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The Role of Signal 3 Cytokines in Costimulation Independent Rejection

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

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B. S. Emory University, 2011

Advisor: Andrew B. Adams, M.D., Ph.D.

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Abstract

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Solid organ transplantation has become the primary treatment for end-stage organ failure, driven by the advent of potent immunosuppressants. Non-specific immunosuppression with calcineurin inhibitors (CNIs) were instrumental in reducing the incidence of early graft failure due to acute rejection. Despite these advances, long-term transplant outcomes have remained largely unchanged over the past thirty years. While this is likely multi-factorial, the non-immune effects of CNIs play a significant role, leading to heart-attack, stroke, diabetes, and allograft vasculopathy which is the leading cause of transplant failure. In 2011, a high affinity variant of the CTLA4-Ig fusion protein, belatacept, was approved as the first alternative to CNIs. Belatacept specifically interrupts CD28-CD80/CD86 mediated T cell costimulation. Compared to patients treated with CNIs, patients treated with belatacept live longer and retain superior renal function, which amounts to the first improvement in long-term outcomes for transplant patients in over thirty years. However, a subset of belatacept treated patients experience increased rates of acute rejection. In order to understand and address costimulation independent rejection, we studied belatacept resistance in a pre-clinical model of non-human primate (NHP) renal transplantation. We found that animals resistant to belatacept had increased frequencies of $CD28^{+}CD8^{+}$ memory T cells prior to transplant. These cells leverage a proliferative advantage in order to prosecute costimulation independent rejection, characterized by a unique more terminally differentiated $CD8^{+}$ T cell graft infiltrate. We hypothesized that signaling through the IL-2/IL-15Rβ (CD122) and/or the IL-7Rα (CD127) may provide alternative pathways for T cell activation in the setting of costimulatory blockade. We found CD122 signaling was critical for costimulation independent memory CD8 T cell mediated graft rejection, and combination therapy improved graft survival in mice and NHPs. We also investigated the role of CD127 in costimulation independent rejection. Combined Costimulation blockade and CD127 blockade gave rise to increased frequencies of graft specific iTregs while controlling the expansion and effector function of graft reactive CD8 T cells. These data provide basic insights into the signaling requirements of T cells and outline a new strategy: targeting unique Signal 3 cytokines for the optimization of clinical costimulatory blockade in transplantation.

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G.K. Chesterton wrote, "The poet only desires exaltation and expansion, a world to stretch himself in. The poet only asks to get his head into the heavens. It is the logician who seeks to get the heavens into his head. And it is his head that splits." I have a great number of people to thank for supporting my creative wanderings into the world of biological investigation; and, in fact, an even greater number of loved ones who kept my head from splitting during this initiation of the journey. First and foremost, I acknowledge my patient and brilliant mentor, Dr. Andrew B. Adams. Dr. Adams's grounded approach to investigation, his pragmatic guidance, his support for innovation and independence, his insightful suggestions, and his dedication as a complete physician-scientist (clinically and scientifically), made this journey to the frontiers of transplant immunology exhilarating, fun and fulfilling. He has fostered a culture of collegiality in the laboratory that has made graduate school a true joy. I could not have performed this work without the help of my laboratory family including Cindy Breeden and Ying Dong, my first teachers, and Taylor Deane who graciously took on more than she asked for. I am thankful for talented, humble and hardworking colleagues and friends in Walter Wakwe, Laura Higginbotham and Allison Stephenson, as well as Steven Kim who indulged me with many meaningful conversations over prolonged coffee breaks and craft beers, providing critical review of data, high-brow fashion and dining recommendations, and indispensable advice.

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The Immunological Barrier to Graft Survival

In 2016, more than thirty-three thousand people in the United States alone received a life-saving organ transplant, according to the Organ Procurement and Transplantation Network. The majority of these patients were kidney transplant recipients. Some would argue that the origin of curative organ transplantation, now a near-routine clinical option for patients suffering from end-stage organ failure, is found just over one-hundred years ago, in the work of a French military surgeon and Nobel laureate. In the early 1900's, Alexis Carrel devised the surgical technique for anastomosing blood vessels and demonstrated that vessels could be preserved days at a time in cold-storage (1). Carrel himself recognized that despite these surgical advancements, serious biological hurdles remained in order to make organ transplantation practical (2). It was the observations of a second European scientist roughly fifty years after Carrel's studies that shed light on the immunological barriers in transplantation. Peter Medawar, similarly motivated by the exigencies of war, observed the rapid rejection of serial skin grafts he termed "second-set phenomenon". Medawar's observations from the battlefield after skin grafting badly burned soldiers were translated to the laboratory (3-5). Along with his trainees, Rupert Billingham and Leslie Brent, their straightforward experiments and investigations laid the foundation for the immunological understanding of immunity and tolerance in transplantation. Even their skin grafting technique has stood the test of time as a model of transplant rejection (6). Their contributions continues to influence investigators and serve as a starting point for a tradition of pharmacological discovery that gives rise to the continuously improving practice of solid organ transplantation today. Numerous studies followed by many groups further underscoring the important barrier posed by the immune system. Medawar's observations in the active acquisition of tolerance, together with Ray Owen's observation that dizygotic

bovine twins did not reject allogeneic tissue led to the persistent quest for the induction of allograft tolerance (7, 8). These observations motivated ongoing studies in donor specific transfusion for the induction of tolerance. In the broadest sense, Medawar's work and legacy opened the field of transplantation immunobiology, foreshadowed many of the research paradigms and therapeutic advances we now consider standard, and perhaps most importantly, these early studies by transplant scientists laid the foundation for significant advances in vaccine development, cancer immunotherapy and autoimmunity research.

Pharmacologic Management of Acute Allograft Rejection

It was the somewhat incidental creation of the anti-mitotic compounds 6-mercapto purine and its imidazole derivative azathioprine, originally intended for use in cancer therapy, that began the odyssey for optimal pharmacologic suppression of the immune system for transplant success. To their credit, Medawar's group foreshadowed the use of corticosteroids for the prolongation of transplanted tissues (9). The purine analogs that were to become the primary mode of transplant immune suppression for the 1970s and 1980s were first synthesized and described by Gertrude ("Trudy") Elion and George Hitchings, who later went on to win the Nobel prize in Physiology "for their discoveries of important principles in drug treatment" (10). As an aside, it was Trudy Elion, brilliant and without an advanced degree in the field at the time, who went on to mentor many of key scientists (such as Emory University Professor Dennis Liotta) who developed powerful anti-virals and anti-retrovirals which have had a global impact and helped halt the unchecked spread of HIV. Yet in the early days of these developments, transplant immunobiologists opened the door to studying these therapies. It was a group in Boston led by R. Schwartz who experimentally

demonstrated the immunologic effects of these purine analogs, and made the connection for a potential benefit in transplantation (11, 12). Surgeons, leaning into the very cutting edge of pharmacology made possible the great leaps to follow. A young English surgeon, Roy Calne, interested in extending these findings to a more translational model performed a series of renal transplants in bilaterally nephrectomized dogs and first demonstrated landmark survival with a transplanted kidney (13). At the same time approaches such as whole-body irradiation, now considered highly dangerous, were attempted by French groups with some success, but were quickly outcompeted by the use of more favorable pharmacologic immune suppression with azathioprine, a derivative of the original 6-MP purine compound (14). Concurrently, Thomas Starzl's group in Denver demonstrated that pairing azathioprine, with massive doses of steroids, could in fact reverse rejection, a paradigm shifting approach borne out of clinically unmet needs and pressures which confronted Starzl as a surgeon (15). The deep connection between surgery and science, and the dialectic relationship between the two is a trademark of transplantation from the field's earliest moments. The use of azathioprine and steroids became the mainstay of transplant immunosuppression, and first-year kidney transplant survival dramatically improved with this course of therapy. However, survival plateaued during the 1970s.

During the course of a large scale screening of fungal extracts at the Sandoz Laboratories in Basle, a novel antilymphocytic extract was isolated from the fungi *Trichoderma polysporum* (16). The drug, called Cyclosporine A (CsA), ushered in a new era of success and improving outcomes in renal transplantation. Cyclosporine demonstrated a potent immunosuppressive effects, and prolonged skin graft survival in mice with lower toxicities compared azathioprine (17, 18). Dr. Calne again extended these results to a rodent model of heterotopic heart transplantation, and these findings were soon translated to pre-clinical and

then clinical trials both in England and in the United States by a number of groups (19-22). Even as cyclosporine utilization was leading to a precipitous decline in the incidence of early acute allograft rejection, there was a serious concern of nephrotoxicity demonstrated in pre-clinical models with the drug. Several groups demonstrated early fibrotic change along with vascular complications during clinical and pre-clinical investigation. These early signs of potent immunosuppressive effects, paired with potentially detrimental toxicities was premonitory of the clinical paradigm that soon became status quo in renal transplant: dramatic improvements in one year survival of transplant patients, with no significant improvement in long-term outcomes.

A new therapy, FK 506, discovered similarly from a screening program, was isolated from *Streptomyces tsukubaensis* and found to be immunosuppressive in rodent models of heart transplantation. This improved graft survival came with the benefit of decreased nephrotoxicity (23). These results were translated into human clinical trials by Thomas Starzl's group where they showed improved patient graft survival and decreased nephrotoxicity compared to cyclosporine in humans (24). The 1990s witnessed a number of publications and multicenter trials leading up to the confirmation of what Starzl's group had initially observed with FK 506. FK 506 or tacrolimus, better known as Prograf, became the standard of immunosuppression for transplant success. Although tacrolimus and cyclosporine are distinct chemical compounds, their effects on IL-2 production, T cell proliferation and IFN-γ production were similar, and their similar mechanism of action as calcineurin inhibitors (CNIs) would later be confirmed (25-27)

Life After Year One: A Failure to Maximize Graft Half-Life

The discovery of novel therapies early on in transplantation immunology often occurred serendipitously, bridged by enthusiastic and dedicated surgeon-scientists and unique observations of antilymphocyte activity. These opportune findings by persistent academicians and courageous patients, formed the reality of practical organ transplantation for end-stage organ failure. Millions of citizens in the United States alone benefit from these advances today. CNIs such as tacrolimus and cyclosporine combined with cell cycle inhibitors such as mycophenalate mofetil and steroids together reduced early allograft rejection dramatically, with roughly 95% first-year transplant survival enjoyed by kidney transplants recipients. These therapies have extended survival of other transplanted organs as well including heart, lung, liver and pancreas as well. Despite these achievements just under fifty years from Medawar's observations of accelerated second-set rejection, long term kidney transplant outcomes remain disappointingly static (28). The half-life of a kidney transplant has not improved in over thirty years. The reality is, after the difficult diagnosis of end stage organ failure, and the miraculous gift of life and much improved health and well-being achieved by organ transplantation, a kidney transplant recipient has just a 50% chance of keeping their allograft after 9 years. The problem of curtailed graft survival is in part due to the non-specific mechanism of action calcineurin inhibitor therapies such as tacrolimus and cyclosporine. Both drugs are notably nephrotoxic, though tacrolimus is less so. Additionally, their wide target distribution, and non-specific mechanism of action is implicated in the generation of metabolic disease, diabetes, and cardiovascular comorbidities (29-31). Another compelling explanation for late graft failure is chronic antibody mediated

rejection (32). Numerous groups have demonstrated that increased donor specific antibody (DSA), particularly anti-HLA antibody, is predictive of accelerated late graft failure (33-36). The presence of complement component C4d in the peritubular capillary of renal allografts, and anti-HLA antibody in peripheral blood has been confirmed, and correlated to decreased long-term graft survival, and augmented graft injury (37-40). This has been remarkably well demonstrated and investigated in non-human primate models of renal transplantation, where chronic antibody mediated rejection (CAMR) featuring the development of DSA, preceding C4d deposition and bona fide chronic antibody mediated rejection pursuant to the cessation of immunosuppression. These models demonstrate that chronic antibody mediated rejection is a relatively slow process taking at least four months and up to two years to eventuate in graft failure (41, 42). Further, they confirm the utility of the non-human primate renal allograft model for developing therapies and investigating immunological barriers to clinical transplantation.

Costimulatory Blockade as a Targeted Approach to Transplant Immunosuppression

The lack of improvement in long term graft survival, and indeed the stagnation of clinical transplantation with suboptimal immune suppression call for dedicated investigation and increased understanding of the rejection process. Building on the work of Medawar and others, studies by Doherty and Zinkernagel described fundamental mechanisms of a cellular basis for self and non-self (or altered-self) recognition (43-46). In a prescient chapter that laid out the discovery of cell mediated immunity and immunologic self/non-self discrimination, Zinkernagel and Doherty are clear to remind readers of the important role of transplant scientists:

> *The biological function of major transplantation antigens has been a puzzle since the discovery of alloreactivity. Much work has been done, both because the problem is experimentally accessible and because of the clinicians' hope that organ transplantation would prove feasible. Graft rejection and the need for genetically homogeneous inbred mouse strains for cancer research led to the development* ***of*** *transplantation immunology and immunogenetics (Gorer,* ***1936;*** *reviewed in Klein,* ***1975;*** *Shreffler and David,* ***1975;*** *Snell* ***et*** *al.,* ***1976).****The result is that the gene complex coding for major transplantation antigens is one of the better understood mammalian genetic regions.*
>
> Zinkernagel & Doherty, Advances in Immunology, 1979

In other words, it was in part the clinicians drive to make organ transplantation a reality that provided the comprehensive mapping system of the so-called "histocompatibility" (i.e. tissue compatibility) gene complex. This foundation allowed for fundamental breakthroughs in how and why immune cells recognize and eradicate pathogens. T cell Signal 1, the interaction of T cell receptors with major histocompatibility complex (MHC) is named in the terms of transplantation immunology, and serves as an homage to the foundational contributions of transplant scientists to the wider field of immunology. T cells are one of the major immunologic barriers in transplantation, and are the primary cause of graft loss due to acute allograft rejection. Beyond T cells recognition (Signal 1), T cells require additional signals for activation. The biology of T cell costimulation and coinhibition (Signal 2) came into focus during the 1990's when the canonical CD28 costimulatory molecule's central role in T cell activation was established definitively (47, 48). T cells express CD28 which binds to B7 family members on antigen presenting cells (APCs), but interestingly CD28 binds with lower affinity than a T cell coinhibitory homologue, CTLA4 (49-51). CD28 and CTLA4 on T cell surfaces compete for CD80/CD86 on APCs, and the sum of signaling, either

inhibitory with CTLA4 or activating with CD28 contributes to the cells decision to activate or regulate itself in response to antigen recognition. The role of CD28/CTLA4 in T cell activation and inhibition was manipulated using selective reagents to target signals in this pathway for amelioration of autoimmune disease and transplant rejection (52, 53). Mice lacking CTLA4 demonstrated lethal autoimmune disease, indicating the role of CTLA4 in regulating T cell responses (51). The elucidation of CD28/B7 for T cell activation and the striking phenotype in mice motivated studies of selective blockade of B7 family members with differential effects (52). The use of CTLA4-Ig, abatacept, was found to be efficacious in treating autoimmune disease, such as rheumatoid arthritis (54). These findings were extended to transplantation where the use of abatacept prolonged graft survival in models of islet transplantation (55).

