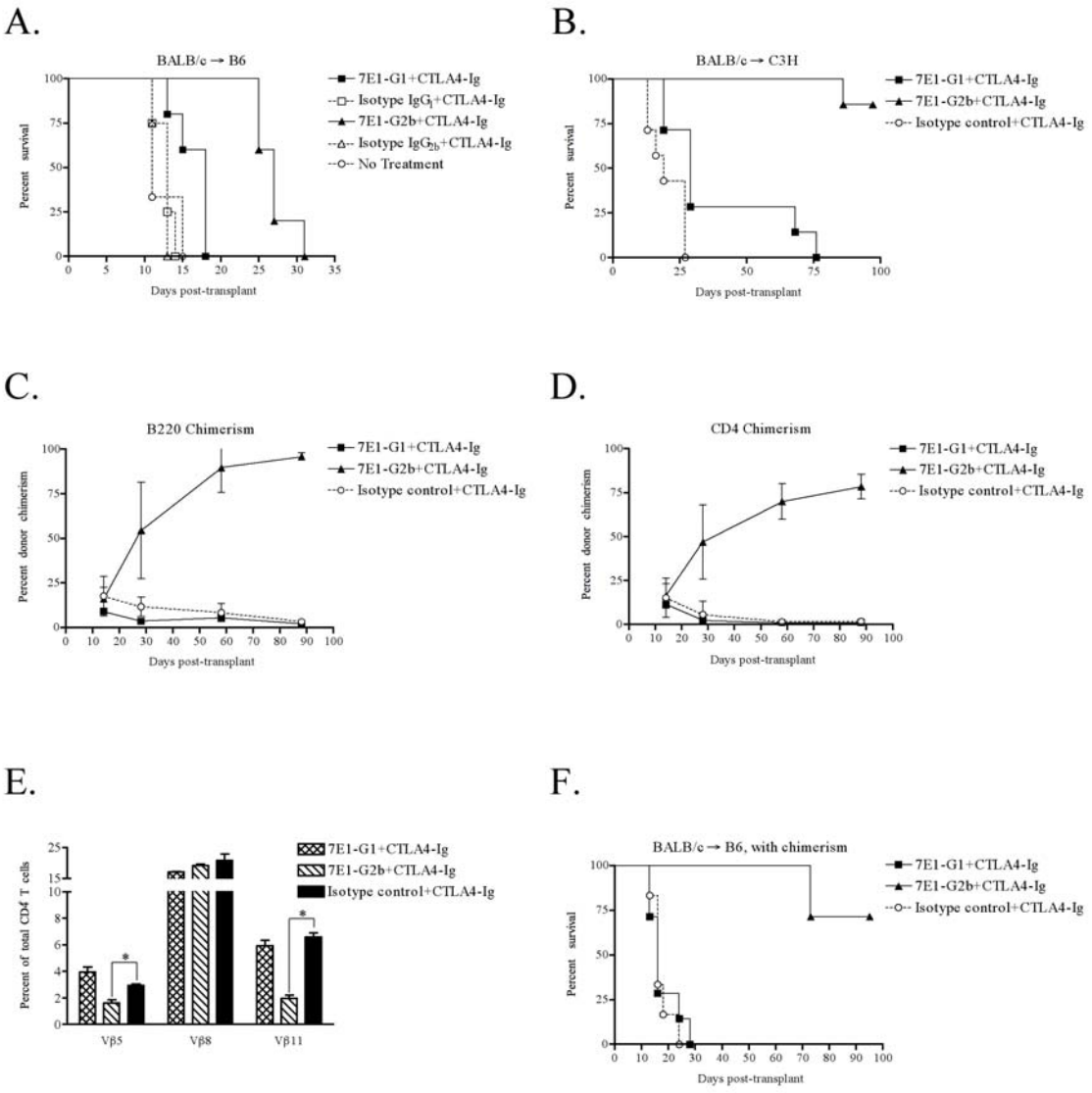
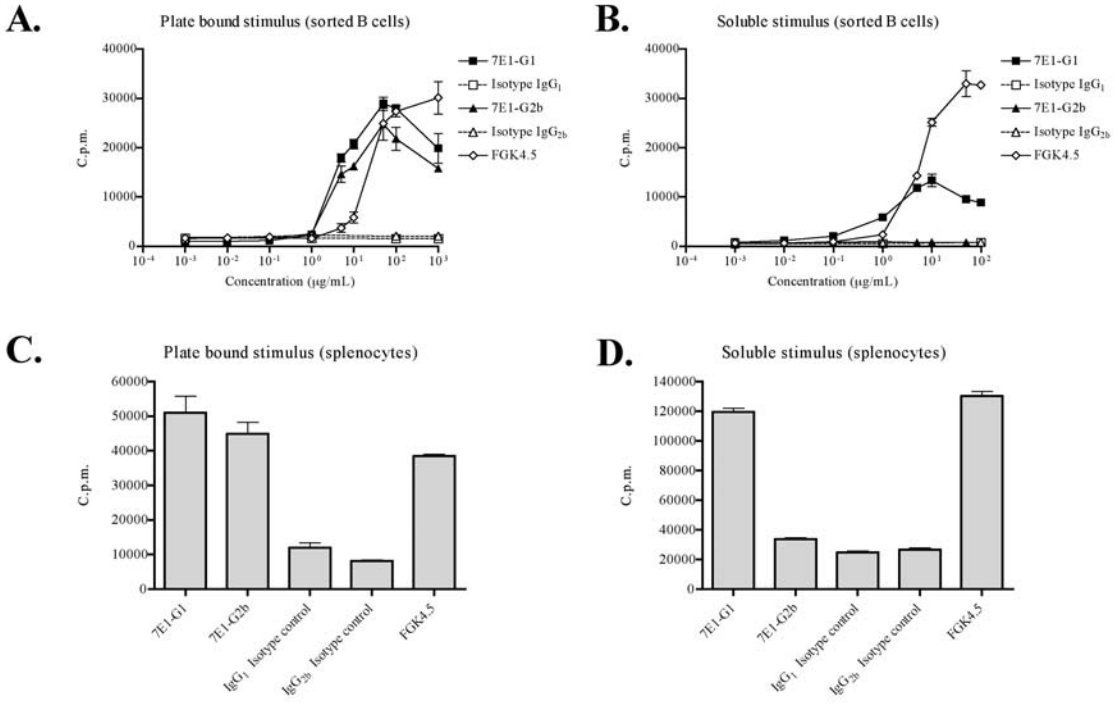

Competitive inhibition
Percent of maximum
100
75
50
25
0
$10^{-5}$ $10^{-4}$ $10^{-3}$ $10^{-2}$ $10^{-1}$ $10^{0}$ $10^{1}$ $10^{2}$ $10^{3}$
Concentration (μg/mL)
7E1-G1
7E1-G2b
FGK4.5

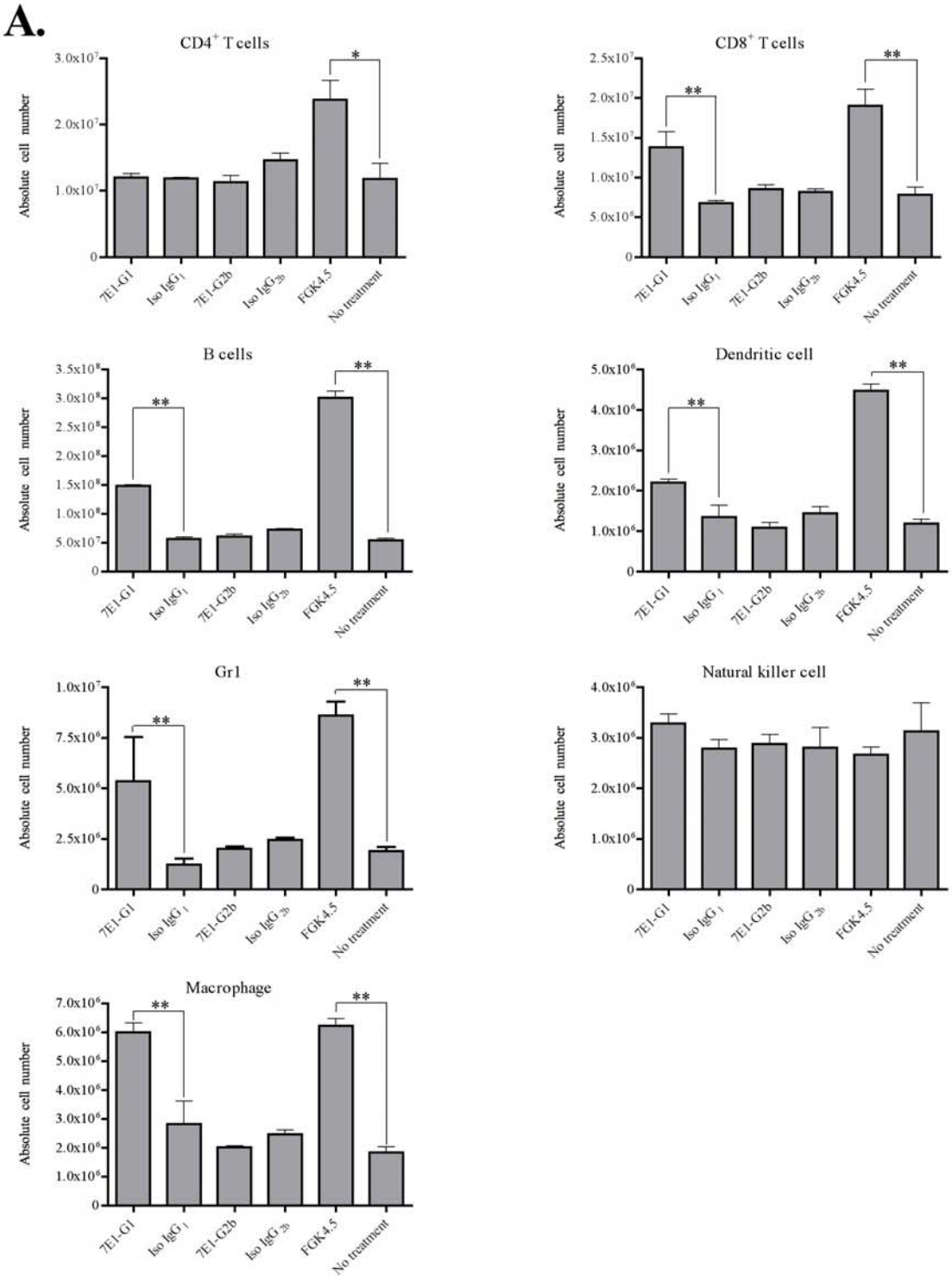
Figure 3.3


A.
BALB/c → B6
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
Isotype IgG1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
No Treatment
B.
BALB/c → C3H
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype control+CTLA4-Ig
C.
B220 Chimerism
Percent donor chimerism
Days post-transplant
7E1-G1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype control+CTLA4-Ig
D.
CD4 Chimerism
Percent donor chimerism
Days post-transplant
7E1-G1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype control+CTLA4-Ig
E.
Percent of total CD4 T cells
Vβ5
Vβ8
Vβ11
7E1-G1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype control+CTLA4-Ig
F.
BALB/c → B6, with chimerism
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype control+CTLA4-Ig