Along with the CD28/B7 pathway, the potency of the CD40/CD40L pathway in T cell activation is a point of great interest in transplant immunology. Seminal studies in murine and non-human primate transplantation established the importance of both the CD28/B7 and CD40L/CD40 pathways in the generation of effective T cell responses. Blockade of both these signaling pathways in the context of transplantation led to durable transplant tolerance in certain models (56, 57). Mechanistic studies revealed that blockade of the CD40 costimulatory pathway potently induced antigen specific regulatory T cell subsets that promote allograft acceptance (58). However, clinical trials with a monoclonal antibody targeting CD40L met with early challenges due to an increased incidence of thromboembolic complications, later attributed to the role of platelet expression of CD40L (59). These instances of negative outcomes notwithstanding, transplant immunologists continued the search for more selective T cell targeted pharmacologic inhibition with the hope of reducing the non-specific effects of calcineurin inhibitors in transplant recipients and prolonging

allograft survival. Novel CD40L domain antibodies have been recently investigated in murine models of transplantation, with promising results (60). Our group demonstrated that a humanized anti-CD154 domain antibody can dramatically prolong allograft survival, both as a monotherapy, and together with conventional agents (61). We found that costimulatory blockade with this novel Fc-silent construct carried no thromboembolic risk, while providing potent immunosuppression and led to the development of regulatory T cells.

In addition to the CD40 and CD28 pathways, the growing array of costimulatory molecules found to be expressed by T cells provide new avenues of research and potential targets for T cell specific transplant immunotherapy (62). Our group and others have investigated the efficacy of blocking the Ox40/Ox40L pathway. We found that blockade of Ox40L synergized with CD28/B7 blockade to dramatically prolong allograft survival in both mice and non-human primate renal transplantation (63). Not all costimulatory pathways demonstrate similar efficacy in these stringent models. Lo et al, demonstrated that blockade of the ICOS/ICOSL pathway, which showed promising resulting in murine models of transplantation, did not improve survival in the non-human primate model of renal transplantation (64).

Important studies into the nature of T cell activation demonstrated that loss of costimulation in the presence of TCR ligation resulted in T cell anergy (65). The efficacy of costimulation blockade in prolonging transplant survival, including blockade of CD28 and CD40L mediated signals, advances the idea that allorecognition minus sufficient costimulation leads to adaptive tolerance. In fact, important studies in murine models reliably induced durable macrochimerism and allograft tolerance utilizing costimulation blockade and busulfan based conditioning (66). These results have been extended to non-human primate models of chimerism, where chimerism can be temporarily induced with

costimulation blockade, and stem cell transplant in order to promote graft acceptance (67). Costimulation blockade is not limited to promising immunosuppression for transplant survival, but has potential for induction of tolerance, a distinct and potentially permanent state, which many consider to be the holy grail of transplant immunology.

Despite the promise in targeting CD28/B7 pathways murine models, use of CTLA4-Ig reagents, abatacept, failed to significantly prolong graft survival in non-human primates. Transplant surgeon-scientists Drs. Christian Larsen and Tom Pearson, along with collaborators at Bristol-Myers Squibb undertook the development of a high-affinity mutant of the CTLA4-Ig fusion protein, LEA29Y or belatacept, which binds with a nearly 100-fold increased affinity to CD80 and CD86, compared to CD28 (68). The use of LEA29Y significantly prolonged allograft survival in non-human primates, and had the additional benefit of significantly blocking the formation of donor specific antibodies compared to calcineurin inhibitors. Promising results from these pre-clinical non-human primate transplantation studies led to clinical trials of belatacept for use in human renal transplantation (69). An open-label, randomized multicenter phase III trial demonstrated that belatacept use was associated with reduced cardiovascular and metabolic toxicities, as well as superior renal function in transplant recipients (70-72). In a watershed moment for the field of transplantation, seven-year post-approval patient follow-up revealed that belatacept treated patients had significantly improved long-term outcomes, with a 43% risk reduction of death or graft loss compared to patients receiving calcineurin inhibitor based therapies (73). These studies demonstrate that the rational development of targeted costimulatory blockade based therapy to suppress alloreactive T cells had at least two major promising consequences: (1) reduction of non-specific toxicities mediated by calcineurin inhibitor based therapies and (2) reduction of alloantibody formation. In the coming years,

the ability of belatacept based immunosuppression to prolong graft half-life will be testable, and we hypothesize, based on the preliminary data, that graft half-life will be significantly improved. This amounts to the first improvement in long-term outcomes for transplant patients since the introduction of CNIs.

Costimulation Independence is a Barrier to Better Outcomes in Transplantation

The use of T cell costimulation blockade, belatacept, as an alternative to non-specific immune suppression with calcineurin inhibitors was a transformational moment in the field of solid organ transplantation. However, despite the number of benefits experienced by patients treated with belatacept, there is an increased incidence of acute allograft rejection associated with belatacept versus calcineurin inhibitor therapy (71). This increased incidence of acute rejection tempered enthusiasm for wider adoption of belatacept in renal transplantation. This was not totally unexpected. In fact, investigators were actively pursuing the biological basis of this so-called costimulation-independent rejection seen in pre-clinical models before clinical costimulation blockade was approved.

Early experiments held the key: different murine strains exhibited a variable susceptibility to costimulation blockade based allograft survival (56, 74). While C3H mice receiving MHC mismatched skin allografts were susceptible to combined costimulatory blockade, C57BL/6 were much more resistant to costimulation blockade, and went on to reject their grafts before 30 days post-transplant (74). Even in translational models, non-human primates treated with belatacept following transplantation exhibited "increased mononuclear cell infiltrate" at the time of sacrifice, and almost half of these primates rejected their allograft during belatacept therapy (68). These animals demonstrated depressed

renal function prior to rejection, consistent with acute allograft rejection. CD28/B7 independent rejection in a subset of mice and non-human primates in these early experiments supports an alternative mechanism (or several mechanisms) of T cell activation in the setting of costimulation blockade. This evidence of costimulation independence predicts that a subset of patients would reject their graft in the presence of CD28 based costimulation blockade. Surprisingly, in the clinical trial which resulted in the approval of belatacept, belatacept treated patients experienced 7% and 6% acute allograft rejection (more intense and less intense regimens) while cyclosporine treated patients experienced 8% indicating equivalent rates of rejection (69). However, in post-trial use, belatacept treated patients demonstrated a markedly increased rate of acute rejection compared to tacrolimus treated patients (50.5% vs 20.5%). Fortunately, these increased rates of rejection did not result in statistically significant increases in death or graft loss, as clinical processes identified and addressed rejection with appropriately augmented immunosuppression. In fact, if censoring for patients who were transitioned off of belatacept therapy, patients who received belatacept had a statistically significant reduction in death or graft loss, even in post-trial use (75).

Several potential non-exclusive explanations for this costimulation-independent, or costimulation blockade resistant rejection exist. To name just a few, immune memory, cytokine activation ("Signal 3"), inflammation, heterologous immunity, NK cells, alternative costimulatory molecules, integrin expression and integrin based activation, and Th17 cells are all implicated in costimulation-independent rejection, and are active areas of investigation by transplant researchers. Given the recent data regarding the long-term benefits and increased survival of belatacept treated patients as well as the currently accruing data on improved graft function in belatacept treated patients, there is a critical clinical purpose in

understanding how and why costimulation independent rejection occurs. Not only do we investigate the basic signaling requirements of T cells in allograft rejection to guarantee and improve survival of patients who undergo transplant surgery, but we also add to the public fund of knowledge, acknowledging that insights into these basic mechanisms have yielded and will likely continue to yield rapid progress in other areas of biomedical research such as cancer biology, infectious disease and autoimmunity (and likely fields considered "unrelated"). Additional incentive in pursuing the overall project of understanding immunologic barriers and costimulation based immunosuppression lies in the promise of inducing tolerance, and one day eradicating the immunologic barrier for patients, if possible. One critical and well described barrier is T cell memory.

Memory as a Barrier to Transplant Tolerance

T cells with prior antigen experience have a decreased requirement for T cell costimulation, and can mount re-call responses quickly upon secondary exposure to cognate antigen (74, 76). In fact, T cell receptors need not recognize a cognate antigen per se, but the concept that T cells are poly-specific, that is a given T cell receptor binds with specificity to multiple biochemically dissimilar peptide:MHC combinations provides yet another distinct mechanism by which priming in the context of viral immunity may potentiate alloimmune memory responses (77). There may be some degeneracy in the binding of T cell receptors to p:MHC, or perhaps two distinct immune challenges (i.e. allografts and infectious pathogens) may bear an identical cognate antigen, but there may also be polyspecific T cell receptors as well. Additionally, there may be prior sensitization from alloimmunization, through red blood cell transfusion, prior transplantation or pregnancy. One can imagine scenarios in

which sufficient TCR ligation promotes alloreactivity by memory T cells, and for many of these memory T cells, costimulation is dispensable. Subsets of human and non-human primate T cells lack CD28 expression altogether and yet retain potent effector function (78-83). Following initial pathogen exposure, T cells give rise to both effector and central memory T cells (84). Central Memory T cells have stem cell like qualities and are able to self-renew. Memory T cells expand and give rise to effector T cells during secondary challenge in order to more rapidly clear pathogens before the pathological effects of microbes can be felt. Effector T cells are less stem-cell like, but have augmented cytolytic capacity. Classic papers from Lanzavecchia and Sallusto first described distinct T cell subsets utilizing CD45 isoforms and CCR7 (81). Advances in multiparametric flow-cyotmetric analyses of T cells give rise to a more nuanced understanding of the heterogenetity of T cell subsets, utilizing CD28 and CD95 (78). New techniques, including Mass Cytometry-Time of Flight (Mass CyTOF) which offer highly multiplexed analyses of T cells are revealing even more diversity than previously appreciated (85). One key differentiating point is the expression of CD28, and mounting evidence describes a transition in T cell function being marked by CD28 loss. Human CD8 T cell CD28 loss is associated with transition from central memory to effector memory, though Azuma et al previously described that a small number of CD28- cells had the ability to re-express CD28 (78, 83, 86). Cells that lack CD28 are by definition relatively inert to CD28 based costimulation blockade. The expression of CD28 on memory subsets, and the role of CD28 costimulation in memory responses are not completely defined, but the tools now exist, with multiparametric phenotyping approaches, to more completely characterize these cells. Indeed, we first set out to explore costimulation resistance, and observed that CD28 expression on memory T cells designated a potently alloproliferative memory subset, which could lose CD28 expression, while maintain remarkable effector

function. These cells, we hypothesize, retain the proliferative capacity to support costimulation independent responses, and likely differentiate in the context of repeated antigenic stimulation via the persistent presence of alloantigen present in transplanted tissue, along with inflammatory cytokines such as IL-15, among others, eventually losing CD28 expression.

CD8 T cell memory is of particular interest because several groups have shown an important role for CD8 T cells, and specifically CD8 central memory T cells in supporting costimulation independent rejection (74, 87, 88) Importantly, Adams et al., demonstrated that adoptive transfer of CD8+ central memory T cells, and not CD4+ T cells nor CD8 effector memory T cells promoted costimulation blockade resistant rejection in a stringent model of allograft rejection (87). In this seminal paper, the group not only demonstrated the critical role of CD8+ central memory T cells in costimulation independent rejection, but advanced the notion of heterologous immunity giving definitive evidence of a critical role for cross-reactive virally primed alloreactive CD8 T cells which were capable of secreting effector cytokines upon heterologous challenge with alloantigen. In an elegant experiment, Adams et al., described how LCMV infected mice more than 100 days post infection were capable of secreting effector cytokines from virally primed CD8 T cells when challenged with alloantigen. Other groups had previously demonstrated that heterologous immunity may pose a barrier to transplant tolerance. For example, groups have demonstrated that virally primed H-2Kb restricted cytotoxic T lymphocytes (CTLs), primed by both vesicular stomatitis virus or lymphocytic choriomeningitis virus gave rise to alloreactive T cells (89, 90). Burrows and colleagues demonstrated that CTLs reactive to a single epitope of Epstein-Barr virus were also alloreactive, and in fact dominated the alloresponse (91). The experiments performed by Adams et al., built on prior evidence and clearly demonstrated

cross-reactive memory CD8 T cells primed by acute lymphocytic choriomeningitis virus were capable of generating an allospecific response, and more importantly could preclude the induction of costimulation blockade based tolerance. These studies shed light on the concept of heterologous immunity, immune memory, and informed future studies exploring the barrier of memory CD8 T cells to transplant tolerance. Reinforcing these basic science data, Peter Heeger's group demonstrated that elevated baseline frequencies of alloreactive lymphocytes correlated with post-transplant rejection episodes in patients (92). A number of other groups demonstrated the role of pathogen primed memory in potentiating rejection (93). Moreover, allospecific memory cells were demonstrated to be resistant to control with costimulation blockade (94-97). More recently, Nadazdin et al., demonstrated in non-human primate renal transplant, tolerance was abrogated by increasing frequencies of pre-transplant alloreactive CD8 T cell central memory (98).

Taken together these data indicate that prior pathogen experience which elicit functional CD8 T cell memory pose a barrier to transplant tolerance and to immunological control with costimulation blockade. Further, memory is a barrier to tolerance induction strategies which rely on costimulation blockade. CD28 mediated costimulation may not be a signaling requirement for memory CD8 T cells, and thus targeting this pathway alone may be less efficacious in prolonging graft survival in animals with increased immune memory. If the quality of the memory is significant, that is to say, the alloreactive memory T cells are not terminally differentiated so as to be exhausted, but rather represent a more fit memory subset with proliferative capacity and effector function, then this subset can pose a serious threat to transplant tolerance and costimulation blockade based immunosuppression. In our own CD8 T cell central memory are significantly elevated in animals who go on to reject their allografts on belatacept therapy, compared to animals who respond to belatacept

therapy (99). To better understand the nature of pre-existing memory, Badell and colleagues demonstrated that not only do the frequency of CD8 T memory T cells play a role in costimulation independent rejection, but the quality of CD8 T cell memory plays an important role (100-102). These experiments demonstrate the nature of prior pathogen experience influences transplant recipient responses to costimulation blockade. In the setting of an invariant antigen, Ova, differences in the generation of antigen experience – acute infection versus latent infection versus persistent infection – suggested that in the setting of persistent infection, more fully differentiated CD8 T cell memory subsets pose a more serious barrier to costimulation blockade based transplant survival (102). In sum, not all memory is created equal, and Badell et al., demonstrate that some memories are more dangerous for transplanted tissue than others.

While CD4 memory T cells, and CD4 T cells more generally, are dispensable for allograft rejection in some models, there is a growing interest in CD4+ CD28- T cell (103). Increases in CD28- cells are associated with advanced age and chronic inflammation (104, 105). More specifically, inflammatory cytokines such as tumor necrosis factor (TNF), IL-2, and IL-15 drive the loss of CD28 (106-108). Antigen exposure and the development of terminally differentiated T cell memory is also marked by the downregulation of CD28 (105). Loss of CD28 is thought to be a part of a program of differentiation and cellular maturation marked by changes in receptor expression and cell functionality. Loss of CD28 has been correlated with immune senescence and aging, exhibited by shortened telomere length in CD28- versus CD28+ cells (109, 110). The loss of CD28 is correlated closely with the gain of an alternative marker, CD57, upon chronic stimulation in humans and non-human primates, but not mice (104, 111). While they may be somewhat less proliferative, CD28- cells have augmented cytotoxicity (86). Critically, signaling through the IL-2Rγ and IL-2Rβ

has been shown to augment loss of CD28, via IL-7 and IL-15 signaling (107, 108). TNF is known to decrease CD28 expression on CD4 T Cells, and as elderly patients have increased levels of circulating TNF, the cause for age related CD28 loss may be related to TNF levels (106). Despite the varied ontogenies of CD28- cells, Espinosa and colleagues have been able to demonstrate an increased frequency of CD28-CD57+ T cells in patients who reject their allograft acutely during belatacept based immunosuppression versus those who respond to belatacept therapy. Taken together these data begin to demonstrate that there are multiple pathways for the development of CD28- T cells, and multiple potential pathways to costimulation independent rejection. The loss of CD28 is associated with a reduction in proliferative capacity, with a maintenance and perhaps an augmented effector function. Studies by Lewis Lanier's group outlined these properties in the early 1990's. The role of these cells in allograft rejection is still being elucidated, but our studies and others have identified increased frequencies of CD28- cells infiltrating the allograft in belatacept resistant rejection (99, 103). However, whether or not the costimulation independent alloreactive T cell subsets begin as more proliferative CD28+ phenotype and then gradually lose CD28 expression as they become dedicated effectors remains to be fully defined, but our data suggest this may be the case. Perhaps these cells are capable of re-expressing CD28 as Azuma et al had previously demonstrated in the early 1990's, or perhaps they represent a cell subset with more stem-like properties. It may be that these findings of pre-transplant peripheral CD28+ memory T cells and phenotypically similar but CD28- graft infiltrate are totally unrelated, and that CD28- graft infiltrate arise from a variety of sources. Perhaps they are an artifact of the rejection process and not causative. The growing heterogeneity we are able to appreciate with multiplexed approaches need to be paired with techniques that allow for the incorporation of two more variables: time and space. Tracking and in vivo imaging

techniques, which allow us to characterize heterogeneous memory subsets *in situ* will be invaluable in understanding the role of distinct phenotypes. Our studies begin to highlight potential answers to the mechanisms of costimulation independence, and we continue to incorporate new technologies, with a temporal, anatomic and multiparametric properties, which will aid us in better understanding the role of distinct memory subsets as they mount costimulation independent responses. Costimulation blockade allows for the study of signaling requirements that may otherwise be confounded due to intracellular signaling redundancies. Our data indicate that the use of CD28 directed costimulation blockade reveals novel and nuanced roles for alternative pathways of activation, such as Signal 3 cytokines, which require further study.

Signal 3 Cytokines in T cell Activation

Costimulation and coinhibition are known as "Signal 2" in the canonical "Three Signal Model of T cell activation". This growing array of molecules has been heavily scrutinized and leveraged for dramatic improvements in human health, most recently in the area of cancer immunotherapy. In the "three signal" model of T cell activation, T cells first bind peptide:MHC complexes with their cognate T cell receptor ("Signal 1"). Next, T cells receive critical costimulation provided by an ever-expanding family of costimulatory (and coinhibitory) molecule. The third signal in this model is provided by cytokines which can augment activation and direct differentiation of T cells. There are a number of cytokines responsible for proliferation, effector function acquisition and differentiation, but the family of γ chain cytokines (gamma chain, γ_c) is one of the most well studied. These cytokines are critical for T cell activation and play an important role in T cell memory and homeostasis.

These cytokines are not limited to their role as signal 3 cytokines, but rather provide biological instruction based on the context in which they are encountered, and the receptor expression and signaling milieu of the cell in question. Here we focus on IL-2/IL-15 as well as IL-7.

Both the cytokine expression and the diversity of receptor expression kinetics, as well as the unique biological niches of these two entities (cytokine and receptors) are important to appreciate in order to more clearly define the role of "Signal 3" in allograft rejection, and in the context of this work. Many of these questions are still outstanding and require further investigation: where, when and what controls cytokine and cytokine receptor expression, and the corollary to this – what impact does that have on the primary and memory immune response (with or without costimulatory signals).

The Shared Biology of Interleukin-2 and Interleukin-15

IL-2 and IL-15 are convergently evolved cytokines, sharing actually very limited sequence homology, but sharing 2 of 3 receptor subunits, and even so, the cytokines have distinctive contact residues with their receptor subunits (112). This picture is further complicated by the divergent phenotypes in IL-2 or IL-2R deficient mice versus IL-15 or IL-15R deficient mice. IL-2 or IL-2R deficient mice demonstrate a loss of peripheral CD4+ regulatory cells, resulting in lethal autoimmunity (113-118). Loss of IL-15 on the other hand plays a more important role in NK, NKT and CD8 memory T cell survival (119, 120). IL-2, first identified as T cell growth factor, is a potent mitogenic cytokine (121). IL-2 is primarily supplied by CD4 T cells in secondary lymphoid organs, but CD8, NK and NKT cells can also produce IL-2 (118, 122). IL-2 expression is reciprocally controlled by levels of B

lymphocyte induced maturation protein (BLIMP1; also termed PRDM1), and persistent T cell activation via IL-2 can lead to Fas dependent cell-death, so called "activation induced cell death" (123, 124).

Elegant experiments conducted by Matt Williams and colleagues demonstrated that IL-2 signals during priming are required for secondary expansion of CD8 memory T cells (125). Further experiments by the same group were aimed at teasing apart the role of IL-2 and IL-15 in T cell priming, utilizing P14 TCR transgenic cells specific for the LCMV gp33 epitope, and P14 mice deficient in IL-2Rα. These antigen specific cells were adoptively transferred to either naïve or IL-15 deficient mice. They asked how the lack of IL-2 signaling versus IL15 availability impacted primary and secondary responses, and their findings suggested that IL-15 was not required for competent secondary responses (126). These results were somewhat surprising given the data surrounding the role of IL-15 in supporting CD8 T cell memory. These data reinforced the notion that IL-2 and IL-15 may be redundant in some capacities, but the unique distribution of their receptor systems, expression in various anatomic compartments and kinetics of binding may confer different roles in supporting memory and recall responses. Our own studies actually suggest that IL-15 is critical for re-call responses, whereas the high-affinity IL-2 receptor on memory CD8 T cells is dispensable for effective re-call responses. Many distinctions in our systems allow for multiple explanations of these divergent findings. One important caveat is the nature of P14 IL-2Rα KO cells, which seemed to generate a qualitatively inferior primary response, and thus may not be the most appropriate comparison to WT P14 cells. Secondly, Mitchell et al utilized a potent heterologous re-challenge, where P14 cells first encounter gp33 in the context of LCMV and then in the context of *Listeria monocytogenes*, a system which would elicit the development of a number of unique and redundant Signal 3 cytokines, including

Type 1 interferons (LCMV) and IL-12 (Lm), which might cloud the evaluation of the unique contributions of IL-2 vs IL-15. Most importantly, the system did not account for the strength of costimulation to support re-call responses in this context, particularly in light of recent studies which demonstrate that LCMV responsive CD8 T cells utilize an array of redundant accessory costimulatory signaling pathways in re-call (127). The blockade of costimulation in our experiments removes an important confounding variable, and thus provides an opportunity to interrogate the unique contributions of IL-2 and IL-15. The defective priming of P14 IL-2Rα KO can account for their poor re-call response. The effective priming of WT P14 during primary infection due to intact IL-2R signaling, along with the presence of intact and redundant costimulatory signals can explain the effective re-call responses seen in this model of heterologous re-challenge. In our model, the absence of costimulation, and the distinct heterologous challenge of a graft after priming with Lm amounts to a host of differences that could account for the seemingly distinct findings.

Studies out of Thomas Malek's group shed some light on the unique aspects of IL-2 and IL-15 signaling (128). They found that proximal signaling events, downstream of IL-2/IL-15R were initially similar, but signaling changes were transient with IL-15 and sustained with IL-2 signaling, which they determined was due to reduction in IL-15/IL-15Rα expression. Again, these in vitro experiments may not approximate the in vivo nature of ubiquitous IL-15/IL-15Rα availability in relevant transplant scenarios. They are however informative for appreciating how downstream signaling impacts phenotype and function. Importantly, CD122 signaling is critical for high quality secondary responses. Secondly, weak signaling through CD122 favors CD8 central memory T cell development.

The topography and signaling of IL-2/IL-15 receptor system lends some insight into key signaling and immunologic outcomes of receptor ligation. The crystal structure of IL15

receptor alpha has only recently been resolved (129, 130). The proposed model of most likely signaling through the IL-2/IL-15 receptor involves sequential recruitment of subunits upon cytokine binding, supporting increasingly high affinity cytokine-receptor complexes, and thus reducing the minimal effective concentration of these cytokines. These studies indicate similar themes but distinct modules in the protein-protein binding of IL-2 and IL-15. As previously highlighted, these cytokines, despite their similar in vivo effects, have divergent amino acid sequences indicating evolutionary convergence. Elegant in vitro studies by Aaron Ring and colleagues indicate that at saturating doses, both cytokines have near identical transcriptomic signatures (112). These studies indicate important kinetic differences in downstream signaling modules that highlight the importance of the alpha receptor expression of IL-15 and IL-2 respectively. These differences may in part explain the divergent phenotypes of IL-2 and IL-15 knockouts. The alpha receptor subunits of IL-2 and IL-15 promoted divergent transcriptomics based on increased receptor-cytokine affinity and augmented signaling capacity. It is important to note that IL-15Rα is widely distributed throughout the body, highly expressed on renal epithelia for example, and is critical in the unique, high affinity trans-presentation of IL-15 to IL-2Rβ and IL-2Rγ on CD8 memory T cells (129, 131). The interaction between trans-presented IL-15 combined with IL-15Rα increases the affinity and signaling of the IL-15 complex nearly 100-fold, and is certainly superior to IL-2 signaling, either in paracrine or autocrine fashion (132, 133). This is not unlike the augmented affinity of combined IL-2 and IL2-Rα (134, 135). IL-2Rα is highly expressed on regulatory T cell subsets whereas IL-2Rβ is highly expressed on CD8 memory T cells and NK cells. These data suggest that while both cytokines play a role in the survival, differentiation and effector function of T cells, their unique receptor expression patterns,

tissue distribution, and availability in various biological niches define their capacity to augment alloreactive CD8 memory T cell. We hypothesized that CD122, the IL-2Rβ may be a more rational target for transplantation than CD25, the IL-2Rα, due to the critical role of CD25 in regulatory T cells. In addition, signaling through this complex is implicated in the loss of CD28 expression (107, 108). Thus, while memory CD8 T cells which either lack or can dispense with CD28 for T cell activation are capable of activation in the presence of CD28 mediated costimulation blockade, these data suggest that signaling through the shared IL-2/IL-15 receptor complex may induce CD28 loss and enhance costimulation independence. These data support a sort of "double-hit" role for IL-15 and IL-2 signaling in costimulation independent rejection: these cytokines activate cells and make them less sensitive to costimulation blockade.

Building on observations by Adams et al., that costimulation independent rejection mediated by central memory CD44hi CD62Lhi CD8 T cells, along with mounting evidence regarding the role of IL-15 in supporting CD8 T cell homeostasis, investigators began to target the shared IL-2/IL-15 receptor pathway in transplantation. Studies by Terry Strom's group utilizing a functionalized IL-15-Fc fusion protein antagonist significantly prolonged graft survival and abrogated CD8 T cell mediated costimulation independent rejection in a model of murine islet transplantation (136-139). Groups investigating the role of IL-15 in autoimmune disease discovered a role for IL-15 in supporting CD8 T cells in mediating pathology in models of Type 1 Diabetes and Graft-Versus Host Disease (GvHD) (140, 141). As early as 2001, our group investigated the role of the IL-2 receptor in promoting costimulation independent rejection, and found anti-CD25 was a promising adjunct therapies to costimulation blockade (142). More recently, Rob Fairchild's group performed studies exploring the relative capacity of IL-15 and IL-2 to support costimulation

independent rejection (143). They demonstrate that CD8 memory phenotype cells from healthy volunteers, when allostimulated, CD28- expanded rapidly and more effectively, with nearly 10 times less supplemental IL-15 cytokine, compared to other cytokine (IL-2, IL-7), and these memory T cells were capable of proliferating despite the presence of CTLA4-Ig. In addition, Francois Villinger's group at the Yerkes National Primate center demonstrated that IL-15 is superior to IL-2 in the generation of long lived antigen specific memory CD4 and CD8 T cells in rhesus macaques (144). These studies highlight important differences in the relative efficacy of these cytokines in augmenting immune function, despite shared receptor systems.

Our group has pursued this promising line of investigation and demonstrated significantly prolonged survival in both murine and non-human primate models of renal transplantation with the addition of anti-CD122 mAb to a costimulation blockade based regimen (manuscript in preparation). We demonstrate that CD8 memory T cell effector function was augmented with the addition of either IL-2 and IL-15, and that blockade of CD122 dramatically ablated effector function. Further, we demonstrated that CD122 signaling was required for expansion and effector function in murine models of CD8 memory mediated costimulation independent rejection. The blockade of anti-CD122 was superior to anti-CD25 in controlling memory CD8 T cell expansion. Improved understanding of the unique roles and niches of IL-2 and IL-15 signaling respectively will no doubt improve our ability to manipulate these pathways in order to prolong graft survival, but also activate T cells optimally in cancer therapy.

Targeting IL-2 Receptor was a clinical reality in the late 1990s, more than ten years prior to the approval of belatacept as a CNI alternative (145). Daclizumab, an early anti-CD25 mAb synergized with CNI therapies to significantly reduce the rate of early allograft

rejection. Studies in the development of costimulation blockade for clinical translation demonstrated that addition of blocking reagents for CD25 augmented survival in non-human primate allotransplantation (68). Targeting CD25 has fallen out of favor due to the high expression of CD25 on regulatory T cell subsets, and the failure of CD25 to improve induction therapy compared to anti-thymocyte globulin (146, 147). Our work in the lab demonstrates that CD122 may be a superior target for the purpose of prolonging graft survival in murine and non-human primate models of transplantation, as it controls the expansion and effector function of CD8 memory T cells, and leaves regulatory T cell frequencies intact.

The role of IL-15 in promoting costimulation independent rejection is not confined to its effect on CD8 T cells alone. Natural Killer cells are known to be costimulation independent, and in a model of cardiac allograft transplantation in CD28 KO mice, Maier et al demonstrated that inhibition of NK cells results in long term graft acceptance (148). Kean and colleagues extended these findings to costimulation blockade resistant rejection, demonstrating that NK cells mediated rejection despite costimulation blockade therapy during non-myeloablative bone-marrow transplant (149). Further investigation revealed that NK cells promote rejection despite costimulation blockade, in part due to their own activating receptors such as NKG2D and Ly49D (150). NK cells are a costimulation independent cell type capable of rejection allografts, and moreover, highly express CD122 and depend on IL-15 for survival and expansion. Indeed NK cells are potently stimulated by IL-15, adopting an activated phenotype and capable of killing allogeneic cells, with potent effector activity in both mice and humans (151, 152). Our own data demonstrates significant NK cell infiltration in the grafts of primates treated with CD28 specific blockade for transplant success (Manuscript in preparation). These data suggest a tangible, significant role

for NK cells in promoting costimulation independent rejection during belatacept based immunosuppression. Further studies are required to fully elucidate the mechanisms underlying NK cell allorecognition and trafficking into the renal allograft, and what part NK cells play in the initiation or perpetuation of costimulation independent rejection.

The Role of Interleukin-7 in T cell Responses

Interleukin-7 is a cytokine produced primarily by non-hematopoietec stromal cells, although dendritic cells (DCs) have been found to secrete a small amount. The production of IL-7 does not have a large dynamic range, its production is not increased or diminished by manipulation of stromal cells (153). IL-7 bioavailability is thought to be controlled by the expression of IL-7Rα primarily on lymphocytes. IL-7 binds to IL-7Rα and the γ_c which transduces signaling through Jak1 and Jak3 respectively, leading to the recruitment of a number of signal transducers, but importantly STAT5. IL-7Rα is not exclusively utilized by IL-7, but can also bind to thymic stromal lymphopoeitin (TSLP) which binds to the combined IL-7Rα-TSLPR dimer (154). The focus here will be on the role of IL-7Rα signaling, but it is important to note that TSLP may play a role in the findings discussed later. The downstream signaling events after IL-7Rα and γ_c dimerization result in the increased expression and distribution of anti-apoptotic intracellular proteins that amount to pro-survival phenotype. The significance of the relationship between IL-7 and IL-7Rα is in the downregulation of IL-7Rα after binding to IL-7, which then leaves the static amount of IL-7 available to other IL-7Rα bearing cells (155).