# Figure 3.4

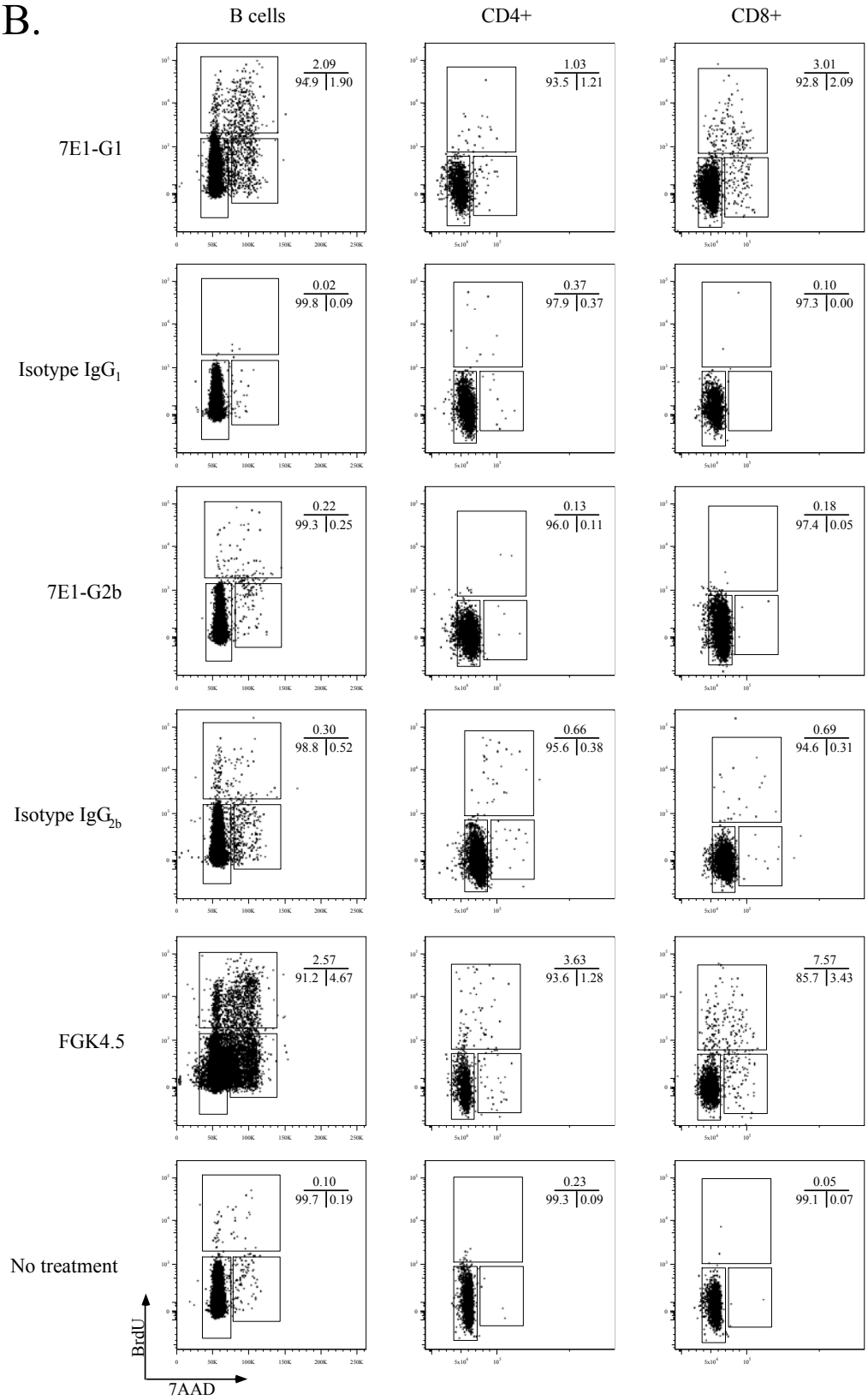

A.
Plate bound stimulus (sorted B cells)
C.p.m.
Concentration (μg/mL)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
B.
Soluble stimulus (sorted B cells)
C.p.m.
Concentration (μg/mL)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
C.
Plate bound stimulus (splenocytes)
C.p.m.
7E1-G1
7E1-G2b
IgG1 Isotype control
IgG2b Isotype control
FGK4.5
D.
Soluble stimulus (splenocytes)
C.p.m.
7E1-G1
7E1-G2b
IgG1 Isotype control
IgG2b Isotype control
FGK4.5

# Figure 3.5


A.
CD4+ T cells
CD8+ T cells
B cells
Dendritic cell
Gr1
Natural killer cell
Macrophage
Absolute cell number
7E1-G1
Iso IgG1
7E1-G2b
Iso IgG2b
FGK4.5
No treatment
*
**

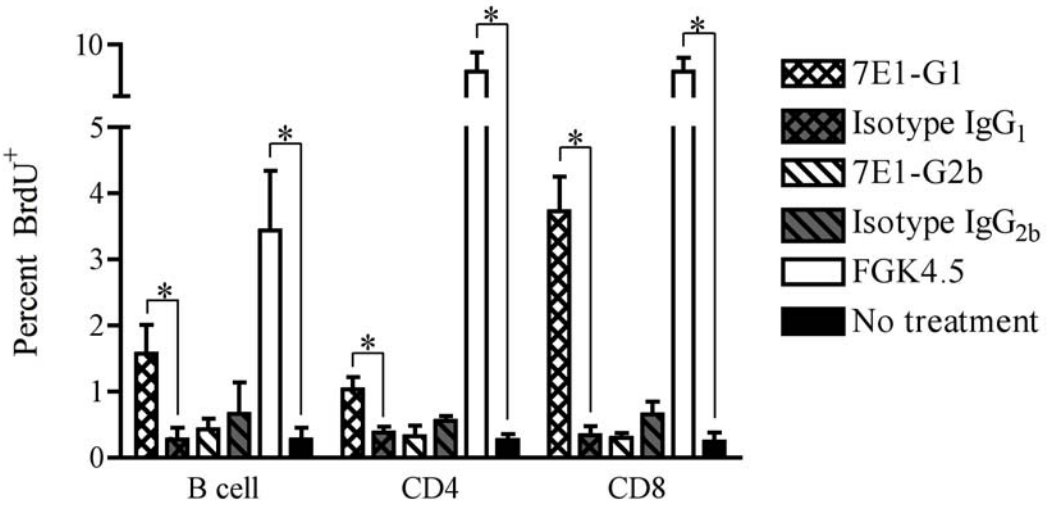
# Figure 3.5

B.


B cells
CD4+
CD8+
7E1-G1
2.09
94.9 | 1.90
1.03
93.5 | 1.21
3.01
92.8 | 2.09
Isotype IgG1
0.02
99.8 | 0.09
0.37
97.9 | 0.37
0.10
97.3 | 0.00
7E1-G2b
0.22
99.3 | 0.25
0.13
96.0 | 0.11
0.18
97.4 | 0.05
Isotype IgG2b
0.30
98.8 | 0.52
0.66
95.6 | 0.38
0.69
94.6 | 0.31
FGK4.5
2.57
91.2 | 4.67
3.63
93.6 | 1.28
7.57
85.7 | 3.43
No treatment
0.10
99.7 | 0.19
0.23
99.3 | 0.09
0.05
99.1 | 0.07
BrdU
7AAD

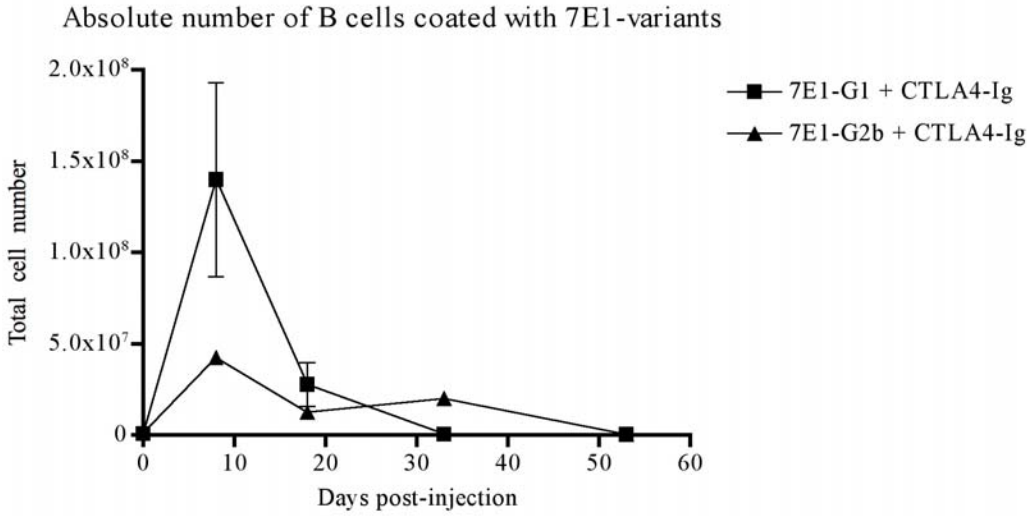
# Figure 3.5

**C.**

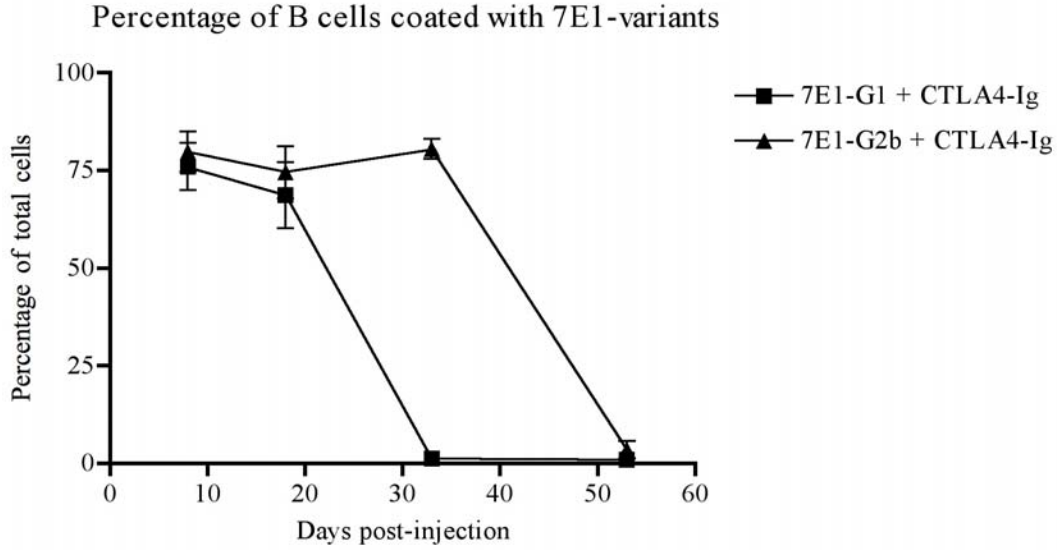

Percent BrdU+
10
5
4
3
2
1
0
*
B cell
CD4
CD8
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
No treatment