Conventionally, IL7-IL7Rα signaling is thought to play a critical role in lymphopoesis, thymopoesis, and T cell homeostasis, particularly central memory T cell

survival. The phenotype of IL-7 and IL-7Rα KO mice are similar, resulting in Severe Combined Immunodeficiency (SCID), observed in humans as well (156-159). These early observations underscored the importance of IL-7 in lymphocyte survival. Studies aimed at the next stage of development, including T cell maturation in the thymus and TCR recombination identified a role for IL-7 in thymopoesis (160-162). Groups then began to investigate the role of IL-7Rα signaling in T cells beyond development. Schluns et al demonstrated that IL-7Rα was dispensable for an antigen induced response, but OT-1 CD8 T cells failed to establish long-lived memory after immunization with VV-OVA. Furthermore, memory OT-1 CD8 T cells had diminished homeostatic proliferation in IL-7 KO hosts. These studies and others highlighted the important role of IL-7Rα signaling in the maintenance of the peripheral memory T cell pool (163). Clinical trials utilizing exogenous recombinant human IL-7 (rhIL7) in HIV infected individuals shed some light on the impact of the cytokine on T cell kinetics (164). Immediately after injection, participants demonstrated a rapid reduction of peripheral CD4 and CD8 T cells (likely due to changes in homing), but by day 4 both CD4 and CD8 T cell numbers were at or above pre-injection baseline. At doses as low as 10ug/kg, CD8 counts were increased, 200 cells/ml above baseline by day 14, and as high as 600 cell/ml above baseline in subjects who received 30 and 60 ug/kg. Less impressive were the changes in CD4 counts, which only demonstrated a marked increase at doses of 60ug/kg, where subjects had 400 cells/ml above baseline by day 14. These numbers persisted for at least 28 days. The increase was reflected in an increased of central memory phenotype cells.

In this rhIL7 trial reported by Sereti and colleagues, they found no relative expansion of regulatory T cells. Other researchers have found an important role for IL-7 and IL-7Rα

signaling in regulatory T cells. Interestingly, Mazzucchelli and colleagues observed that mice deficient in IL-7Rα had 22.7 fold reduction in CD4+CD25+FoxP3+ Tregs, whereas Treg levels were relatively stable in IL-7 KO and TSLPR KO animals. These data suggest that either IL-7 or TSLP are sufficient to support the development of Tregs. The researchers went on to define the role of this cytokine-receptor network in supporting development and persistence of regulatory T cells. Our own work demonstrates that CD4+CD25+FoxP3+ regulatory T cells can expand in the setting of combined short-term costimulation blockade and IL-7Rα blockade. The findings by Mazzucchelli and others outline a redundancy in IL-7, IL-7Rα , TSLP and TSLPR signaling. This group identified that IL-7Rα was important for the development, but not the persistence of peripheral Tregs. Other groups have found that even in the absence of IL-7Rα a relatively normal frequency of Tregs can develop (165). In our own work we do not find an absolute increase in the number of Tregs, suggesting that these cells are not expanding (although this remains to be tested). Rather, we hypothesize that Tregs can rely on alternative signals to persist, including TSLP or IL-2, which is concordant with the above findings, and we hypothesize that the relative increase of Tregs is beneficial for graft survival (166). These hypotheses require further testing.

The role of IL-7 as a signal 3 cytokine is gaining interest, by those intent on utilizing this pathway as a vaccine adjuvant or in cancer immunotherapy. Studies utilizing IL-7 as a vaccine adjuvant show both augmented expansion of effector cells as well as durable improvements in the quality and quantity of memory. In these studies, researchers found that immunization with IL-7 or IL-15 augmented the quality and quantity of memory, but adjuvant IL-7 elicited more tetramer positive memory cells than IL-15, and also resulted in better tumor growth control than IL-15 (167). A number of studies in cancer vaccine development and cancer immunotherapy made similar observations, and went so far as to

utilize a strategies that provided both costimulation with engineered CD80 expression as well as signal 3 with IL-7 expression, to promote potent antitumor immunity (168-170). Researchers interested in leveraging these effects for control of chronic infection found promising, reinvigorating effects of IL-7 administration. Immunotherapy with IL-7 in the context of chronic LCMV Clone 13 infection resulted in expansion of naïve and antigen specific T cells, decreased PD1 expression and changes in activation status that suggests a reversal or prevention of exhaustion and limited Treg development (171, 172). In addition to these cell intrinsic effects, the ex-vivo administration of rhIL7 in humans has resulted in increased diversity of the T cell repertoire (173).

Mounting evidence suggests that IL-7 may augment protective immunity through a number of mechanisms. In the context of undesirable immune responses such as in transplantation and autoimmunity, IL-7 has been identified as a potential mediator of disease. Genetic studies revealed polymorphisms in IL-7Rα were highly associated with increased incidence of autoimmune disease, demonstrating that polymorphisms in IL-7Rα had a significant correlation with incidence of multiple sclerosis (MS, 12% etiologic risk, compared to 40% etiologic risk of HLA-DR15), and furthermore this group found elevated IL-7 and IL-7Rα mRNA in CSF of MS patients with progressive disease compared to healthy controls (174, 175). In the setting of GvHD, researchers found elevated IL-7 levels to be associated with and predictive of acute disease and poor outcomes following bone marrow transplantation (176-178). In our own studies, anti-IL7Rα therapy synergizes with costimulatory blockade to constrain allostimulated T cell proliferation in a model of GvHD, but further studies are needed to assess whether a physiologic benefit exists in this model. In the setting of autoimmune disease, blockade of IL-7Rα resulted in inhibition of collagen

induced arthritis, and chronic colitis, likely by limiting T cell activation and expansion of pathogenic T cells (179). Our studies suggest that indeed the IL-7Rα pathway provides important signaling which supports alloreactive immune responses, and together with costimulation blockade, anti-IL7Rα therapy results in prolonged graft survival, which will be discussed more completely in subsequent chapters.

Costimulation Independence – A Pressing Clinical Need with Potential Solutions

Since Medawar's observations of second-set rejection in man and mice, the immunological barrier in transplantation has continued to be the source of great curiosity, creativity and the seed of profound scientific advances, rewarding its students with principles that have informed (at least) biology broadly. The unmet clinical needs for patients suffering from end stage organ failure demand focused investigation, but these studies promise to yield broadly applicable knowledge. The field is at a turning point, with the advent of biologics, and specifically the clinical utilization of costimulation blockade based therapies, which foreshadow the opportunity for the induction of durable tolerance, and tailored therapies that might account for a patient's personal pre-transplant immunophenotype.

However, the steep challenge of costimulation independent rejection remains. A number of immunologic culprits have been identified and are actively being investigated. Immune memory in particular, with increased capacity for trafficking into peripheral tissues, augmented adhesion molecule expression and unique cytokine requirements, along with a change in the sensitivity to costimulatory blockade, is an important area of ongoing research. Specific memory subsets will require careful evaluation of their unique contributions to costimulation independent allograft rejection. Signal 3 cytokines IL-2/IL-15 and IL-7 have a

demonstrated impact in costimulation independent responses, but also require further study to elucidate their mechanism of action. The universe of Signal 3 cytokines is by no means limited to those in the γ_c family, and in fact exciting preliminary data in our lab indicate a number of inflammatory cytokines may support costimulation independent responses in certain models, but these studies are ongoing.

The availability of sophisticated inducible knockout systems will help researchers temporally isolate the contributions of cytokines and receptors and more specifically identify the cell subsets responsible for responses. In other words, we will have the ability to more specifically define when, how and which cells support costimulation independent rejection. In tandem with this approach which obviates the confounding effects of developmental defects, techniques in discretely visualizing whole-body anatomic distribution will give insight into the trafficking patterns and anatomic niches that necessarily play a role in immunopathology. Together, temporal and spatial tools will help support and contextualize studies conducted at specific time-points in immune responses. For the study of allograft rejection, and the role of Signal 3 cytokines in costimulation independent rejection, these inducible knockout systems and whole-body visualization approaches will provide invaluable insight into the basic mechanisms of T cell responses that give rise to immunopathology.

Chapter 2. Belatacept Resistant Rejection is Associated with CD28$^+$ Memory CD8 T cells

Introduction

Solid organ transplantation has become the primary treatment for end-stage organ failure. Success over the last 30 years has largely been driven by the advent of increasingly potent immunosuppressants. In particular, calcineurin inhibitors (CNIs) such as cyclosporine and tacrolimus were instrumental in reducing the incidence of early graft failure due to acute rejection. Despite these advances, long-term transplant outcomes have remained largely unchanged over the past 20 years (28). Although this is likely multifactorial, the nonimmune side effects of CNIs, including nephrotoxicity, diabetes, hyperlipidemia, and increased overall cardiovascular risk, play an important role in diminishing long-term outcomes (30, 31, 180). In 2011 the first non-CNI alternative, belatacept, was approved for use in kidney transplantation (69). Belatacept is a high-affinity variant of the fusion protein CTLA4-Ig, which specifically blocks CD28-mediated T cell costimulation, providing a more targeted and less toxic form of immunosuppression than CNIs (68). Kidney transplant patients treated with belatacept live longer and enjoy better renal function than those patients treated with cyclosporine (43% reduction in the risk of death or graft loss at 7-year follow-up (73, 181-183). Despite these improvements, use of belatacept has to date been tepid, mostly due to its association with more frequent and severe rejection episodes in a subset of patients (71). Costimulation blockade–based strategies hold immense promise for improved long-term outcomes, but resistance to belatacept-based immunosuppression in a subset of patients demands further investigation into the underlying mechanisms of costimulation independence. Indeed, wider utilization of this more targeted, less toxic approach to

transplant immunosuppression may hinge on the ability to phenotypically identify costimulation-independent cell subsets and understand their unique signaling requirements (184). This knowledge will enhance both the clinical utility of belatacept as well as inform future strategies to optimize belatacept-based therapy.

Belatacept binds to CD80 and/or CD86, preventing the ligation of the CD28 costimulatory molecule expressed on the majority of T cells. The ability of cells to dispense with CD28 costimulation, potentially marked by CD28 loss, is one mechanism by which T cells may become belatacept resistant. Increases in CD28− cells are associated with advanced age and chronic inflammation (104, 105). More specifically, inflammatory cytokines such as tumor necrosis factor (TNF), IL-2, and IL-15 drive the loss of CD28 (106-108). Antigen exposure and the development of terminally differentiated T cell memory is also marked by the downregulation of CD28 (82, 185). Contextually, loss of CD28 or CD28 independence is part of a program of differentiation and cellular maturation marked by changes in receptor expression and cell functionality. Classic models of memory classification define CD4 and CD8 T cells by CCR7 and CD45RA or by CD28 and CD95 (81, 186, 187). Multiparametric flow cytometry has revealed the phenotypic and functional heterogeneity of T cell subsets (78, 79, 187-189). These studies reveal functional differences between subsets defined by four or more phenotypic markers at a time, with a potentially critical transition marked by CD28 loss (83). Previous studies demonstrate that CD28+ cells retain enhanced antiviral capacity and proliferative potential, whereas loss of CD28 is associated with increased cytotoxicity, reduced responsiveness to T cell activation via the T cell receptor, and the development of a dependence on homeostatic cytokine signaling for survival and effector function (82, 86, 144, 190). One study suggests that CD28−CD57+CD4+ T cells may be associated with increased risk of belatacept-resistant rejection (103). How and when CD28 is

lost following transplantation, and whether the loss of CD28 potentiates belatacept resistance, remain formally untested.

We investigated the mechanisms that underlie belatacept resistance in a preclinical nonhuman primate (NHP) model of kidney transplantation. Therapy was withdrawn day 140 posttransplantation, giving rise to two distinct study populations: belatacept-resistant animals that experienced early acute rejection during belatacept therapy, and belatacept-susceptible animals that demonstrated excellent graft function for the duration of belatacept treatment. Together with data from a separate clinical study of patients treated with belatacept (191), we observe that a critical threshold frequency of CD28+, not CD28−, memory T cells is associated with belatacept resistance. These CD28+ memory cells retain proliferative capacity and may eventually lose CD28 expression as they fully differentiate into cytotoxic effector T cells. These findings suggest that pretransplant immunophenotyping using the frequency of CD8+CD28+TEMRA T cells may provide a strategy to identify individuals who are susceptible to belatacept therapy, thereby reducing the risk of rejection.

Materials and Methods

Donor–recipient pair selection and kidney transplantation. All experiments described herein were performed in compliance with the principles set forth in The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services). Outbred rhesus monkeys (Macaca mulatta) ranging between 3 and 5 years old were obtained from AlphaGenesis, Inc. (Yemassee, SC) and Yerkes National Primate Research Center (Lawrenceville, GA). Donor–recipient pairs were chosen to maximize genetic disparity at both MHC class I and class II alleles based on 454 deep sequencing analysis (University of Wisconsin, Madison, WI). Kidney transplantation was performed using standard microvascular techniques . Animals were heparinized (100 U/kg) during organ procurement and implantation. Left native nephrectomy was performed at least 3 weeks prior to transplantation, and a completion right native nephrectomy was performed at the time of transplantation. All transplanted animals were monitored with daily clinical assessment and serial laboratory evaluations, including complete blood count and serum chemistry.

NHP experimental groups and immunomodulation. All animals received methylprednisolone (subcutaneous injection according to the following schedule, day 0 (d0): 20 mg, d1: 16 mg, d2: 12 mg, d3: 8 mg, d4: 4 mg, d5–14: 3 mg, d15–140: 1 mg) and mycophenolate mofetil (30 mg/kg bid, d0–d140) to recapitulate clinically relevant immunosuppression strategy. A subset (n = 8/16) of belatacept-treated animals received basiliximab induction therapy (0.3 mg/kg d0, d4). All tacrolimus-treated animals received

basiliximab induction (Figure 2.1). Belatacept therapy was discontinued at day 140 (d0: 10 mg/kg, d4: 15 mg/kg, d14–d56: 20 mg/kg biweekly, d56–d140: 20 mg/kg every 4 weeks). Tacrolimus levels were monitored weekly (8–12 ng/mL d0–d56, 5–8 ng/mL d57–d168, Figure S4.1). Expanded analysis of pretransplant immunophenotype included animals treated with CD28 domain antibody (manuscript in preparation) or CD154 domain antibody (61).

Mixed lymphocyte reaction and cell sorting. Peripheral blood mononuclear cells (PBMCs) were isolated from unmanipulated rhesus monkeys and fluorescently sorted based on CD28, CD45RA, and CCR7 expression. Responder cells were labeled with Cell Trace Violet (C34557; Invitrogen, Carlsbad, CA), and stimulators were labeled with carboxyfluorescein succinimidyl ester (Invitrogen, C34554). 1 × 105 Responder cells were plated in 96-well flat-bottom plates with 1×105 irradiated MHC-mismatched stimulators, and cultured for 5 days. Cells were cultured in RPMI 1640 (Corning cellgro, Manassas, VA) supplemented with 10% fetal bovine serum ± 100 μg/mL belatacept (Bristol Myers-Squibb, Princeton, NJ).

Isolation of graft-infiltrating lymphocytes. To isolate graft-infiltrating lymphocytes, rejected allografts were mechanically disrupted, filtered through 70-μM cell strainers, washed in phosphate-buffered saline, and then filtered again through 40-μM cell strainers. Cell suspensions were separated by Ficoll-Paque density gradient centrifugation (GE Healthcare Life Sciences, Pittsburgh, PA) to isolate mononuclear cells. Mononuclear cells were washed twice and counted prior to antibody staining.

Antibodies and flow cytometric analysis. Flow cytometric analysis was performed up to three times pretransplant and serially posttransplant to characterize peripheral blood immune cell phenotypes. Total T cells and T cell subsets were quantified by complete blood cell count and flow cytometry. Fresh PBMCs were isolated by Ficoll density gradient centrifugation (BD Biosciences, Franklin Lakes, NJ). PBMCs were stained with the following mAbs: CD3 PacBlue, CD95 V450, CD3 Alexa 700, CD4 PerCP-Cy5.5, CD8 V500, CD28 PE-Cy7, CD25 PE-Cy7, IFNy PE-Cy7, CD28 APC, TNF APC, VLA-4 APC, CD11a PE, CD45RA FITC, CD40 FITC, CCR7 APC, and CD20 APC (all BD Biosciences). PBMCs (1.5 × 106) were incubated with appropriately titered antibodies for 15 min at 20°C and washed twice. Samples were acquired immediately on a BD LSR II multicolor flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA). For the stimulation assay, 1.5 × 106 PBMCs were cultured in RPMI 1640 (Corning cellgro) supplemented with 10% fetal bovine serum and stimulated with 10-μM phorbol 12-myristate 13-acetate (PMA) and 200 nM ionomycin (Sigma-Aldrich, St. Louis, MO), with 1 μL/mL GolgiPlug protein transport inhibitor for 5 h, ± IL-15 (10 ng/mL). PBMCs were processed with BD Cytofix/Cytoperm Plus kit (BD 555028) per the manufacturer's recommendation prior to data acquisition.

Statistics. Survival statistics were calculated using a log-rank test. T cell frequencies were compared using a parametric unpaired t-test. Decision tree analysis was performed with the programming language and statistical software R (v. 3.2.3), and utilized the "rpart" package. Data were analyzed using Prism 6 (GraphPad Software, La Jolla, CA). A two-tailed p-value of <0.05 was considered statistically significant.

Transcriptome analysis. RNA was prepared from biopsies of transplanted kidneys (at time of euthanizing), as well as from isolated graft-infiltrating cells. The quality of the total RNA from samples was monitored by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and RNA quantity was measured with NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE) following the manufacturer's instructions. Two hundred nanograms of total RNA were amplified and labeled with 3' IVT Express Kit (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized on Affymetrix GeneChip Rhesus Macaque Genome Array (Cat. 900657, Affymetrix). Scanned images were subjected to visual inspection and a chip quality report was generated by Expression console (Affymetrix). The image data were processed using the Rhesus Macaque Array to determine the specific hybridizing signal for each gene. All chip data were loaded into BMS database (Bristol Myers-Squibb) for further analysis. Gene expression data were also analyzed utilizing DAVID Bioinformatics Database as described (192, 193)

Results

Transplant recipients segregated based on response to belatacept therapy

In order to investigate the mechanisms of rejection during belatacept treatment, 24 rhesus macaques were randomized to receive either belatacept- or tacrolimus-based immunosuppression following life-sustaining kidney transplant (Figure 2.1). Animals continued to receive immunosuppressive therapy until day 140, at which time therapy was discontinued. We observed two distinct populations within each treatment group: belatacept-"resistant" animals that experienced rejection and graft failure during treatment, and belatacept-"susceptible" animals that maintained excellent graft function during administration of therapy (Figure 2.1A). Tacrolimus therapy similarly gave rise to resistant (n = 2) and susceptible (n = 6) animals (Figure 2.1B). Once all treatments were stopped, the remaining "susceptible" animals eventually succumbed to immune-mediated graft destruction in the absence of immunosuppression.

IL-2Rα blockade is utilized clinically as induction therapy and has demonstrated efficacy in combination with primary immunosuppressive agents such as CNIs or belatacept (69, 194). There has been some concern that the addition of an antibody targeting CD25 may impact beneficial cell populations such as regulatory T cells and may negatively impact outcomes, particularly with newer strategies such as costimulatory blockade (147).The addition of basiliximab to a subset of the belatacept-treated animals (n = 8) did not have a significant impact on the rate of rejection (rejectors on therapy n = 3/group) when compared to belatacept-treated animals that did not receive basiliximab induction (n = 3/group). Similarly there was no significant difference in survival between belatacept (mean survival time [MST] = 167 days), belatacept with basiliximab induction (MST = 189.5 days), or tacrolimus (MST = 183 days)-based immunosuppression (Figure S2.1). All animals

demonstrated excellent renal function as measured by serum creatinine until just prior to allograft rejection, defined as two consecutive serum creatinine values >5 (Figure S2.1).

Elevated frequency of CD28+CD8+TEMRA is associated with costimulation-resistant rejection

An important question in the field of transplantation is the role of pretransplant immune memory status in transplant recipients, particularly in the context of costimulatory blockade–based immunosuppression. We and others have demonstrated elevated pre-existing immune memory, and more broadly, increasing prior pathogen exposure constitutes a potent barrier to transplant tolerance in murine and NHP models (87, 98, 101). Other groups have found unique regulatory memory subsets in alternative models of transplantation (195).To assess whether pretransplant memory immune phenotype could predict whether animals would respond to belatacept-based immunosuppression, we examined peripheral blood samples from all recipients prior to kidney transplantation using multiparameter flow cytometry. We then examined the association of the frequency of memory cell subsets and rejection. We found that belatacept-resistant animals, which rejected while on therapy, had higher baseline frequencies of CD28+CD95+CD8+ T cells prior to transplantation, compared to nonrejectors (Figure 2.3C, $p = 0.0402$). A similar trend was not observed in the tacrolimus-treated animals (Figure 2.3D, $p = 0.2005$). Interestingly, the pretransplant frequency of CD28− memory T cells (CD28−CD95+ CD8+ T cells), which have been implicated in costimulation-resistant rejection [46], was not significantly different between therapy-resistant or -susceptible animals in any treatment group (Figure 2.3E and F).

The use of CD28 and CD95 is a standard approach for identifying naïve (CD28+CD95−), central memory (CD28+CD95+), and effector/effector memory (CD28−CD95+) T cells when phenotyping subsets in primates. For a more granular analysis of the heterogeneous and functionally distinct memory and effector populations within CD3+ T cell compartment, we included additional conventional memory markers CD45RA and CCR7. Utilizing a gating strategy involving these four memory markers (Figure S2.6) revealed that belatacept-resistant rejection was highly associated with elevated pretransplant frequencies of CD28+CD95+CD45RA+CCR7− CD8 T cells, so-called CD28+ CD8+ TEMRA (Figure 2.4C and D, $p < 0.0001$). The pretransplant frequencies of CD28+ CD8+ TEMRA were significantly elevated in belatacept-resistant animals irrespective of whether they received basiliximab induction (Figure S2.2C–F). In contrast, tacrolimus-resistant animals did not exhibit similarly elevated pretransplant frequencies of CD28+ CD8+ TEMRA (Figure 2.4A and B, $p = 0.2060$).

In order to assess the generalizability of this biomarker to predict costimulation blockade–resistant rejection beyond belatacept treatment, we examined additional animals that had been treated with various costimulation blockade reagents that target either the CD28-CD80/86 or CD40-CD154 pathways. Similar to the belatacept-treated animals, we saw a clear distinction between "susceptible" and "resistant" animals using the pretransplant level of CD28+CD8+T effector memory CD45RA+cells (TEMRA) (Figure 2.4E and F, $n = 37$, $p < 0.0001$). We next performed a decision tree analysis on this same larger cohort utilizing all CD4 and CD8 T cell subsets, including memory subsets such as CD4+ and CD8+, effector memory T cells (TEM) or TEMRA, accounting for CD28 expression. We found that a frequency of >3% CD28+CD95+CD45RA+CCR7− (CD28+TEMRA) of total CD8+ T cells provided the strongest predictor of costimulation blockade–resistant

rejection in our model with an 87.5% sensitivity and 95.2% specificity (Figure 2.4G). For this model, the positive predictive value was 93.3% and the negative predictive value was 90.1% (Figure 2.4G). Tacrolimus-treated animals did not similarly segregate based on baseline frequencies of memory T cell subsets (Figure 2.4A and B), and thus we limited our decision tree analysis to the belatacept-treated cohort.

CD28+ CD8+TEMRA cells lose CD28 expression and have superior proliferative capacity

We investigated the kinetics of peripheral blood CD28+ CD8+ TEMRA T cells over time and interestingly observed that while the overall frequency of CD45RA+CCR7− CD8+ T cells (CD8+ TEMRA) remained unchanged following transplantation, the percentage of CD28+ cells within the CD45RA+CCR7− CD8+ T cell (CD8+ TEMRA) compartment precipitously declined in the peripheral blood in belatacept-"resistant" animals (Figure 2.5A and B). The rapid decline of the CD28+ fraction of CD8+ TEMRA was observed in both belatacept-resistant animals irrespective of basiliximab induction (Figure S2.3). When we examined the frequency of CD28+ cells within the CD8+ TEMRA subset 7 days prior to rejection, we found a significant decrease compared to baseline values (−19.24%, $p = 0.0029$, Figure 2.5C).

In an effort to measure the proliferative capacity and effector function of alloreactive NHP T cell subsets, we sorted PBMCs from rhesus macaques into CD28+ and CD28− TEMRA and performed a standard mixed lymphocyte reaction using allogeneic stimulators. We found that CD28+CD8+ TEMRA had superior proliferative capacity compared to CD28−CD8+ TEMRA cells (77.5% relative reduction in proliferative capacity, Figure 2.6A, $p < 0.001$). This difference in proliferative capacity was not affected by belatacept treatment.

CD28+ CD8+ TEMRA exhibited a 71% proliferative advantage over conventional CD8+ TEM, CD8+CD45RA−CCR7−, as well (Figure 2.6B, $p = 0.0016$). Of note, after 96 h in coculture, allo-stimulated CD8+ TEMRA failed to produce effector cytokines with classic restimulation reagents such as PMA and ionomycin. Instead, exogenous administration of IL-15 to the culture was required to elicit effector function for either CD28+ or CD28− TEMRA. IL-15 provoked a potent response in both CD28+ and CD28− TEMRA (Figure 2.6C, increased frequency of Interferon-γ+TNF+ cells no cytokine vs cytokine CD28+ TEMRA 0.11% to 11.51%, $p = 0.0013$, and CD28−TEMRA, 0.21% to 15.77%, $p < 0.0001$). While there is a clear distinction between CD28+ and CD28− TEMRA in their proliferative capacity, both subsets seem to be reliant on exogenous IL-15 for effector cytokine production.

Increased adhesion molecule expression is temporally associated with rejection

Mounting evidence has demonstrated a critical role for adhesion molecules such as CD49d (VLA-4) and CD11a (LFA-1) in mediating costimulation-independent allograft rejection (196-198). Analysis of adhesion marker expression on T cell subsets prior to transplantation revealed no significant difference in the frequency or intensity of VLA-4 or LFA-1 expression on CD8+ or CD4+ T cells between belatacept-"resistant" or -"susceptible" animals. After transplantation, however, circulating CD8+ and CD4+ T cells increased expression of VLA-4 and CD11a early in those animals resistant to belatacept therapy, particularly in the weeks preceding allograft rejection (Figure 2.7A and B). Graft-infiltrating cells analyzed at the time of rejection in all treatment groups expressed uniformly high levels of VLA-4 and CD11a compared to baseline peripheral blood samples (Figure 2.7C and D). Taken together, these data suggest that CD28+TEMRA may drive a

costimulation-independent response where donor reactive cells rapidly expand and acquire effector function and then lose CD28 expression while upregulating adhesion molecules such as LFA-1 and VLA-4, allowing for access to the donor compartment. At the time of rejection, graft infiltrate of all animals demonstrated uniformly elevated levels of VLA-4 and CD11a expression, suggesting that expression of these molecules is part of "final common pathway" of allograft infiltration regardless of treatment.

Belatacept-resistant graft infiltrate is characterized by CD28− CD8+ TEMRA

As described above, treatment with belatacept gave rise to two distinct groups: those animals that rejected on therapy (<140 days): "resistant" and those that enjoyed excellent graft function with minimal graft infiltrate on biopsy until therapy was withdrawn, at which time rejection ensued: "susceptible." In other words, "resistant" animals rejected on belatacept treatment while "susceptible" animals did not reject until therapy had been discontinued. We were interested to see whether the character of the rejection response differed between these two groups. Accordingly, we more closely examined the rejection response between belatacept-"resistant" and -"susceptible" groups at the time of graft failure. In general, allograft infiltrate was principally CD8+ T cells (60–70%), with a smaller CD4+ component (20–30%), in both groups (data not shown). These frequencies in graft infiltrate were reciprocal to the frequencies found in the peripheral blood where CD4+ T cells typically outnumbered CD8+ T cells 3 to 1. Belatacept-"resistant" animals exhibited a significant increase in CD8+ TEMRA graft-infiltrating cells extracted from rejected kidneys, whereas belatacept-susceptible animals exhibited a larger frequency of less fully differentiated CD8+ TEM, even though no difference in the frequency of these subsets existed in the peripheral blood before transplant (Figure 2.8A and B). Further analysis

including CD28 expression status revealed a significant increased CD28− CD8+ TEMRA in graft-infiltrating cells in resistant animals (Figure 2.8E), whereas susceptible animals exhibited a higher frequency of CD28+ CD8+ TEM (Figure 2.8F). These differences were not observed in tacrolimus-treated animals (Figure 2.8C and D, G–H). In summary, when examining the character of the infiltrate at the time of rejection in "resistant" animals, the predominant cell subset was CD28− CD8+ TEMRA compared to CD28+ CD8+ TEM in "susceptible" animals, suggesting that a more terminally differentiated subset was responsible for rejection in belatacept-"resistant" animals (representative flow plot, Figure 2.8I).

Graft-infiltrating cells in belatacept-"resistant" rejection exhibit a transcriptional signature consistent with exhaustion

In an effort to further characterize the graft infiltrate in belatacept-resistant animals, we analyzed the gene-expression profile of graft tissue from belatacept-"resistant" versus -"susceptible" animals at the time of rejection. We identified unique modules of coordinated gene expression that were significantly divergent between the two groups. Unique chemokine and cytokines, increased trafficking and adhesion molecules, and increased expression of CD8+ T cell memory and exhaustion genes were associated with belatacept resistance (Figure 2.9A–C). Pro-inflammatory chemokines and cytokines such as CXCL12, IL-6, and IL-15 were coordinately upregulated in belatacept-resistant animals compared to those that were susceptible to therapy (Figure 2.9A). Cytokine and chemokine receptors IL-17RA, IL12RB2, and CCR6 were also upregulated in belatacept-resistant rejection, while CD25 (IL2RA), critical for regulatory T cell function, was downregulated. Genes associated with adhesion and trafficking such as ITGA4 and ITGB2 were upregulated in belatacept

resistance (Figure 2.9B). Belatacept-resistant animals demonstrated significant upregulation of a number of coinhibitory receptors and memory transcription factors characteristic of and critical for terminally differentiated CD8+ T cells, such as several of the killer cell lectin-like receptors, EOMES, TBX21, BTLA, CTLA-4, and FAS (Figure 2.9C). Consistent with the flow cytometric analyses, these gene expression data suggest that the infiltrate associated with belatacept resistance is of a more fully differentiated T cell phenotype.