# Figure 3.6

**A.**

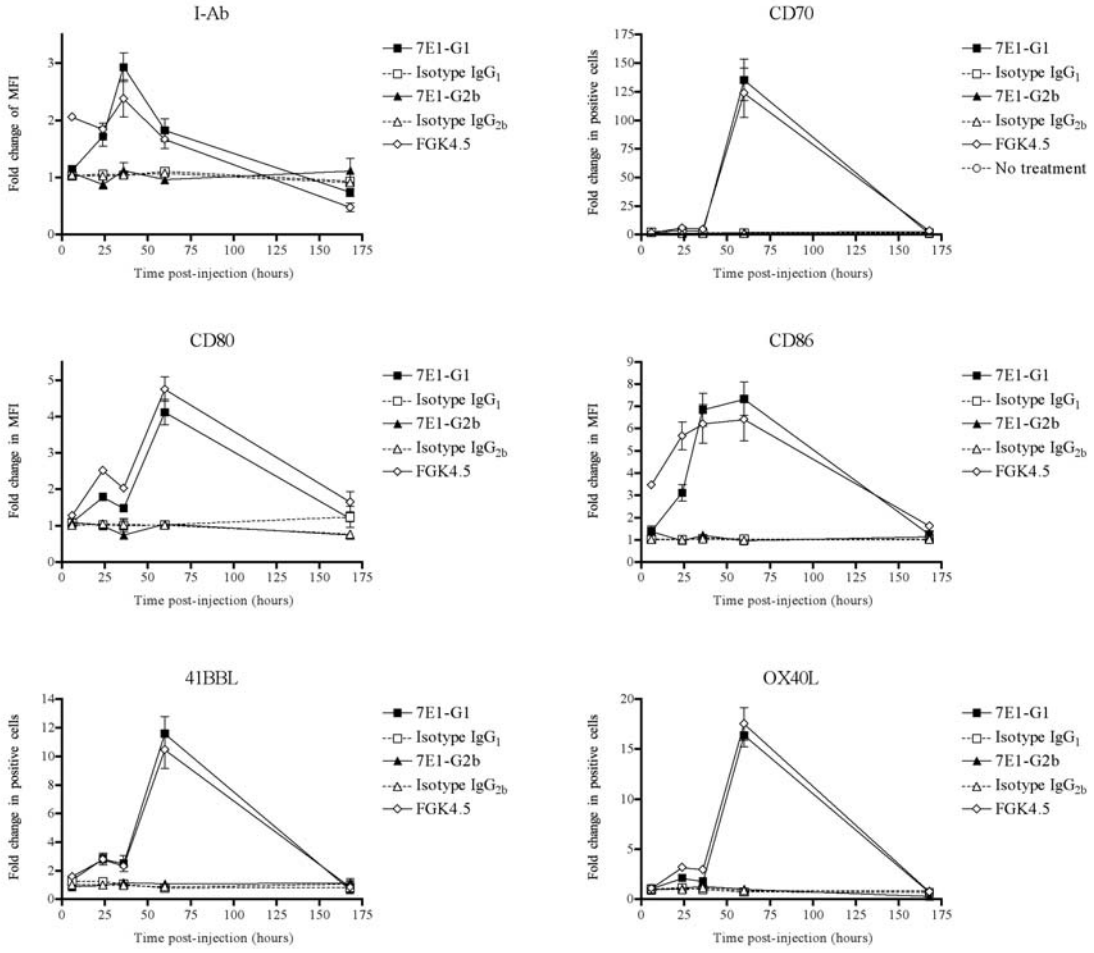

Absolute number of B cells coated with 7E1-variants
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
0
Total cell number
0
10
20
30
40
50
60
Days post-injection
7E1-G1 + CTLA4-Ig
7E1-G2b + CTLA4-Ig

**B.**


Percentage of B cells coated with 7E1-variants
100
75
50
25
0
Percentage of total cells
0
10
20
30
40
50
60
Days post-injection
7E1-G1 + CTLA4-Ig
7E1-G2b + CTLA4-Ig

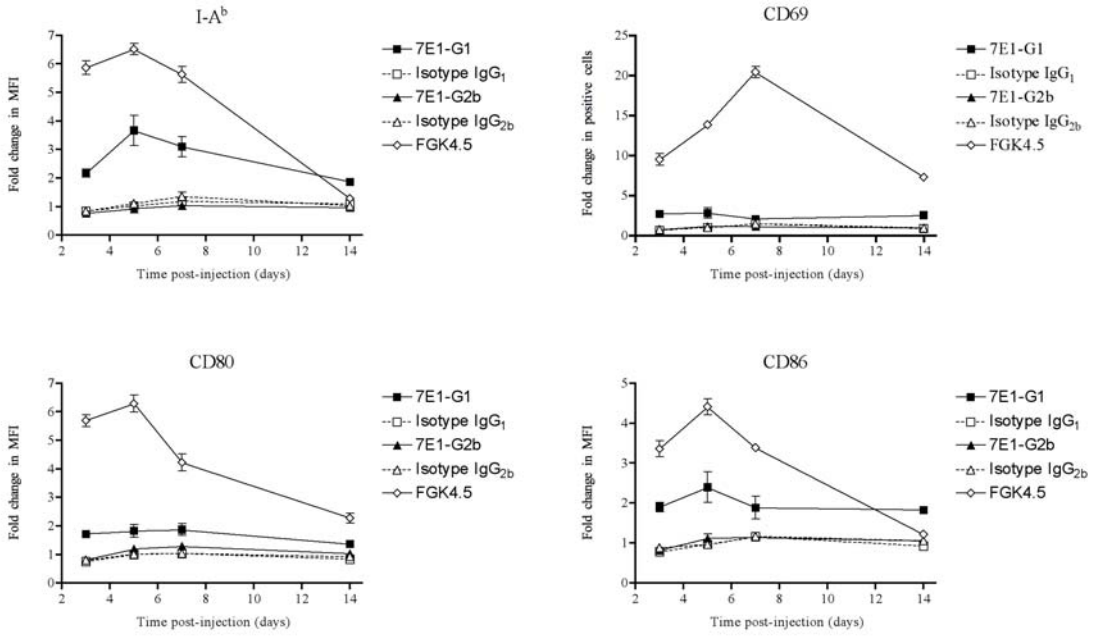
# Figure 3.7

**A.**

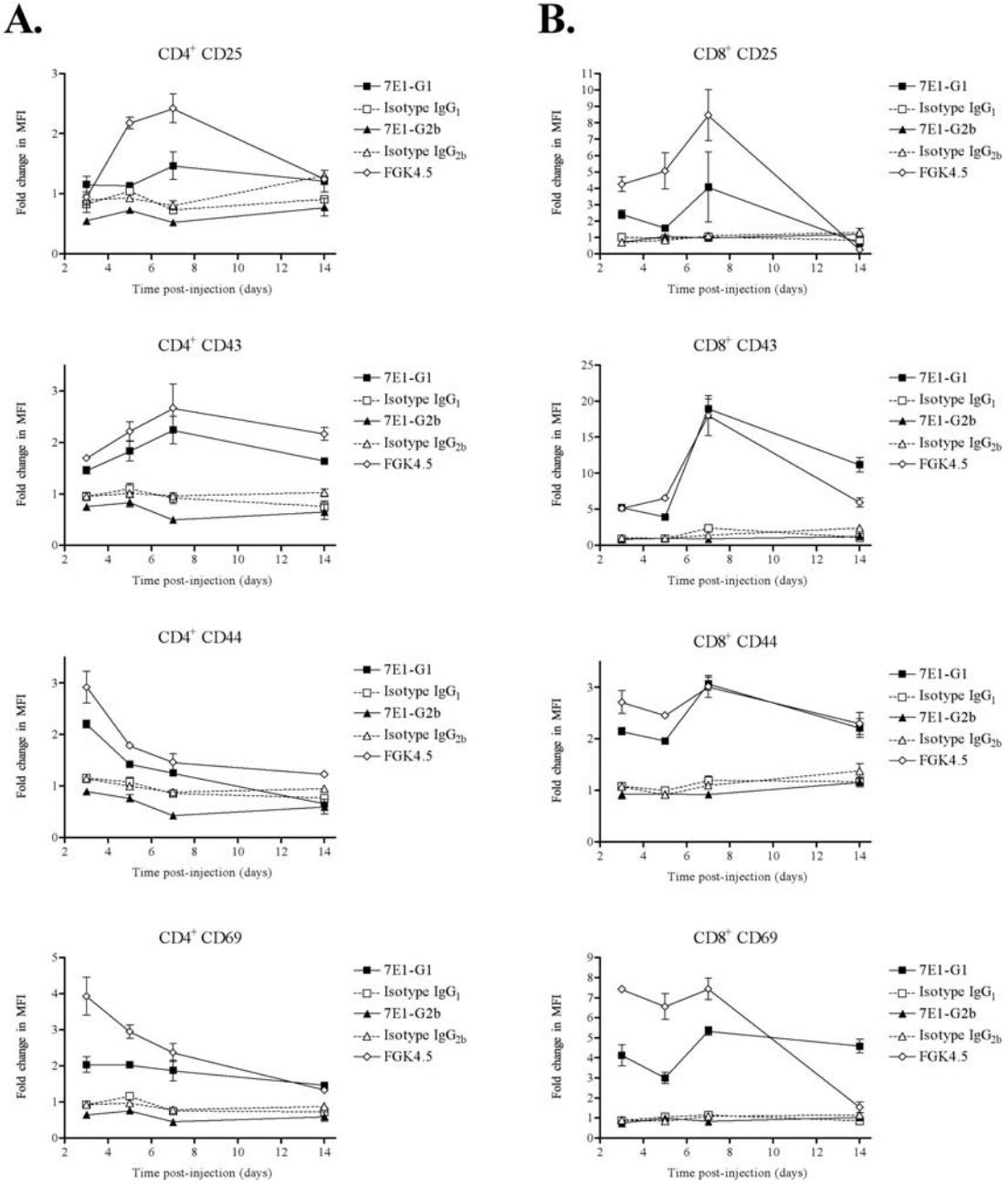

I-Ab
Fold change of MFI
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
CD70
Fold change in positive cells
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
No treatment
CD80
Fold change in MFI
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
CD86
Fold change in MFI
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
41BBL
Fold change in positive cells
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5
OX40L
Fold change in positive cells
Time post-injection (hours)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5

# Figure 3.7

## B.


I-A$^b$
Fold change in MFI
Time post-injection (days)
7E1-G1
Isotype IgG$_1$
7E1-G2b
Isotype IgG$_{2b}$
FGK4.5
CD69
Fold change in positive cells
Time post-injection (days)
7E1-G1
Isotype IgG$_1$
7E1-G2b
Isotype IgG$_{2b}$
FGK4.5
CD80
Fold change in MFI
Time post-injection (days)
7E1-G1
Isotype IgG$_1$
7E1-G2b
Isotype IgG$_{2b}$
FGK4.5
CD86
Fold change in MFI
Time post-injection (days)
7E1-G1
Isotype IgG$_1$
7E1-G2b
Isotype IgG$_{2b}$
FGK4.5

# Figure 3.8


A.
B.
CD4+ CD25
CD8+ CD25
CD4+ CD43
CD8+ CD43
CD4+ CD44
CD8+ CD44
CD4+ CD69
CD8+ CD69
Fold change in MFI
Time post-injection (days)
7E1-G1
Isotype IgG1
7E1-G2b
Isotype IgG2b
FGK4.5

# Figure 3.9

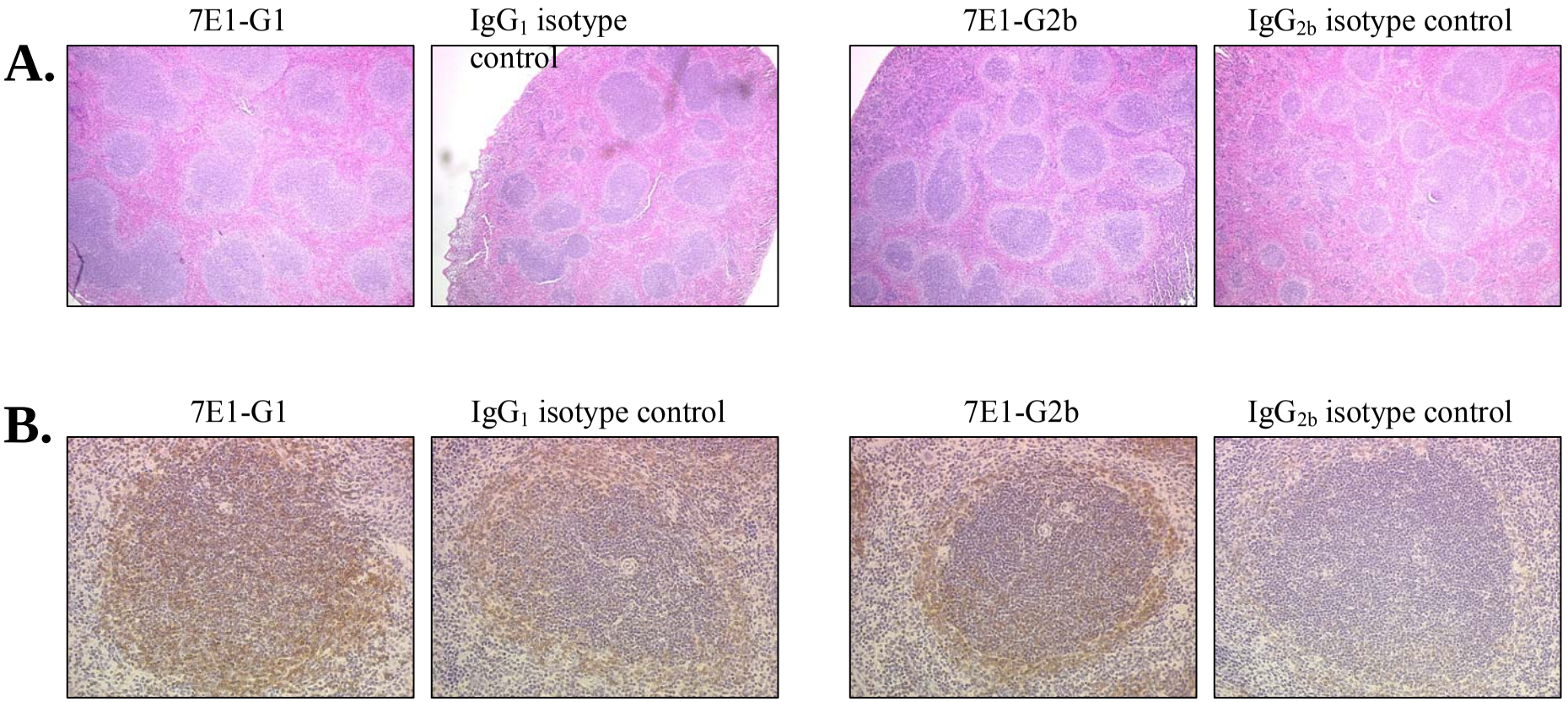
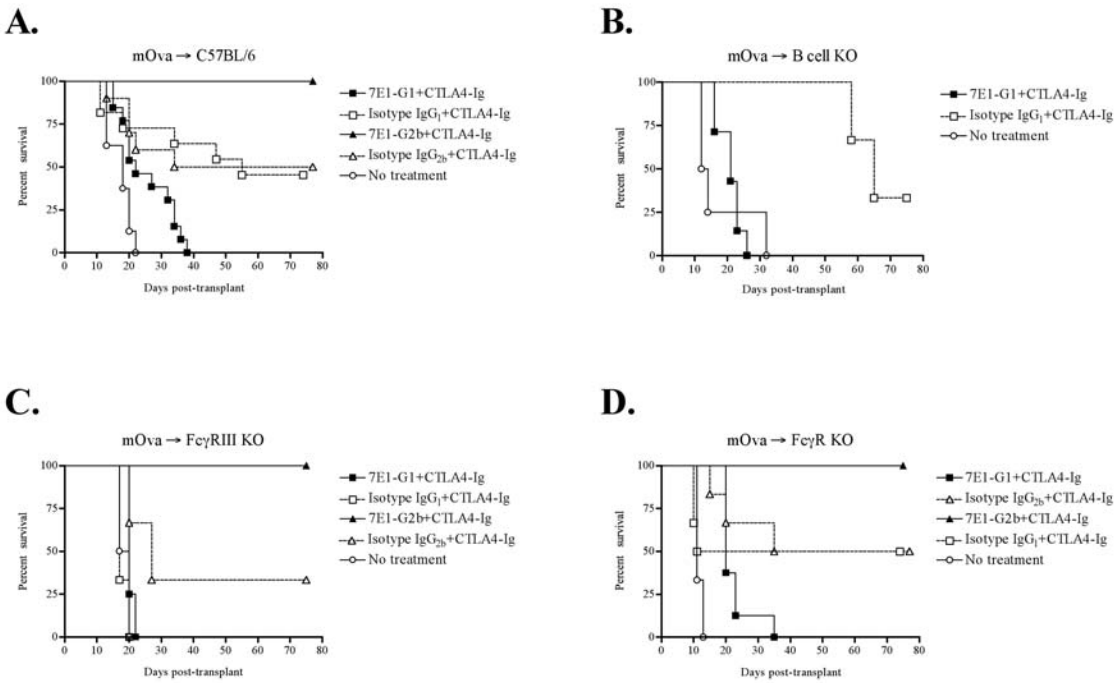

A.
7E1-G1
IgG1 isotype control
7E1-G2b
IgG2b isotype control
B.
7E1-G1
IgG1 isotype control
7E1-G2b
IgG2b isotype control

**Figure 3.10**


A.
mOva → C57BL/6
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
Isotype IgG1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
No treatment
B.
mOva → B cell KO
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
Isotype IgG1+CTLA4-Ig
No treatment
C.
mOva → FcγRIII KO
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
Isotype IgG1+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
No treatment
D.
mOva → FcγR KO
Percent survival
Days post-transplant
7E1-G1+CTLA4-Ig
Isotype IgG2b+CTLA4-Ig
7E1-G2b+CTLA4-Ig
Isotype IgG1+CTLA4-Ig
No treatment

# Chapter 4

# Discussion

*Introduction.*

This thesis examined two issues surrounding the blockade of the CD40 signaling pathway for the experimental induction of murine transplantation tolerance. First, anti-CD40 blockade can synergize with CTLA4-Ig to induce and maintain immune tolerance as effectively as historically seen with anti-CD154 and CTLA4-Ig. Secondly, there are distinct isotype specific differences with regards to function of anti-CD40 binding antibodies.

Considerable interest and effort has been placed in blocking costimulation via the CD40/CD154 signaling pathway using anti-CD154 antibodies. Although this approach is effective in mice, thromboembolic side effects observed in humans have hampered its progression through clinical trials. Nevertheless, the impact on graft survival of blocking this pathway has shown compelling utility, thus, appropriately designed anti-CD40 antibodies may provide a suitable alternative. We investigated two isoforms of a novel monoclonal rat anti-murine CD40 antibody, 7E1-G1, an $IgG_1$ isotype, and 7E1-G2b, an $IgG_{2b}$ isotype, for characteristics mirroring anti-CD154. 7E1-G2b was as effective as anti-CD154 when used in concert with CTLA4-Ig in promoting both allogeneic bone marrow chimerism and skin graft survival, while 7E1-G1 was not. The protection was not due to depletion of CD40 bearing antigen-presenting cells. Naïve mice injected with 7E1-G1, but not anti-CD154 or 7E1-G2b, show maturation of B cells and dendritic cells along with marked proliferation of B cells and T cells. Although B cells showed significant activation in 7E1-G1 treated mice and may have contributed to the overall stimulated immune environment, this may not have been sufficient for graft rejection as both wild-type and B cell deficient mice promptly rejected allogeneic skin grafts when

treated with 7E1-G1 and CTLA4-Ig. *In vitro* proliferation assays to measure the agonist properties of the two 7E1 variants revealed similar weak responses when plate-bound. However, when added as a soluble stimulus, 7E1-G1 but not 7E1-G2b led to strong proliferation, suggesting interactions mediated by the Fc region. Mice deficient in either the inhibitory FcγRIIb receptor, the activating FcγRIII receptor, or the common Fc signaling chain, FcεRγ1, showed no difference in skin graft survival compared to wild type mice when treated with 7E1-G1 or 7E1-G2b and CTLA4-Ig. These data suggest that an appropriately designed anti-CD40 antibody can promote graft survival as well as anti-CD154, making 7E1-G2b an attractive substitute in murine models of costimulation blockade-based tolerance regimens.

*Blockade of the CD40 Signaling Pathway: Optimistic Consternation.*

The CD40 pathway has been a therapeutic target of great interest, but also one of considerable frustration. Interactions between CD40 and its ligand CD154 play crucial roles in many aspects of the immune response including induction of B7 molecules and IL-12 by APC, macrophage effector functions and immunoglobulin class switching of B cells (176). In rodents, anti-CD154 monoclonal antibodies potently inhibit rejection and induce some measure of tolerance in stringent models (121, 141, 177-186). Several groups have used transient CD40 and CD28 blockade to induce mixed chimerism and robust donor-specific tolerance in mice (131, 187-189). This has been particularly effective in promoting selective deletion of naïve donor-reactive T cells (187-189). Similar approaches show promise in non-human primates, but challenges remain (95, 96, 103, 122, 190).

It has been postulated that antibodies bound to CD154 serve to specifically deplete activated allogeneic T cells (137). Monk, et al. provided evidence to support this hypothesis, showing that the prolongation of graft survival using anti-CD154 and rapamycin is dependant on both complement and Fc receptor mediated mechanisms in a fully mismatched skin transplant model. Using a traceable population of H-Y specific transgenic $CD8^+$ T cells, they went on to describe that $F(ab')_2$ fragments of anti-CD154 were unable to prevent the development of the H-Y response. However, the half-life of $F(ab')_2$ fragments *in vivo* are known to be much shorted than fully intact immunoglobulin (191). This often necessitates using much higher quantities of the $F(ab')_2$ to achieve the same effect therapeutic effects (192). Another possible confounder to the interpretation could be the use of rapamycin in conjunction with anti-CD154 in this study. Rapamycin diminishes the response to IL-2 by binding the cytosolic protein FKBP12 and inhibiting the mTOR pathway (193). This not only prevents T cell activation and proliferation, but has also been shown to promote apoptosis (194). The combined effects of anti-CD154 and rapamycin may present a phenotype that can be interpreted as antibody mediated depletion, even if this is not an intrinsic characteristic of the anti-CD154 monoclonal antibody itself. The data presented in this thesis strongly supports the interpretation that anti-CD40 binding antibodies can act in the absence of direct cellular depletion, thus likely resulting in regulation and not direct depletion of allospecific T cell.

Interrupting CD40 signals to antigen presenting cells during T cell priming has been shown to have profound impacts on the dynamics of the T cell-APC interaction. Among these include limiting the up-regulation of activation markers such as CD80 and CD86 (141), decreasing the production of proinflammatory cytokines such as IL-8, IL-

12, MIP-1α and TNFα (142, 143) as well as shortening the duration of the cognate interaction (144). CD40 signaling is important to APC survival and is believed to involve the up-regulation of Bcl-2 and Bcl-xl (195-197). Although 7E1-G2b did not lead to the active depletion of CD40 expressing APCs, the blockade of survival signals normally provided by the CD40 pathway may be just as detrimental to APC longevity and the duration of antigen presentation (198). Further studies are clearly warranted to characterize the phenotype of antigen presenting cells during the establishment of tolerance using either anti-CD40 or anti-CD154 antibodies.

*Therapeutic targets down-stream of the CD40 pathway.*

The CD40 pathway influences a variety of cellular processes including cell division, survival and differentiation. The cytoplasmic tail of CD40 has three TRAF-interacting motifs (TIMs). Activation of CD40 leads to the recruitment of tumor necrosis factor receptor-associated factor (TRAF) family members, TRAF2, TRAF3, and TRAF6 as well as JAK3 (176). The binding of these adaptor proteins activate kinase cascades that in turn activate signal transduction pathways such as NIK/NF-kB, JNK, p38, ERK, and PI3K (61). The specific function of the TRAFs during CD40 signaling has been described for B cells and dendritic cells. The binding of TRAF2 and/or TRAF3 but not TRAF6 is essential for CD40-mediated class switching in B cells (62), while TRAF6 has been implicated in affinity maturation and plasma cell generation (63). In dendritic cells, TRAF6 activates the MAP kinases JNK, ERK and p38. Whereas p38 enhances the production of IL-12 p40 (64), ERK signals enhance IL-10 secretion (199-201). TRAF2 and TRAF3 function in concert through NIK and PI3 kinase leading to activation of

NFκB pathways that have been implicated in regulating the expression of activation genes and influencing cell survival (176, 202). There is a role for TRAF-independent signaling via CD40 as that many CD40 dependent functions are retained in the absence of TRAF signaling, including up-regulation of CD80 and CD86. Thus, the CD40 cascade appears to be cell-specific and these studies highlight the need to perform experiments directly on the cells of interest *in vivo* to define the signaling events that are responsible for the functional outcome.

There is an expanding list of selective pharmacological inhibitors that interrupt signaling downstream from CD40, including inhibitors of p38, ERK, and JNK (203-205). While these pathways are not completely unique to CD40 signaling, they nonetheless offer potential therapeutic targets for tolerance induction. If the specific signaling events that are vital to the promotion of T cell inactivation can be defined, this may provide the opportunity to more selectively interrupt the CD40 costimulatory pathway.