Discussion

Current immunosuppressive strategies most commonly employ a CNI, such as tacrolimus or cyclosporine, as the primary agent to prevent rejection following organ transplant. While these reagents provide for excellent initial outcomes, including 1-year patient and graft survival, long-term outcomes remain less than desirable (28, 31, 180). There has been a concerted effort to develop new strategies to avoid unwanted side effects and improve late outcomes. While belatacept has established the promise of costimulation blockade to improve long-term outcomes, widespread adoption of this therapy has been limited by, among other things, increased rates of rejection and a perceived lack of efficacy. In an effort to better understand the underlying mechanisms leading to the resistance of costimulation blockade therapy, we designed a study in a preclinical model of NHP kidney transplantation comparing animals treated with tacrolimus and belatacept. We identified a pretransplant immunophenotype in the peripheral blood of elevated CD28+ CD8+ TEMRA cells that discriminates animals that go on to experience costimulation blockade–resistant rejection from those that are susceptible to therapy. These CD28+ TEMRA retain proliferative capacity in addition to other effector functions, unlike their CD28− counterparts. Furthermore, we show that these cells likely downregulate CD28 or give rise to a population of CD28− effector/TEM, which constitute the major component of the infiltrate in rejected kidneys in resistant animals. Along with data presented in our companion study in human patients, we suggest that this relatively straightforward test could potentially be used as a pretransplant screen to determine eligibility for belatacept therapy (191).

There are several differences between our findings in the NHP model and what was observed in patients. First, there tended to be a tighter link between increased frequencies of CD28+CD4+ TEM/EMRA and a higher likelihood of rejection in human patients, whereas in our NHP study we found the pretransplant level of CD28+CD8+ TEMRA was more highly associated with costimulation-independent rejection. There are many differences between NHPs and humans, including age and immune experience that are likely contributors. Animals used in our studies are captive-bred juveniles and may not have the same degree of immune exposures as older adult humans. Moreover, human transplant recipients, unlike the healthy primates, all have end-stage renal disease with its accompanying uremia and almost universal dialysis requirement, factors that can contribute to an inflammatory environment causing immune dysregulation, perhaps differentially affecting some cell subsets more than others.

We initially hypothesized that higher baseline levels of CD28− memory T cells may be predictive of costimulation-independent rejection. On a cursory level, this subset would appear to be poised to avoid the effects of belatacept, given the lack of the receptor for the targeted pathway. However, a more comprehensive view of this subset suggests that the lack of CD28 expression accompanies cells that have a more differentiated phenotype, usually following antigen exposure and activation (109). These cells often demonstrate diminished proliferative capacity despite their immediate effector capabilities. While not completely clear, we suggest from our studies that CD28− cells, although potentially potent effector cells, may not possess the capacity to sustain a response that would lead to rejection of the transplanted organ. Rather we believe that CD28 expression on TEM/TEMRA designates a cells subset that has a combination of proliferative reserve and cytolytic capacity. In fact, we

showed that CD28+ CD8+ TEMRA proliferate readily in mixed lymphocyte reaction despite belatacept treatment, and that CD28− CD8+ TEMRA lack proliferative potential. Interestingly, we observed that while CD8+ TEMRA cell frequencies remain stable during transplantation, there is a rapid loss of the CD28+ fraction. We further demonstrate CD28−CD8+ TEMRA uniquely compose a predominate fraction of the allograft infiltrate at the time of rejection in belatacept-resistant rejection.

The stability of this phenotype is unknown (i.e. higher frequency of CD28+CD8+ TEMRA). Multiple factors may drive increased CD28 expression on TEMRA and TEM. Viral-specific memory T cells from prior pathogen exposure exhibit distinct memory phenotypes based on CD45RA, CCR7, CD28, and CD95 expression. T cell memory phenotype is subject to change based on chronicity of antigen exposure, acuity of infection, or heterologous challenge (82, 84). Consistent with our work, previous studies have identified a highly proliferative and potently cytolytic CD28+ CD8+ TEMRA cell subset in patients vaccinated for yellow fever (199). Additional investigation into the factors contributing to rejection associated with a higher level of CD28+ TEMRA is required. Work by our group and others suggests that differential expression of coinhibitory molecules such as CTLA-4 may be responsible for preferential activation of some T cell subsets when belatacept is used. Further studies are needed to examine the longitudinal stability of this phenotype and whether interventions such as cytokine deprivation via antibody administration or periods of lower overall inflammation/immune stimulation may allow for a decrease in this subset. More importantly, if the level of CD28+ TEMRA changes over time and drops below the 3% level, are animals that were once "resistant" to costimulation

blockade now "susceptible" to therapy? These important questions require additional investigation.

Transcriptomic analysis of the allograft infiltrate suggests that an augmented pro-inflammatory milieu leads to a more fully differentiated T cell infiltrate consistent with the phenotype obtained from flow cytometry. Taken together, these data suggest that one mechanism of belatacept resistance is mediated by CD28-bearing memory cells that leverage a proliferative advantage in order to sustain alloreactivity, while further differentiating and increasingly relying on alternative signals (i.e. killer immunoglobulin-like receptors, γ-chain cytokines, inflammatory chemokines, adhesion molecules) to prosecute costimulation-independent rejection. Studies aimed at high-resolution spatial and temporal tracking of memory T cells will increase our understanding of the ontology, kinetics, and plasticity of T cell memory subsets. These studies may inform how and when to leverage dynamic T cell signaling sensitivities to promote transplant tolerance.

Figures

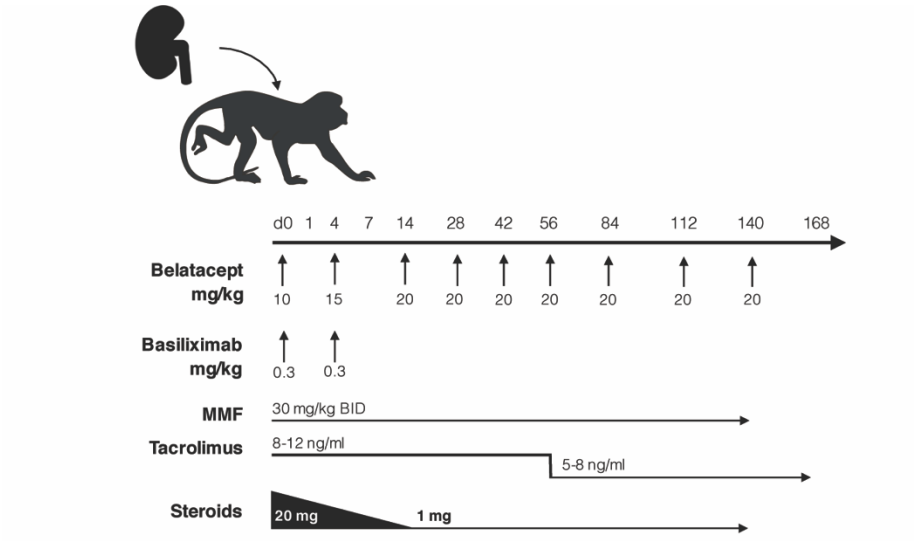


Figure 2.1 Kidney Transplant Treatment Schema. All animals received methylprednisolone (subcutaneous injection according to the following schedule, d0: 20 mg, d1: 16 mg, d2: 12 mg, d3: 8 mg, d4: 4 mg, d5–14: 3mg, d15–140: 1mg) and mycophenalate mofetil (30mg/kg bid, d0-d140) to recapitulate clinically relevant immunosuppression strategy. A subset (n=8/16) of belatacept treated animals received basiliximab induction therapy (0.3mg/kg d0, d4). All Tacrolimus treated animals received basiliximab induction. Belatacept therapy was discontinued at day 140 (d0: 10 mg/kg, d4: 15 mg/kg, d14-d56: 20 mg/kg bi-weekly, d56-d140: 20 mg/kg every 4 weeks). Tacrolimus trough levels were monitored weekly (8-12ng/ml d0-56, 5-8ng/ml d57-168, Supplemental Figure 2.4).

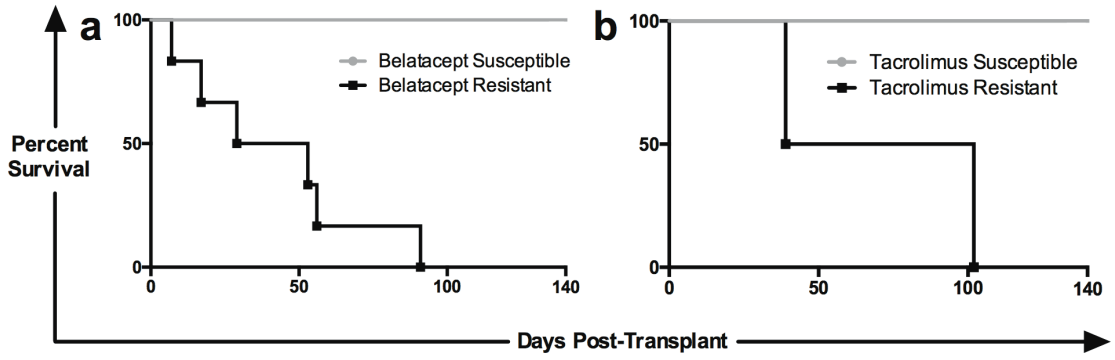


Figure 2.2 Survival and Therapeutic Resistance. Belatacept and tacrolimus based immunosuppression gave rise to two distinct study populations: those animals which were "susceptible" to therapy (grey lines) and those which were "resistant" to treatment (black lines). Resistant animals experienced rejection and graft loss while treatment was ongoing (between day 0 and day 140). Susceptible animals experienced prolonged allograft survival for the entire duration of the therapy and only experienced rejection after withdrawal of therapy (after day 140). (a) Survival with belatacept therapy (n=6 "resistant' animals and n=10 "susceptible" animals). (b) Survival with tacrolimus treatment (n=2 "resistant" animals and n=6 "susceptible" animals).

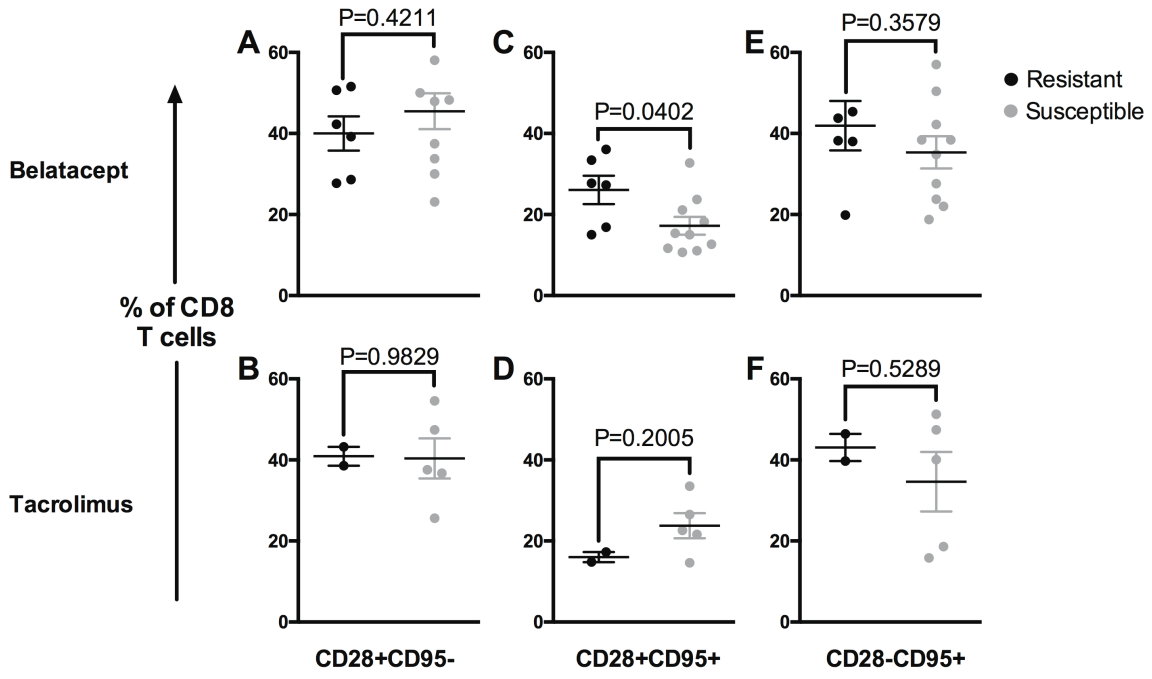
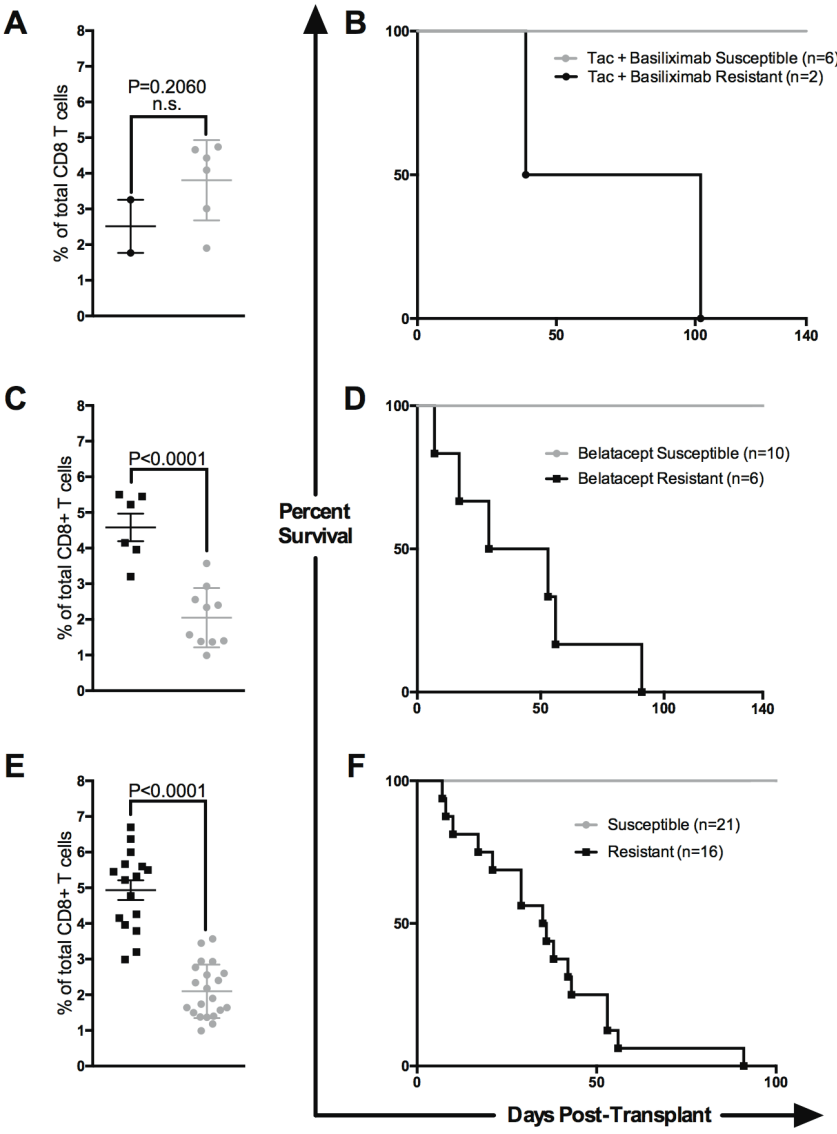


Figure 2.3 Pre-Transplant Immune Phenotyping with CD28 and CD95. Prior to transplantation, CD8 T cells were analyzed by CD28 and CD95 expression (top row: belatacept treated animals, bottom row: tacrolimus treated animals). Subsets are expressed as a frequency of total CD8 T cells as follows: (a & b) CD28+CD95- (c & d) CD28+CD95+ and (e & f) CD28-CD95+. Mean values of pre-transplant samples from resistant animals (black circles) were compared to those from susceptible animals (grey circles). Only the difference in the frequency of CD28+CD95+CD8+ T cells between resistant and susceptible animals treated with belatacept were significantly different (Figure 3c, P=0.0402).