*On the role of antibody isotype.*

We compared the efficacy of two isoforms of a novel murine anti-CD40 antibody, 7E1-G1 and 7E1-G2b. Both antibodies have an equivalent affinity for their ligand CD154, and are able to inhibit the interaction between CD40 and CD154 to the same extent. This was confirmed by both peptide sequencing and PCR (173). Studies have proposed that the position of antibody binding on CD40 relative to the natural ligand CD154 is an accurate predictor of whether it will function as an agonist or antagonist (155-157). If the possibility remains that there are differences in the variable region, it does not appear to alter the binding epitope. The different biological functions of these

two antibodies, 7E1-G2b effectively promotes allogeneic graft survival while 7E1-G1 does not, is likely due to other mechanisms.

Predicting how an antibody will function *in vivo* is confounded by many factors, most of which are beyond the scope of this study but still bear mentioning. Studies have shown that the flexibility of the antibody hinge region significantly differs between the various murine isotypes (167, 206). $IgG_1$ was found to be the most rigid, while $IgG_{2b}$ had the greatest rotational freedom and flexibility. Due to the restrictions of steric hindrance, these characteristics can impact the biological function. In certain systems, antibody mediated CD40 signaling has been found to be epitope-independent and occurs predominantly by aggregating the normally monomeric CD40 molecules on the cell membrane (158).

A study by Scallon, et al. described two neutralizing anti-TNF monoclonal antibodies that appeared to be functionally monovalent for TNF binding despite containing two antigen binding sites (166). They hypothesized that this was due to steric hindrance from one TNF molecule binding to one arm of the antibody, which then prevented the binding of a second TNF molecule to the other. When the antibodies were engineered with an additional immunoglobulin domain near the hinge region, which introduced extra space and flexibility, the modified antibodies were then capable of binding two TNF molecules simultaneously. A related physical effect could be occurring for the 7E1 variants. 7E1-G1 would be expected to have less rotational freedom and be more rigid than would 7E1-G2b. This may translate to 7E1-G1 having a greater ability to aggregate and pull together the membrane associated CD40 monomers into close enough proximity to initiate signal transduction.

Although we did not find any significant effects associated with the deficiency of either the inhibitory or the activating Fc receptors alone, this does not completely exclude the possibility that Fc receptors are involved. Fc receptors may interact with the bound 7E1 variant antibody either on the same cell or on neighboring cells. This could aid in the antibody-mediated aggregation of CD40 initiating signaling of the pathway. Although going against the reported affinity of Fc receptors to the $IgG_1$ verses $IgG_{2b}$ isotypes, this interaction may preferentially favor 7E1-G1, causing the apparent agonism seen with this antibody and not 7E1-G2b.

Several indirect measures of CD40 agonism of the 7E1 variants were used in this thesis, including the induction of proliferation *in vivo* and *in vitro*, activation marker expression, and graft rejection. To more accurately define the characteristics of these agents western blot analysis of the downstream signal transduction events, as outlined above, could be performed to directly determine if CD40 triggering is actually occurring. It is possible that due to isotype specific differences, these two antibodies differentially activate the CD40 signaling pathway. The human anti-CD40 antibody Chi220 has been shown to be a partial CD40 agonist and this incomplete activation has been proposed to induce apoptosis and immune attenuation (113). 7E1-G2b was shown to be capable of supporting *in vitro* proliferation when plate bound, but not when soluble. Thus, the mechanism of 7E1-G2b may be to function as a partial CD40 agonist. Real-time PCR or gene-chip analysis of the cellular changes occurring during stimulation with either isotype could help to present a full picture of what is occurring. Further investigation of the possible differences in CD40 signaling induced by these 7E1 variants may help to better explain their divergent *in vivo* efficacies.

Additional hypotheses could be proposed to explain the distinct characteristics of the 7E1 variants, however testing the validity of them was often beyond the scope of the current study. The flow cytometry based assay used to comparing the binding affinity was developed as a substitute for the more accurate and definitive BiaCore technique that was unavailable. The role of complement was not explored, although the two isotypes have different abilities to interact and activate this system. The analysis of Fc receptor interaction was also incomplete. The complete Fc receptor deficient transgenic mouse, lacking all expression of both activating and inhibitory receptors, was not readily available. This mouse would have allowed the discrete examination of whether Fc receptors are involved in the antibody-mediated aggregation of CD40 monomers to initiate signaling. Finally, generating $F(ab')_2$ of each of the 7E1 variants would have allowed for a focused comparison of the variable regions of each antibody in the absence of either Fc receptor or complement mediated confounding effects. However, due to limiting quantities of the antibodies performing these worthwhile experiments were not possible.

*General conclusions.*

Most immunosuppressive drugs seek to completely prevent all immunological responses from occurring, whether helpful or harmful. However, the robustness and diversity of the immune system makes this often an impossibility. Transient clinical strategies must be designed to preserve immunocompetency against infection and malignancy, but also to preserve specific graft tolerance. The costimulatory blockade strategies seek to play an active role in altering the programming of the responding T cell,

as opposed to ablating them all together. Current therapeutics, such as CTLA4-Ig and anti-CD154 monoclonal antibodies, has shown great promise in achieving these outcomes. However, clinical translation of anti-CD154 has been hampered by serious safety concerns. The work presented in this thesis suggests that an appropriately designed anti-CD40 antibody, with proper examination of the isotype, can serve as a suitable alternative. These findings further emphasize the overall importance of the immunosuppressive effects of blocking the CD40 pathway itself as compared to the specific mode with which it is accomplished. Further research exploring what elements of the CD40 signaling pathway are most vital to tolerance may provide targets for even more focused reagents.

*Epilogue*

*The interaction between a T cell and its antigen-presenting cell can be envisioned as akin to a conversation amongst long-lost friends. It begins with a seemingly chance encounter somewhere in the lymphatics. The APC, having just arrived from an exciting journey, longs to find just the right T cell with which to share its story. In spite of a terribly crowded, chaotic room, that special T cell is somehow found. The dialog begins with an intense discussion over a bit of peptide between MHC and TCR. The T cell shares its initial set of costimulatory opinions on the subject and the APC responds in kind. The issues become more focused and the tête-à-tête takes on a synaptic quality. There is a passionate exchange of receptors, ligands and an indulgence of cytokines. And then, alas, the T cell makes the fateful decision that is it time to move on, to go out into the efferent beyond and see for itself these wonders the APC has spoken so fondly of. The APC is, sadly, remains behind. Alone, in a crowded room, it is left to ponder the meaning of it all, as it succumbs to its own inevitable, apoptotic demise.*

## References


1. Toledo-Pereyra, L.H., and A.H. Toledo. 2005. 1954. *J Invest Surg* 18:285-290.
2. 2007. 2007 Annual Report of the U.S. Organ Procurement and Transplantation Network and the Scientific Registry of the Transplant Recipients: Transplant Data 1997-2006. In Health Resources and Services Administration, Healthcare Systems Bureau, Division of Transplantation, Rockville, MD.
3. Meier-Kriesche, H.U., J.D. Schold, T.R. Srinivas, and B. Kaplan. 2004. Lack of Improvement in Renal Allograft Survival Despite a Marked Decrease in Acute Rejection Rates Over the Most Recent Era. *American Journal of Transplantation* 4:378-383.
4. Paul, L.C. 1995. Chronic renal transplant loss. *Kidney Int* 47:1491-1499.
5. Streblow, D.N., S.L. Orloff, and J.A. Nelson. 2007. Acceleration of allograft failure by cytomegalovirus. *Curr Opin Immunol* 19:577-582.
6. Reichrath, J., and B. Nurnberg. 2008. Solar UV-radiation, vitamin D and skin cancer surveillance in organ transplant recipients (OTRs). *Adv Exp Med Biol* 624:203-214.
7. Abbasoglu, O., M.F. Levy, B.B. Brkic, G. Testa, D.R. Jeyarajah, R.M. Goldstein, B.S. Husberg, T.A. Gonwa, and G.B. Klintmalm. 1997. Ten years of liver transplantation: an evolving understanding of late graft loss. *Transplantation* 64:1801-1807.
8. Reding, R., and H.F. Davies. 2004. Revisiting liver transplant immunology: from the concept of immune engagement to the dualistic pathway paradigm. *Liver Transpl* 10:1081-1086.

9. Puccetti, P. 2007. On watching the watchers: IDO and type I/II IFN. *Eur J Immunol* 37:876-879.

10. Wood, K.J., and S. Sakaguchi. 2003. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* 3:199-210.

11. von Rango, U. 2008. Fetal tolerance in human pregnancy--a crucial balance between acceptance and limitation of trophoblast invasion. *Immunol Lett* 115:21-32.

12. Matzinger, P. 1998. An innate sense of danger. *Seminars in Immunology* 10:399-415.

13. Bhandari, M., and A. Tewari. 1997. Is transplantation only 100 years old? *Br J Urol* 79:495-498.

14. Peltier, L.F. 1997. Patron saints of medicine. *Clin Orthop Relat Res* 374-379.

15. Tewari, M.S., H.S. 2005. Sushruta: 'The Father of Indian Surgery'. *Indian Journal of Surgery* 67:229-230.

16. Zimbler, M.S. 2001. Gaspare Tagliacozzi (1545-1599): renaissance surgeon. *Arch Facial Plast Surg* 3:283-284.

17. Brent, L. 1997. A History of Transplantation Immunology. Academic Press, San Diego, CA.

18. Medawar, P.B. 1944. The Behavior and Fate of Skin Autografts and Skin Homografts in Rabbits. *The Journal of Anatomy* 78:176-199.

19. Medawar, P.B. 1945. A Second Study of the Behavior and Fate of Skin Homografts in Rabbits. *The Journal of Anatomy* 79:157-176.

20. Mitchison, N.A. 1955. Studies on Immunological Response to Foreign Tumor Transplants in Mouse; Role of Lymph Node cells in Conferring Immunity by Adoptive Transfer. *J. Exp. Med.* 102:157-177.

21. Billingham, R.E., L. Brent, and P.B. Medawar. 1954. Quantitative Studies on Tissue Transplantation Immunity. II. The Origin, Strength, and Duration of Actively and Adoptively Acquired Immunity. *Proceedings of the Royal Society of London Series B* 143:58-80.

22. Kohler, G., and C. Milstein. 1975. Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. *Nature* 256:495-497.

23. Krieger, N.R., D.P. Yin, and C. Garrison Fathman. 1996. CD4+ but not CD8+ cells are essential for allorejection. *J Exp Med* 184:2013-2018.

24. Hall, B.M. 1991. Cells mediating allograft rejection. *Transplantation* 51:1141-1151.

25. Shelton, M.W., L.A. Walp, J.T. Basler, K. Uchiyama, and D.W. Hanto. 1992. Mediation of skin allograft rejection in scid mice by CD4+ and CD8+ T cells. *Transplantation* 54:278-286.

26. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination: Paralysis and induction involve the recognition of one and two determinants on an antigen, respectively. *Science* 169:1042-1049.

27. Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21:139-176.

28. Heath, W.R., and F.R. Carbone. 2001. Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol* 1:126-134.

29. Medzhitov, R. 2001. CpG DNA: security code for host defense. *Nat Immunol* 2:15-16.

30. Sporri, R., and C. Reis e Sousa. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* 6:163-170.

31. Rescigno, M., F. Granucci, S. Citterio, M. Foti, and P. Ricciardi-Castagnoli. 1999. Coordinated events during bacteria-induced DC maturation. *Immunol Today* 20:200-203.

32. Sallusto, F., and A. Lanzavecchia. 1999 Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

33. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol* 21:685-711.

34. Schwartz, R.H. 2003. T cell anergy. *Annu Rev Immunol* 21:305-334.

35. Williams, M.E., A.H. Lichtman, and A.K. Abbas. 1990. Anti-CD3 antibody induces unresponsiveness to IL-2 in Th1 clones but not in Th2 clones. *J. Immunol.* 144:1208-1214.

36. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302-319.

37. Radvanyi, L.G., Y. Shi, H. Vaziri, A. Sharma, R. Dhala, G.B. Mills, and R.G. Miller. 1996. CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response. *J. Immunol.* 156:1788-1798.

38. June, C.H., J.A. Ledbetter, P.S. Linsley, and C.B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol Today* 11:211-216.

39. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 251:313-316.

40. Harding, F., J. McArthur, J. Gross, D. Raulet, and J. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T cell clones. *Nature* 356:607-609.

41. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small resting T lymphocytes. *J. Exp. Med.* 175:353-360.

42. Wenner, C.A., M.L. Guler, S.E. Macatonia, A. O'Garra, and K.M. Murphy. 1996. Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. *J. Immunol.* 156:1442-1447.

43. Thompson, C.B., T. Lindsten, J.A. Ledbetter, S.L. Kunkel, H.A. Young, S.G. Emerson, J.M. Leiden, and C.H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA* 86:1333-1337.

44. Sperling, A.I., J.A. Auger, B.D. Ehst, I.C. Rulifson, C.B. Thompson, and J.A. Bluestone. 1996. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J. Immunol.* 157:3909-3917.

45. Noel, P.J., L.H. Boise, J.M. Green, and C.B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J. Immunol.* 157:636-642.

46. Linsley, P.S., W. Brady, M. Urnes, L. Grosmaire, N. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174:561-569.

47. Brunet, J.F., F. Denizot, M.F. Luciani, D.M. Roux, M. Suzan, M.G. Mattei, and P. Golstein. 1987. A new member of the immunoglobulin superfamily--CTLA-4. *Nature* 328:267-270.

48. 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.

49. Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.

50. De Smedt, T., B. Pajak, G.G. Klaus, R.J. Noelle, J. Urbain, O. Leo, and M. Moser. 1998. Antigen-specific T lymphocytes regulate lipopolysaccharide-induced apoptosis of dendritic cells in vivo. *J Immunol* 161:4476-4479.

51. Van Parijs, L., V.L. Perez, A. Biuckians, R.G. Maki, C.A. London, and A.K. Abbas. 1997. Role of interleukin 12 and costimulators in T cell anergy in vivo. *Journal of Experimental Medicine* 186:1119-1128.

52. Grewal, I.S., and R.A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annual Review of Immunology* 16:111-135.

53. Andre, P., K.S. Prasad, C.V. Denis, M. He, J.M. Papalia, R.O. Hynes, D.R. Phillips, and D.D. Wagner. 2002. CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nat Med* 8:247-252.

54. Cayabyab, M., J.H. Phillips, and L.L. Lanier. 1994. CD40 preferentially costimulates activation of CD4+ T lymphocytes. *J. Immunol.* 152:1523-1531.

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

56. Kiener, P.A., D.P. Moran, B.M. Rankin, A.F. Wahl, A. Aruffo, and D. Hollenbaugh. 1995. Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J. Immunol.* 155:4917-4925.

57. Tian, L., R. Noelle, and D. Lawrence. 1995. Activated T cells enhance nitric oxide production by murine splenic macrophages through gp39 and LFA-1. *Eur. J. Immunol.* 25:306-309.

58. Armitage, R.J., C.R. Maliszewski, M.R. Alderson, K.H. Grabstein, M.K. Spriggs, and W.C. Fanslow. 1993. CD40L: a multi-functional ligand. *Semin Immunol* 5:401-412.

59. Suttles, J., M. Evans, R.W. Miller, J.C. Poe, R.D. Stout, and L.M. Wahl. 1996. T cell rescue of monocytes from apoptosis: role of the CD40-CD40L interaction and

requirement for CD40-mediated induction of protein tyrosine kinase activity. *Journal of Leukocyte Biology* 60:651-657.

60. Stout, R.D., and J. Suttles. 1996. The many roles of CD40 in cell-mediated inflammatory responses. *Immunol today* 17:487-492.

61. Dempsey, P.W., S.E. Doyle, J.Q. He, and G. Cheng. 2003. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev* 14:193-209.

62. Jabara, H., D. Laouini, E. Tsitsikov, E. Mizoguchi, A. Bhan, E. Castigli, F. Dedeoglu, V. Pivniouk, S. Brodeur, and R. Geha. 2002. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity* 17:265-276.

63. Ahonen, C., E. Manning, L.D. Erickson, B. O'Connor, E.F. Lind, S.S. Pullen, M.R. Kehry, and R.J. Noelle. 2002. The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. *Nat Immunol* 3:451-456.

64. Mackey, M.F., Z. Wang, K. Eichelberg, and R.N. Germain. 2003. Distinct contributions of different CD40 TRAF binding sites to CD154-induced dendritic cell maturation and IL-12 secretion. *Eur J Immunol* 33:779-789.

65. Grewal, I.S., and R.A. Flavel. 1996. A central role of CD40 ligand in the regulation of CD4+ T-cell responses. *Immunology Today* 17:410-414.

66. Borrow, P., A. Tishon, S. Lee, J. Xu, I.S. Grewal, M.B. Oldstone, and R.A. Flavell. 1996. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J. Exp. Med.* 183:2129-2142.

67. Schoenberger, S.P., R.E. Toes, E.I. van der Voort, R. Offringa, and C.J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480-483.

68. Rhen, T., and J.A. Cidlowski. 2005. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med* 353:1711-1723.

69. Allison, A.C. 2000. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology* 47:63-83.

70. Oka, T., and N. Yoshimura. 1996. Immunosuppression in organ transplantation. *Jpn J Pharmacol* 71:89-100.

71. Sayegh, M.H., and C.B. Carpenter. 2004. Transplantation 50 years later--progress, challenges, and promises. *N Engl J Med* 351:2761-2766.

72. Kahan, B.D. 1989. Cyclosporine. *New England Journal of Medicine* 321:1725-1738.

73. Allison, A.C., and E.M. Eugui. 2000. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* 47:85-118.

74. Liu, J., J.D. Farmer, Jr., W.S. Lane, J. Friedman, I. Weissman, and S.L. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 66:807-815.

75. Sehgal, S.N. 2003. Sirolimus: its discovery, biological properties, and mechanism of action. *Transplant Proc* 35:7S-14S.

76. Pritchard, D.I. 2005. Sourcing a chemical succession for cyclosporin from parasites and human pathogens. *Drug Discov Today* 10:688-691.

77. Lubel, J.S., A.G. Testro, and P.W. Angus. 2007. Hepatitis B virus reactivation following immunosuppressive therapy: guidelines for prevention and management. *Intern Med J* 37:705-712.

78. Gutierrez-Dalmau, A., and J.M. Campistol. 2007. Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. *Drugs* 67:1167-1198.

79. Chan, M., and G.J. Pearson. 2007. New advances in antirejection therapy. *Curr Opin Cardiol* 22:117-122.

80. Penfornis, A., and S. Kury-Paulin. 2006. Immunosuppressive drug-induced diabetes. *Diabetes Metab* 32:539-546.

81. Lafferty, K.J., and A.J. Cunningham. 1975. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci* 53:27-42.

82. Lenschow, D., Y. Zeng, J. Thistlethwaite, A. Montag, W. Brady, M. Gibson, P. Linsley, and J. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science* 257:789-792.

83. Linsley, P.S., P.M. Wallace, J. Johnson, M. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh, and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science* 257:792-795.

84. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J.A. Ledbetter, and P.S. Linsley. 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* 177:165-173.

85. Bluestone, J.A., E.W. St Clair, and L.A. Turka. 2006. CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* 24:233-238.

86. Metz, D.P., D.L. Farber, T. Taylor, and K. Bottomly. 1998. Differential role of CTLA-4 in regulation of resting memory versus naive CD4 T cell activation. *J Immunol* 161:5855-5861.

87. Vincenti, F. 2008. Costimulation blockade in autoimmunity and transplantation. *J Allergy Clin Immunol* 121:299-306; quiz 307-298.

88. Reynolds, J., K. Shojania, and C.A. Marra. 2007. Abatacept: a novel treatment for moderate-to-severe rheumatoid arthritis. *Pharmacotherapy* 27:1693-1701.

89. Kuek, A., B.L. Hazleman, and A.J. Ostor. 2007. Immune-mediated inflammatory diseases (IMIDs) and biologic therapy: a medical revolution. *Postgrad Med J* 83:251-260.

90. Larsen, C.P., T.C. Pearson, A.B. Adams, P. Tso, N. Shirasugi, E. Strobertm, D. Anderson, S. Cowan, K. Price, J. Naemura, J. Emswiler, J. Greene, L.A. Turk, J. Bajorath, R. Townsend, D. Hagerty, P.S. Linsley, and R.J. Peach. 2005. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* 5:443-453.

91. McAdam, A.J., A.N. Schweitzer, and A.H. Sharpe. 1998. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol Rev* 165:231-247.

92. Pentcheva-Hoang, T., J.G. Egen, K. Wojnoonski, and J.P. Allison. 2004. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 21:401-413.

93. Vincenti, F., C. Larsen, A. Durrbach, T. Wekerle, B. Nashan, G. Blancho, P. Lang, J. Grinyo, P.F. Halloran, K. Solez, D. Hagerty, E. Levy, W. Zhou, K. Natarajan, and B. Charpentier. 2005. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 353:770-781.

94. Kirk, A.D., P.J. Blair, D.K. Tadaki, H. Xu, and D.M. Harlan. 2001. The role of CD154 in organ transplant rejection and acceptance. *Philos Trans R Soc Lond B Biol Sci* 356:691-702.

95. Kirk, A.D., D.M. Harlan, N.N. Armstrong, T.A. Davis, Y. Dong, G.S. Gray, X. Hong, D. Thomas, J.H. Fechner, and S.J. Knechtle. 1997. CTLA4-Ig and anti-CD40L prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. USA* 94:8789-8794.

96. Kenyon, N.S., L.A. Fernandez, R. Lehmann, M. Masetti, A. Ranuncoli, M. Chatzipetrou, G. Iaria, D. Han, J.L. Wagner, P. Ruiz, M. Berho, L. Inverardi, R. Alejandro, D.H. Mintz, A.D. Kirk, D.M. Harlan, L.C. Burkly, and C. Ricordi. 1999. Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes* 48:1473-1481.

97. Montgomery, S.P., H. Xu, D.K. Tadaki, A. Celniker, L.C. Burkly, J.D. Berning, F. Cruzata, E.A. Elster, G. Gray, R.L. Kampen, S.J. Swanson, D.M. Harlan, and A.D. Kirk. 2002. Combination induction therapy with monoclonal antibodies specific for CD80, CD86, and CD154 in nonhuman primate renal transplantation. *Transplantation* 74:1365-1369.

98. Buhler, L., I.P. Alwayn, J.Z. Appel, 3rd, S.C. Robson, and D.K. Cooper. 2001. Anti-CD154 monoclonal antibody and thromboembolism. *Transplantation* 71:491.

99. Preston, E.H., H. Xu, K.K. Dhanireddy, J.P. Pearl, F.V. Leopardi, M.F. Starost, D.A. Hale, and A.D. Kirk. 2005. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. *Am J Transplant* 5:1032-1041.

100. Xu, H., S.P. Montgomery, E.H. Preston, D.K. Tadaki, D.A. Hale, D.M. Harlan, and A.D. Kirk. 2003. Studies investigating pretransplant donor-specific blood transfusion, rapamycin, and the CD154-specific antibody IDEC-131 in a nonhuman primate model of skin allotransplantation. *J Immunol* 170:2776-2782.

101. Kean, L.S., S. Gangappa, T.C. Pearson, and C.P. Larsen. 2006. Transplant tolerance in non-human primates: progress, current challenges and unmet needs. *Am J Transplant* 6:884-893.

102. Kanmaz, T., J.J. Fechner, Jr., J. Torrealba, H.T. Kim, Y. Dong, T.D. Oberley, J.M. Schultz, D.D. Bloom, M. Katayama, W. Dar, J. Markovits, W. Schuler, H. Hu, M.M. Hamawy, and S.J. Knechtle. 2004. Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. *Transplantation* 77:914-920.