A
% of total CD8 T cells
P=0.2060
n.s.
B
Tac + Basiliximab Susceptible (n=6)
Tac + Basiliximab Resistant (n=2)
C
% of total CD8+ T cells
P<0.0001
D
Belatacept Susceptible (n=10)
Belatacept Resistant (n=6)
Percent Survival
E
% of total CD8+ T cells
P<0.0001
F
Susceptible (n=21)
Resistant (n=16)
Days Post-Transplant

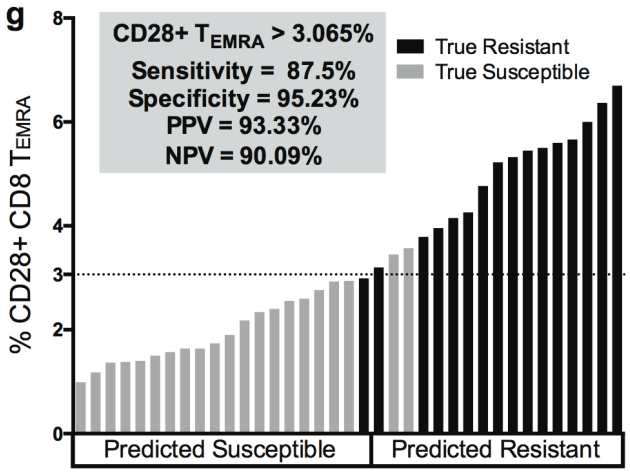

g
CD28+ TEMRA > 3.065%
Sensitivity = 87.5%
Specificity = 95.23%
PPV = 93.33%
NPV = 90.09%
True Resistant
True Susceptible
% CD28+ CD8 TEMRA
Predicted Susceptible
Predicted Resistant

Figure 2.4 Pre-transplant Multi-parametric Immune Phenotyping of CD8 T cells. Pre-transplant immunophenotyping of therapy resistant (black circles) versus therapy susceptible animals (grey circles), with corresponding survival curves. (a) Tacrolimus treated animals demonstrate no significant difference in pre-transplant CD28+CD95+CD45RA+CCR7- cells as a frequency of total CD8+ T cells between therapy susceptible (4a grey circles) and therapy resistant animals (4a black circles, 4b corresponding survival curve). (c) Elevated CD28+CD95+CD45RA+CCR7- (CD28+TEMRA) as a frequency of total CD8+ T cells in pre-transplant peripheral blood samples discriminates belatacept resistant animals (black circles) from those susceptible to treatment (grey circles) with (d) corresponding survival curve ($P<0.0001$). (e) In an expanded cohort of animals treated with costimulatory blockade reagents (either CD28 directed or CD154 directed blockade) we observed a similar pre-transplant immunophenotype between resistant (black circles) and susceptible animals (grey circles), (f) corresponding aggregate survival curve. (g) Decision tree analysis determined that a cut-off value of 3.065% CD28+ CD8+ TEMRA segregated animals who would go on to experience costimulation resistant rejection from those animals susceptible to costimulation blockade therapy (87.5% sensitivity, 95.23% specificity, 93.33% PPV and 90.09% NPV).

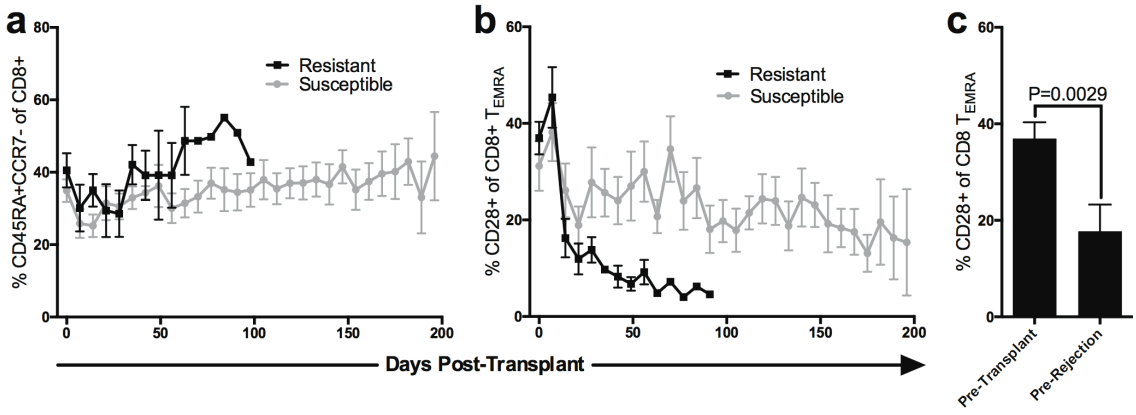


Figure 2.5 Kinetics of CD8+ TEMRA. (a) overall percentage of CD45RA+CCR7- (CD8+ TEMRA, all CD95+) as a subset of CD8 T cells remained stable over time in both groups (b) while the fraction of CD8+ TEMRA which were CD28+ decreased rapidly post-transplantation and remained low in belatacept resistant animals (black lines), compared to animals susceptible to therapy (grey lines). (c) The frequency of CD28+ cells within the CD8 TEMRA subset is decreased by 19.24% (SEM= 3.543%, P=0.0029) 7 days prior to rejection compared to pre-transplant peripheral blood samples.

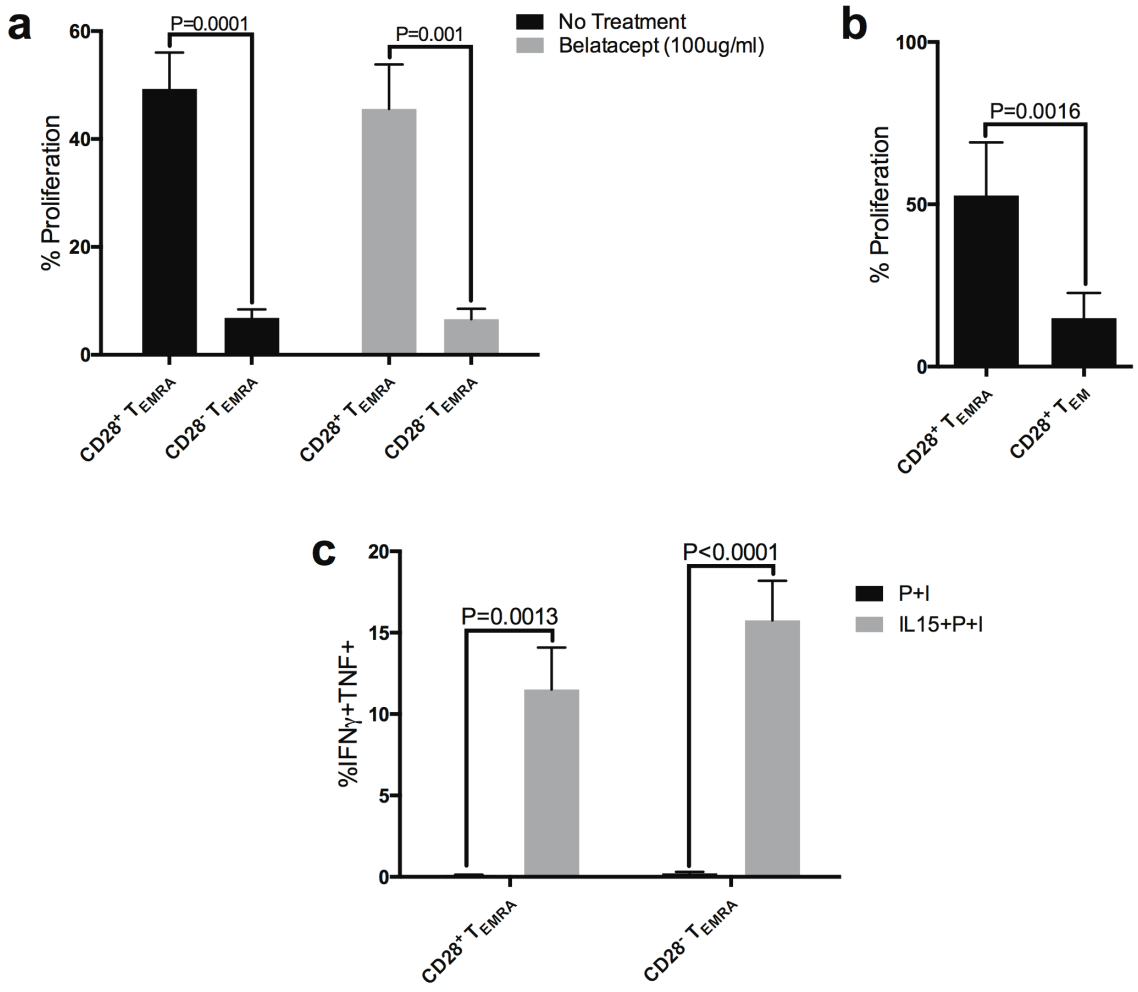


Figure 2.6 In-Vitro Functional Assessment of Alloreactive CD28+ CD8+ TEMRA.

(a) Depletion of CD28+ cells from sorted CD8+ TEMRA results in a 77.5% relative reduction in proliferative capacity (P=0.0001, black bars). Proliferation of CD28+ TEMRA is unaffected by belatacept treatment (100ug/ml, grey bars). (b) CD28+ CD8+ TEMRA exhibited a 71% relative proliferative advantage over CD8+CD45RA-CCR7-, conventional CD8+ TEM (P=0.0016). (c) PMA and Ionomycin (P+I) was insufficient to elicit effector function from CD8+ TEMRA, but addition of IL-15 results in IFNγ+TNF+ CD8+ TEMRA in both CD28+ (P=0.0013) and CD28- TEMRA (P<0.0001).

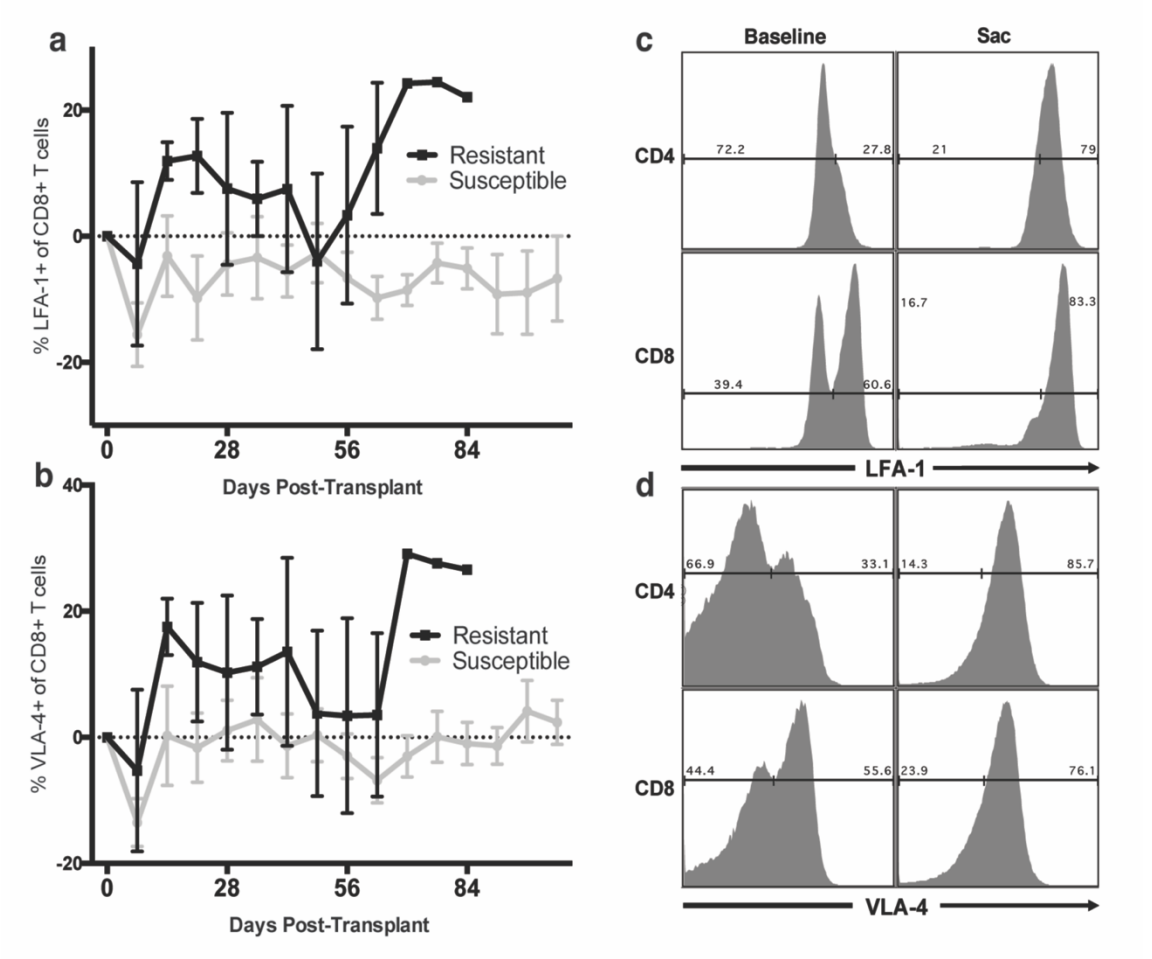


Figure 2.7 Increased Adhesion Molecule Expression in Rejection. Belatacept resistance demonstrates a trend towards increased expression of (a) LFA-1 and (b) VLA-4 early post-transplantation, particularly in the weeks preceding allograft rejection (resistant animals black lines, susceptible animals grey lines). Graft Infiltrating Cells isolated from rejecting allografts (Sac) uniformly express high levels of both (c) LFA-1 and (d) VLA-4 irrespective of response to therapy, compared to peripheral blood at baseline, representative histogram.