103. Kean, L.S., A.B. Adams, E. Strobert, R. Hendrix, S. Gangappa, T.R. Jones, N. Shirasugi, M.R. Rigby, K. Hamby, J. Jiang, H. Bello, D. Anderson, K. Cardona, M.M. Durham, T.C. Pearson, and C.P. Larsen. 2007. Induction of Chimerism in

Rhesus Macaques through Stem Cell Transplant and Costimulation Blockade-Based Immunosuppression. *Am J Transplant* 7:320-335.

104. Czapiga, M., A.D. Kirk, and J. Lekstrom-Himes. 2004. Platelets deliver costimulatory signals to antigen-presenting cells: a potential bridge between injury and immune activation. *Exp Hematol* 32:135-139.

105. Inwald, D.P., A. McDowall, M.J. Peters, R.E. Callard, and N.J. Klein. 2003. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circ Res* 92:1041-1048.

106. Crow, A.R., V. Leytin, A.F. Starkey, M.L. Rand, and A.H. Lazarus. 2003. CD154 (CD40 ligand)-deficient mice exhibit prolonged bleeding time and decreased shear-induced platelet aggregates. *J Thromb Haemost* 1:850-852.

107. Haanstra, K.G., J. Ringers, E.A. Sick, S. Ramdien-Murli, E.M. Kuhn, L. Boon, and M. Jonker. 2003. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* 75:637-643.

108. de Vos, A.F., M.J. Melief, D. van Riel, L. Boon, M. van Eijk, M. de Boer, and J.D. Laman. 2004. Antagonist anti-human CD40 antibody inhibits germinal center formation in cynomolgus monkeys. *Eur J Immunol* 34:3446-3455.

109. Pearson, T.C., J. Trambley, K. Odom, D.C. Anderson, S. Cowan, R. Bray, A. Lin, D. Hollenbaugh, A. Aruffo, A.W. Siadak, E. Strobert, R. Hennigar, and C.P. Larsen. 2002. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* 74:933-940.

110. Imai, A., T. Suzuki, A. Sugitani, T. Itoh, S. Ueki, T. Aoyagi, K. Yamashita, M. Taniguchi, N. Takahashi, T. Miura, T. Shimamura, H. Furukawa, and S. Todo.

2007. A novel fully human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys. *Transplantation* 84:1020-1028.

111. Boon, L., J.D. Laman, A. Ortiz-Buijsse, M.T. den Hartog, S. Hoffenberg, P. Liu, F. Shiau, and M. de Boer. 2002. Preclinical assessment of anti-CD40 Mab 5D12 in cynomolgus monkeys. *Toxicology* 174:53-65.

112. Haanstra, K.G., E.A. Sick, J. Ringers, J.A. Wubben, E.M. Kuhn, L. Boon, and M. Jonker. 2005. Costimulation blockade followed by a 12-week period of cyclosporine A facilitates prolonged drug-free survival of rhesus monkey kidney allografts. *Transplantation* 79:1623-1626.

113. Adams, A.B., N. Shirasugi, T.R. Jones, M.M. Durham, E.A. Strobert, S. Cowan, P. Rees, R. Hendrix, K. Price, N.S. Kenyon, D. Hagerty, R. Townsend, D. Hollenbaugh, T.C. Pearson, and C.P. Larsen. 2005. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* 174:542-550.

114. Jenkins, M.K. 1994. The ups and downs of T cell costimulation. *Immunity* 1:443-446.

115. Jenkins, M.K., P.S. Taylor, S.D. Norton, and K.B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147:2461-2466.

116. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248:1349-1356.

117. Yamada, A., A.D. Salama, M. Sho, N. Najafian, T. Ito, J.P. Forman, R. Kewalramani, S. Sandner, H. Harada, M.R. Clarkson, D.A. Mandelbrot, A.H.

Sharpe, H. Oshima, H. Yagita, G. Chalasani, F.G. Lakkis, H. Auchincloss, Jr., and M.H. Sayegh. 2005. CD70 signaling is critical for CD28-independent CD8+ T cell-mediated alloimmune responses in vivo. *J Immunol* 174:1357-1364.

118. Ozkaynak, E., W. Gao, N. Shemmeri, C. Wang, J.C. Gutierrez-Ramos, J. Amaral, S. Qin, J.B. Rottman, A.J. Coyle, and W.W. Hancock. 2001. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat Immunol* 2:591-596.

119. Demirci, G., F. Amanullah, R. Kewalaramani, H. Yagita, T.B. Strom, M.H. Sayegh, and X.C. Li. 2004. Critical role of OX40 in CD28 and CD154-independent rejection. *J Immunol* 172:1691-1698.

120. Wang, J., Z. Guo, Y. Dong, O. Kim, J. Hart, A. Adams, C.P. Larsen, R.S. Mittler, and K.A. Newell. 2003. Role of 4-1BB in allograft rejection mediated by CD8+ T cells. *Am J Transplant* 3:543-551.

121. Larsen, C.P., E.T. Elwood, D.Z. Alexander, S.C. Ritchie, R. Hendrix, C. Tucker-Burden, H.R. Cho, A. Aruffo, D. Hollenbaugh, P.S. Linsley, K.J. Winn, and T.C. Pearson. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381:434-438.

122. Kirk, A.D., L.C. Burkly, D.S. Batty, R.E. Baumgartner, J.D. Berning, K. Buchanan, J.H. Fechner, Jr., R.L. Germond, R.L. Kampen, N.B. Patterson, S.J. Swanson, D.K. Tadaki, C.N. TenHoor, L. White, S.J. Knechtle, and D.M. Harlan. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 5:686-693.

123. Kawai, T., D. Andrews, R.B. Colvin, D.H. Sachs, and A.B. Cosimi. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6:114.

124. Weaver, T.A., A.H. Charafeddine, and A.D. Kirk. 2008. Costimulation blockade: towards clinical application. *Front Biosci* 13:2120-2139.

125. Schoenberger, S.P., R.E.M. Toes, E.I.H. van der Voort, R. Offringa, and C.J.M. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480-483.

126. Staveley-O'Carroll, K., T.D. Schell, M. Jimenez, L.M. Mylin, M.J. Tevethia, S.P. Schoenberger, and S.S. Tevethia. 2003. In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes CD8+ T cell effector function in SV40 T antigen transgenic mice. *J Immunol* 171:697-707.

127. Maxwell, J.R., J.D. Campbell, C.H. Kim, and A.T. Vella. 1999. CD40 activation boosts T cell immunity in vivo by enhancing T cell clonal expansion and delaying peripheral T cell deletion. *Journal of Immunology* 162:2024-2034.

128. Bennett, S.R.M., F.R. Carbone, F. Karamalis, R.A. Flavell, J.F.A.P. Miller, and W.R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393:478-480.

129. Dullforce, P., D.C. Sutton, and A.W. Heath. 1998. Enhancement of T cell-independent immune responses in vivo by CD40 antibodies. *Nat Med* 4:88-91.

130. Hale, G., S.P. Cobbold, H. Waldmann, G. Easter, P. Matejtschuk, and R.R. Coombs. 1987. Isolation of low-frequency class-switch variants from rat hybrid myelomas. *J Immunol Methods* 103:59-67.

131. Adams, A.B., M.M. Durham, L. Kean, N. Shirasugi, J. Ha, M.A. Williams, P.A. Rees, M.C. Cheung, S. Mittelstaedt, A.W. Bingaman, D.R. Archer, T.C. Pearson, E.K. Waller, and C.P. Larsen. 2001. Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *J Immunol* 167:1103-1111.

132. Salomon, B., and J.A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol* 19:225-252.

133. Wekerle, T., J. Kurtz, S. Bigenzahn, Y. Takeuchi, and M. Sykes. 2002. Mechanisms of transplant tolerance induction using costimulatory blockade. *Curr Opin Immunol* 14:592-600.

134. Trambley, J., A.W. Bingaman, A. Lin, E.T. Elwood, S.Y. Waitze, J. Ha, M.M. Durham, M. Corbascio, S.R. Cowan, T.C. Pearson, and C.P. Larsen. 1999. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest* 104:1715-1722.

135. Williams, M.A., J. Trambley, J. Ha, A.B. Adams, M.M. Durham, P. Rees, S.R. Cowan, T.C. Pearson, and C.P. Larsen. 2000. Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. *J Immunol* 165:6849-6857.

136. Tomita, Y., A. Khan, and M. Sykes. 1994. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow

transplantation in mice conditioned with a nonmyeloablative regimen. *J Immunol* 153:1087-1098.

137. Monk, N.J., R.E. Hargreaves, J.E. Marsh, C.A. Farrar, S.H. Sacks, M. Millrain, E. Simpson, J. Dyson, and S. Jurcevic. 2003. Fc-dependent depletion of activated T cells occurs through CD40L-specific antibody rather than costimulation blockade. *Nat Med* 9:1275-1280.

138. Ehst, B.D., E. Ingulli, and M.K. Jenkins. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3:1355-1362.

139. Ravetch, J.V., and S. Bolland. 2001. IgG Fc receptors. *Annu Rev Immunol* 19:275-290.

140. Blair, P.J., J.L. Riley, D.M. Harlan, R. Abe, D.K. Tadaki, S.C. Hoffmann, L. White, T. Francomano, S.J. Perfetto, A.D. Kirk, and C.H. June. 2000. CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. *J Exp Med* 191:651-660.

141. Hancock, W.W., M.H. Sayegh, X.G. Zheng, R. Peach, P.S. Linsley, and L.A. Turka. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc. Natl. Acad. Sci. USA* 93:13967-13972.

142. Cella, M., D. Scheidegger, L.K. Palmer, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of

interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184:747-752.

143. Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184:741-746.

144. Miga, A.J., S.R. Masters, B.G. Durell, M. Gonzalez, M.K. Jenkins, C. Maliszewski, H. Kikutani, W.F. Wade, and R.J. Noelle. 2001. Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur J Immunol* 31:959-965.

145. Kurtz, J., H. Ito, T. Wekerle, J. Shaffer, and M. Sykes. 2001. Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive CD4+ cells. *Am J Transplant* 1:339-349.

146. Quezada, S.A., K. Bennett, B.R. Blazar, A.Y. Rudensky, S. Sakaguchi, and R.J. Noelle. 2005. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol* 175:771-779.

147. Wells, A.D., X.C. Li, Y. Li, M.C. Walsh, X.X. Zheng, Z. Wu, G. Nunez, A. Tang, M. Sayegh, W.W. Hancock, T.B. Strom, and L.A. Turka. 1999. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nature Medicine* 5:1303-1307.

148. Graca, L., K. Honey, E. Adams, S.P. Cobbold, and H. Waldmann. 2000. Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J Immunol* 165:4783-4786.

149. Bruggemann, M., C. Teale, M. Clark, C. Bindon, and H. Waldmann. 1989. A matched set of rat/mouse chimeric antibodies. Identification and biological properties of rat H chain constant regions mu, gamma 1, gamma 2a, gamma 2b, gamma 2c, epsilon, and alpha. *J Immunol* 142:3145-3150.

150. Hale, G., M. Clark, and H. Waldmann. 1985. Therapeutic potential of rat monoclonal antibodies: isotype specificity of antibody-dependent cell-mediated cytotoxicity with human lymphocytes. *J. Immunol.* 134:3056-3061.

151. Bruggemann, M., C. Teale, M. Clark, C. Bindon, and H. Waldmann. 1989. A matched set of rat/mouse chimeric antibodies. *J. Immunol.* 142:3145-3150.

152. Meier-Kriesche, H.U., J.D. Schold, and B. Kaplan. 2004. Long-Term Renal Allograft Survival: Have we Made Significant Progress or is it Time to Rethink our Analytic and Therapeutic Strategies? *American Journal of Transplantation* 4:1289-1295.

153. Paulie, S., B. Ehlin-Henriksson, H. Mellstedt, H. Koho, H. Ben-Aissa, and P. Perlmann. 1985. A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. *Cancer Immunol Immunother* 20:23-28.

154. 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, W.C. Fanslow, J. Belmont, and M.K. Spriggs. 1993. CD40 ligand gene defects

responsible for X-linked hyper-IgM syndrome [see comments]. *Science* 259:990-993.

155. Heath, A.W., W.W. Wu, and M.C. Howard. 1994. Monoclonal antibodies to murine CD40 define two distinct functional epitopes. *Eur J Immunol* 24:1828-1834.

156. Malmborg Hager, A.C., P. Ellmark, C.A. Borrebaeck, and C. Furebring. 2003. Affinity and epitope profiling of mouse anti-CD40 monoclonal antibodies. *Scand J Immunol* 57:517-524.

157. Barr, T.A., and A.W. Heath. 2001. Functional activity of CD40 antibodies correlates to the position of binding relative to CD154. *Immunology* 102:39-43.

158. Schwabe, R.F., S. Hess, J.P. Johnson, and H. Engelmann. 1997. Modulation of soluble CD40 ligand bioactivity with anti-CD40 antibodies. *Hybridoma* 16:217-226.

159. Kaminski, M.S., K. Kitamura, D.G. Maloney, M.J. Campbell, and R. Levy. 1986. Importance of antibody isotype in monoclonal anti-idiotype therapy of a murine B cell lymphoma. A study of hybridoma class switch variants. *J Immunol* 136:1123-1130.

160. Denkers, E.Y., C.C. Badger, J.A. Ledbetter, and I.D. Bernstein. 1985. Influence of antibody isotype on passive serotherapy of lymphoma. *J Immunol* 135:2183-2186.

161. Liu, A.Y., R.R. Robinson, E.D. Murray, Jr., J.A. Ledbetter, I. Hellstrom, and K.E. Hellstrom. 1987. Production of a mouse-human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity. *J Immunol* 139:3521-3526.

162. Waldmann, H., and S. Cobbold. 1998. How do monoclonal antibodies induce tolerance? A role for infectious tolerance? *Annu Rev Immunol* 16:619-644.

163. Fossati-Jimack, L., A. Ioan-Facsinay, L. Reininger, Y. Chicheportiche, N. Watanabe, T. Saito, F.M. Hofhuis, J.E. Gessner, C. Schiller, R.E. Schmidt, T. Honjo, J.S. Verbeek, and S. Izui. 2000. Markedly different pathogenicity of four immunoglobulin G isotype-switch variants of an antierythrocyte autoantibody is based on their capacity to interact in vivo with the low-affinity Fcgamma receptor III. *J Exp Med* 191:1293-1302.

164. Uchida, J., Y. Hamaguchi, J.A. Oliver, J.V. Ravetch, J.C. Poe, K.M. Haas, and T.F. Tedder. 2004. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 199:1659-1669.

165. Nimmerjahn, F., and J.V. Ravetch. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310:1510-1512.

166. Scallon, B., A. Cai, J. Radewonuk, and M. Naso. 2004. Addition of an extra immunoglobulin domain to two anti-rodent TNF monoclonal antibodies substantially increased their potency. *Mol Immunol* 41:73-80.

167. Dangl, J.L., T.G. Wensel, S.L. Morrison, L. Stryer, L.A. Herzenberg, and V.T. Oi. 1988. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J* 7:1989-1994.

168. Kohl, J. 2006. Self, non-self, and danger: a complementary view. *Adv Exp Med Biol* 586:71-94.

169. Deitch, E.A., and M.C. Mancini. 1993. Complement receptors in shock and transplantation. *Arch Surg* 128:1222-1226.

170. Ryan, U.S. 1986. The endothelial surface and responses to injury. *Fed Proc* 45:101-108.

171. Kaneko, Y., F. Nimmerjahn, and J.V. Ravetch. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313:670-673.

172. Clark, E.A., and J.A. Ledbetter. 2005. How does B cell depletion therapy work, and how can it be improved? *Ann Rheum Dis* 64 Suppl 4:iv77-80.

173. Aruffo, A.A., D. Hollenbaugh, A.W. Siadak, K.K. Berry, L. Harris, B.A. Thorne, and J. Bajorath. 1998. Methods of using antibodies against human CD40. In U.S.P. Office, editor Bristol-Myers Squibb Company, United States.

174. Rolink, A., F. Melchers, and J. Andersson. 1996. The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching. *Immunity* 5:319-330.

175. Nimmerjahn, F., and J.V. Ravetch. 2006. Fcgamma receptors: old friends and new family members. *Immunity* 24:19-28.

176. Quezada, S.A., L.Z. Jarvinen, E.F. Lind, and R.J. Noelle. 2004. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* 22:307-328.

177. Beilke, J.N., N.R. Kuhl, L. Van Kaer, and R.G. Gill. 2005. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. *Nat Med* 11:1059-1065.

178. Rayat, G.R., and R.G. Gill. 2005. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* 54:443-451.

179. Beilke, J., Z. Johnson, N. Kuhl, and R.G. Gill. 2004. A major role for host MHC class I antigen presentation for promoting islet allograft survival. *Transplant Proc* 36:1173-1174.

180. Nanji, S.A., W.W. Hancock, B. Luo, C.D. Schur, R.L. Pawlick, L.F. Zhu, C.C. Anderson, and A.M. Shapiro. 2006. Costimulation blockade of both inducible costimulator and CD40 ligand induces dominant tolerance to islet allografts and prevents spontaneous autoimmune diabetes in the NOD mouse. *Diabetes* 55:27-33.

181. Nanji, S.A., W.W. Hancock, C.C. Anderson, A.B. Adams, B. Luo, C.D. Schur, R.L. Pawlick, L. Wang, A.J. Coyle, C.P. Larsen, and A.M. Shapiro. 2004. Multiple combination therapies involving blockade of ICOS/B7RP-1 costimulation facilitate long-term islet allograft survival. *Am J Transplant* 4:526-536.

182. Ozkaynak, E., W. Gao, N. Shemmeri, C. Wang, J.C. Gutierrez-Ramos, J. Amaral, S. Qin, J.B. Rottman, A.J. Coyle, and W.W. Hancock. 2001. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat Immunol* 2:591-596.

183. Rothstein, D.M., M.F. Livak, K. Kishimoto, C. Ariyan, H.Y. Qian, S. Fecteau, M. Sho, S. Deng, X.X. Zheng, M.H. Sayegh, and G.P. Basadonna. 2001. Targeting signal 1 through CD45RB synergizes with CD40 ligand blockade and promotes

long term engraftment and tolerance in stringent transplant models. *Journal of Immunology* 166:322-329.

184. Kishimoto, K., V.M. Dong, S. Issazadeh, E.V. Fedoseyeva, A.M. Waaga, A. Yamada, M. Sho, G. Benichou, H. Auchincloss, Jr., M.J. Grusby, S.J. Khoury, and M.H. Sayegh. 2000. The role of CD154-CD40 versus CD28-B7 costimulatory pathways in regulating allogeneic Th1 and Th2 responses in vivo. *J Clin Invest* 106:63-72.

185. Zheng, X.X., T.G. Markees, W.W. Hancock, Y. Li, D.L. Greiner, X.C. Li, J.P. Mordes, M.H. Sayegh, A.A. Rossini, and T.B. Strom. 1999. CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J Immunol* 162:4983-4990.

186. Parker, D.C., D.L. Greiner, N.E. Phillips, M.C. Appel, A.W. Steele, F.H. Durie, R.J. Noelle, J.P. Mordes, and A.A. Rossini. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc. Natl. Acad. Sci. USA* 92:9560-9564.

187. Wekerle, T., J. Kurtz, H. Ito, J.V. Ronquillo, V. Dong, G. Zhao, J. Shaffer, M.H. Sayegh, and M. Sykes. 2000. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* 6:464-469.

188. Taylor, P.A., C.J. Lees, H. Waldmann, R.J. Noelle, and B.R. Blazar. 2001. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-

CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood* 98:467-474.

189. Durham, M.M., A.W. Bingaman, A.B. Adams, J. Ha, S.Y. Waitze, T.C. Pearson, and C.P. Larsen. 2000. Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J Immunol* 165:1-4.

190. Kawai, T., H. Sogawa, S. Boskovic, G. Abrahamian, R.N. Smith, S.L. Wee, D. Andrews, O. Nadazdin, I. Koyama, M. Sykes, H.J. Winn, R.B. Colvin, D.H. Sachs, and A.B. Cosimi. 2004. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant* 4:1391-1398.

191. Sedlacek, H.H., P. Gronski, T. Hofstaetter, E.J. Kanzy, H.U. Schorlemmer, and F.R. Seiler. 1983. The biological properties of immunoglobulin G and its split products [F(ab')2 and Fab]. *Klin Wochenschr* 61:723-736.

192. Sevcik, C., V. Salazar, P. Diaz, and G. D'Suze. 2007. Initial volume of a drug before it reaches the volume of distribution: pharmacokinetics of F(ab')2 antivenoms and other drugs. *Toxicon* 50:653-665.

193. Jozwiak, J., S. Jozwiak, and M. Oldak. 2006. Molecular activity of sirolimus and its possible application in tuberous sclerosis treatment. *Med Res Rev* 26:160-180.

194. Law, B.K. 2005. Rapamycin: an anti-cancer immunosuppressant? *Crit Rev Oncol Hematol* 56:47-60.

195. Chen, M., L. Huang, Z. Shabier, and J. Wang. 2007. Regulation of the lifespan in dendritic cell subsets. *Mol Immunol* 44:2558-2565.

196. Dallman, C., P.W. Johnson, and G. Packham. 2003. Differential regulation of cell survival by CD40. *Apoptosis* 8:45-53.

197. Nopora, A., and T. Brocker. 2002. Bcl-2 controls dendritic cell longevity in vivo. *J Immunol* 169:3006-3014.

198. Obst, R., H.M. van Santen, R. Melamed, A.O. Kamphorst, C. Benoist, and D. Mathis. 2007. Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation. *Proc Natl Acad Sci U S A* 104:15460-15465.

199. Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T.L. Denning, K. Oswald-Richter, D.J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, S. Ziegler, D. Unutmaz, and B. Pulendran. 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J Clin Invest* 116:916-928.

200. Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke, and B. Pulendran. 2003. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol* 171:4984-4989.

201. Agrawal, A., S. Dillon, T.L. Denning, and B. Pulendran. 2006. ERK1-/- Mice Exhibit Th1 Cell Polarization and Increased Susceptibility to Experimental Autoimmune Encephalomyelitis. *J Immunol* 176:5788-5796.

202. Yu, Q., C. Kovacs, F.Y. Yue, and M.A. Ostrowski. 2004. The role of the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and

phosphoinositide-3-OH kinase signal transduction pathways in CD40 ligand-induced dendritic cell activation and expansion of virus-specific CD8+ T cell memory responses. *J Immunol* 172:6047-6056.

203. Nakahara, T., H. Uchi, K. Urabe, Q. Chen, M. Furue, and Y. Moroi. 2004. Role of c-Jun N-terminal kinase on lipopolysaccharide induced maturation of human monocyte-derived dendritic cells. *Int Immunol* 16:1701-1709.

204. Xie, J., J. Qian, S. Wang, M.E. Freeman, 3rd, J. Epstein, and Q. Yi. 2003. Novel and detrimental effects of lipopolysaccharide on in vitro generation of immature dendritic cells: involvement of mitogen-activated protein kinase p38. *J Immunol* 171:4792-4800.

205. Kriehuber, E., W. Bauer, A.S. Charbonnier, D. Winter, S. Amatschek, D. Tamandl, N. Schweifer, G. Stingl, and D. Maurer. 2005. Balance between NF-kappaB and JNK/AP-1 activity controls dendritic cell life and death. *Blood* 106:175-183.

206. Schneider, W.P., T.G. Wensel, L. Stryer, and V.T. Oi. 1988. Genetically engineered immunoglobulins reveal structural features controlling segmental flexibility. *Proc Natl Acad Sci U S A* 85:2509-2513.