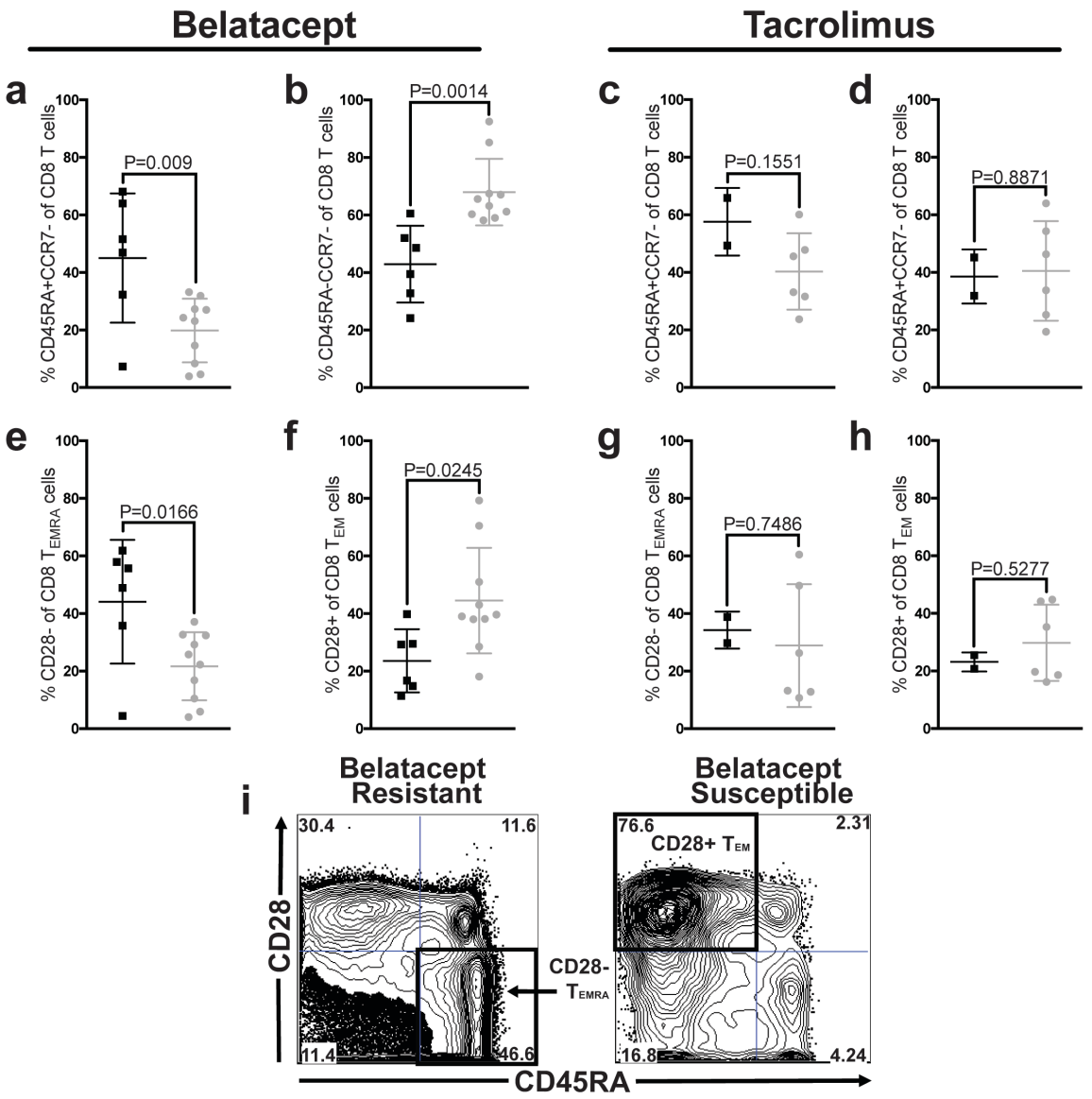
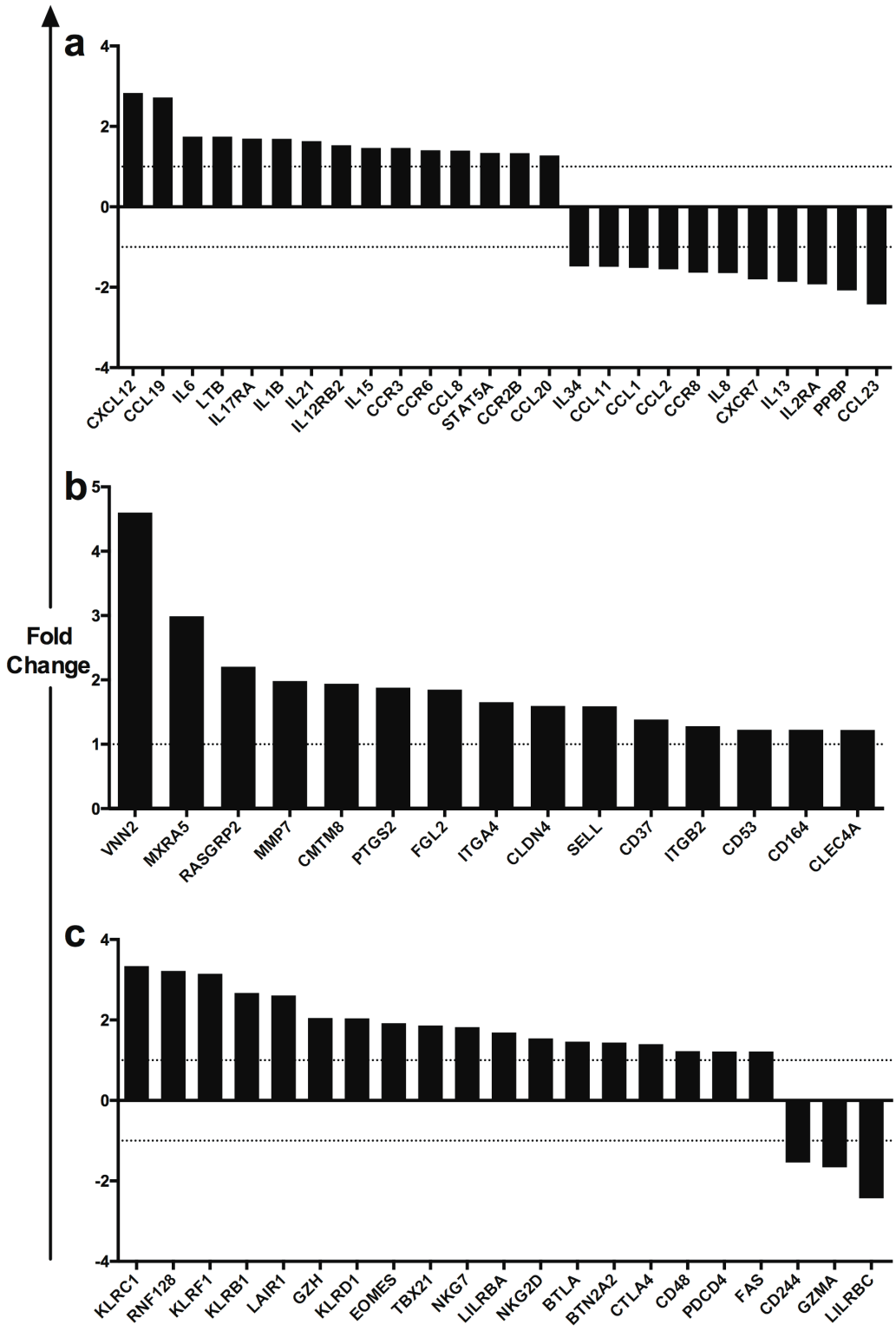


Figure 2.8 Belatacept Resistance is Marked by Terminally Differentiated T cells in the Graft Infiltrate. CD3+ CD8+ T cells extracted from rejected allografts were characterized by memory phenotype. (a) Belatacept resistant animals (black squares) demonstrate a unique, increased frequency of CD8 TEMRA infiltrate within the graft compared to belatacept susceptible animals (grey circles, P=0.009). (b) Belatacept susceptible animals (grey circles) who mount a rejection response in the absence of belatacept demonstrate a less differentiated CD8+ TEM infiltrate compared to belatacept resistant

animals (black squares, P=0.0014). We next investigated the CD28 expression pattern on memory subsets. (e) Belatacept resistant animals (black squares) demonstrated increased CD28- CD8+ TEMRA compared to Belatacept susceptible animals (grey circles, (P=0.0166). (f) In contrast, belatacept susceptible animals (grey circles) had increased levels of less fully differentiated CD28+ CD8+ TEM compared to belatacept resistant animals (black squares, P=0.0245) (i) Example plots of graft infiltrating CD8 T cells in belatacept resistant rejection and belatacept susceptible animals who rejected after the withdrawal of therapy with the predominating phenotype distinctive of belatacept resistance (CD28-CD8+ CD45RA+ TEMRA, lower right quadrant) vs belatacept susceptibility (CD28+ CD8+ CD45RA- TEM, upper left quadrant). These differences were not observed in tacrolimus treated animals (c-d, g-h).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STAT5A
CCR2B
CCL20
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CCL11
CCL1
CCL2
CCR8
IL8
CXCR7
IL13
IL2RA
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MXRA5
RASGRP2
MMP7
CMTM8
PTGS2
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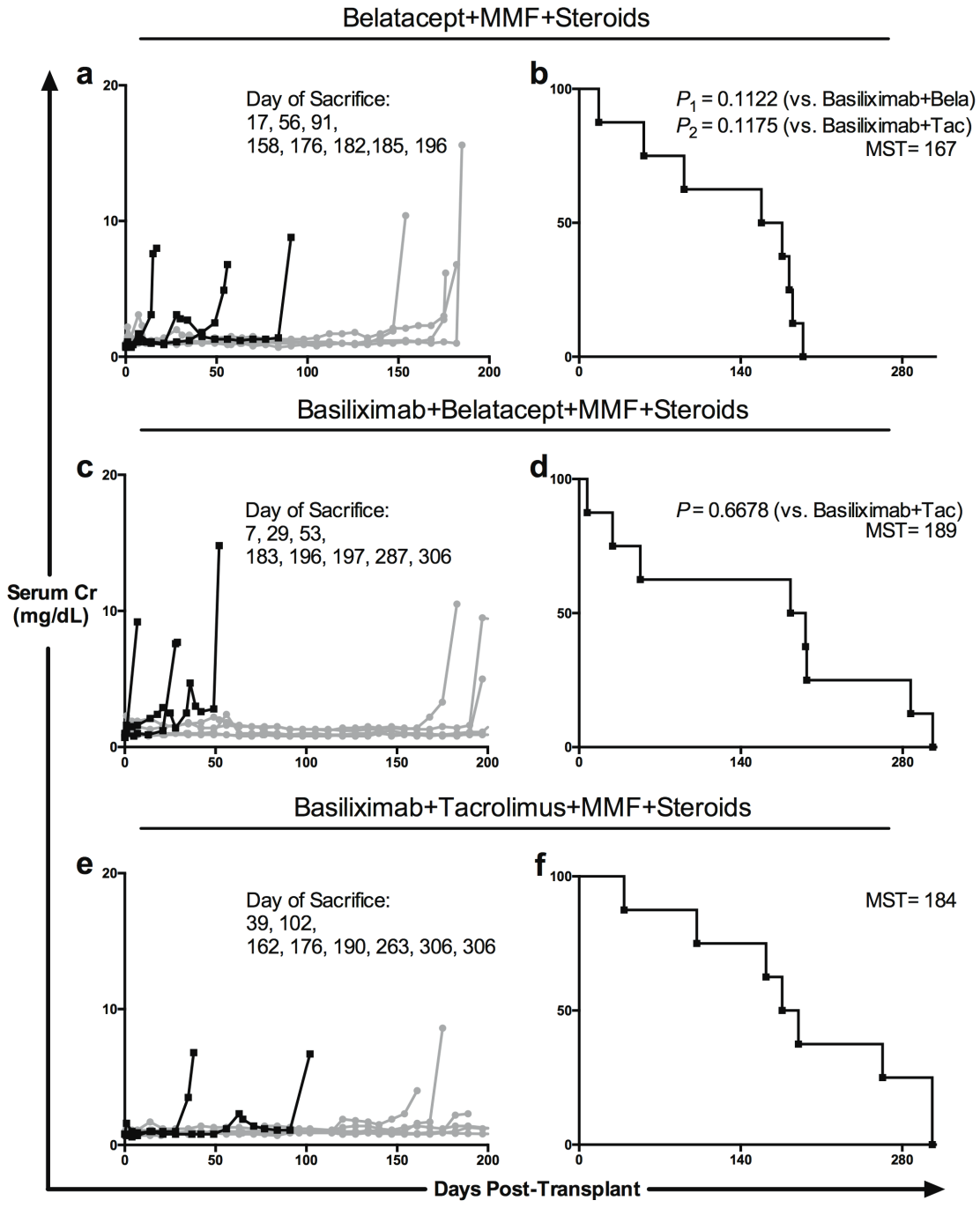
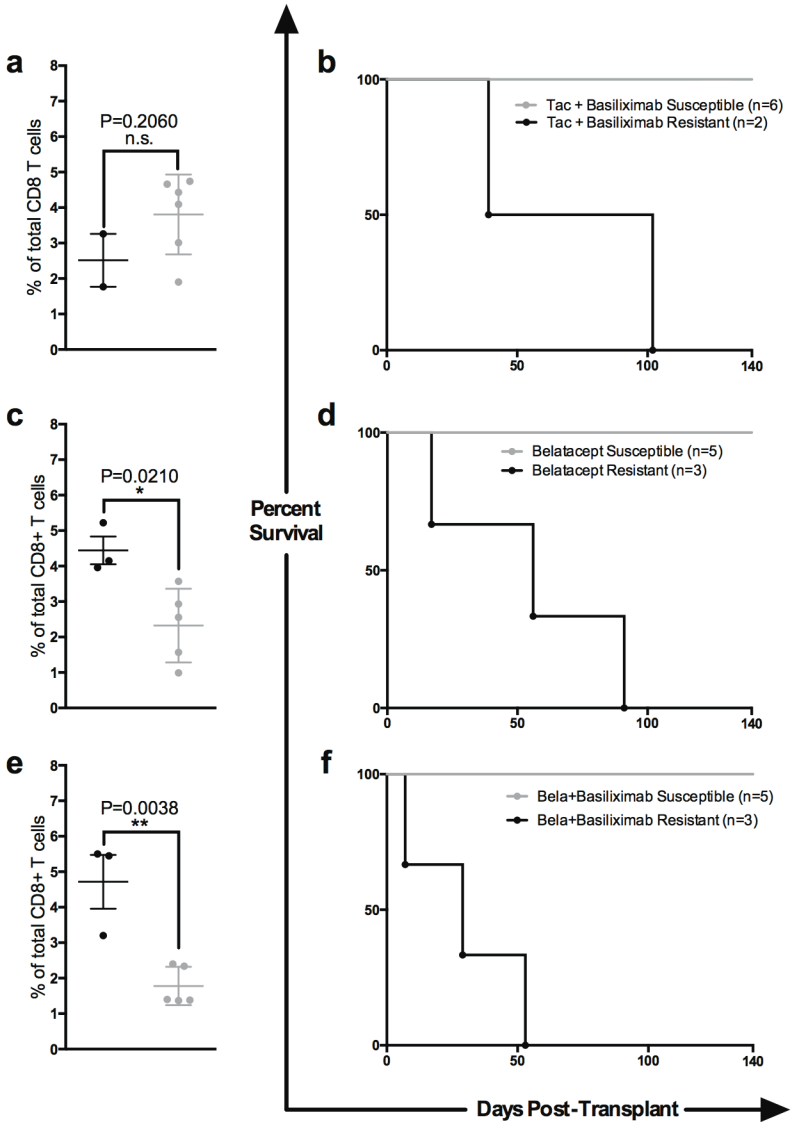
KLRF1
KLRB1
LAIR1
GZH
KLRD1
EOMES
TBX21
NKG7
LILRBA
NKG2D
BTLA
BTN2A2
CTLA4
CD48
PDCD4
FAS
CD244
GZMA
LILRBC

Figure 2.9 Distinct Intragraft Transcriptome Modules Define Belatacept Resistant Rejection. Transcriptome arrays from tissue acquired at the time of rejection revealed unique pathways augmented in belatacept resistance compared to belatacept susceptibility, as defined by KEGG and/or Biocarta pathway analysis. Analysis was restricted to differential gene expression between on-therapy rejection (belatacept resistant) compared off-therapy rejection (belatacept susceptible). Genes involved in (a) Cytokine and Chemokine systems, (b) Adhesion and Migration and (c) Exhaustion and Memory were up-regulated in belatacept resistant rejection. For example, CXCL12 was expressed 2.83 fold higher in belatacept resistant graft tissue compared to belatacept susceptible graft tissue, at the time of rejection.

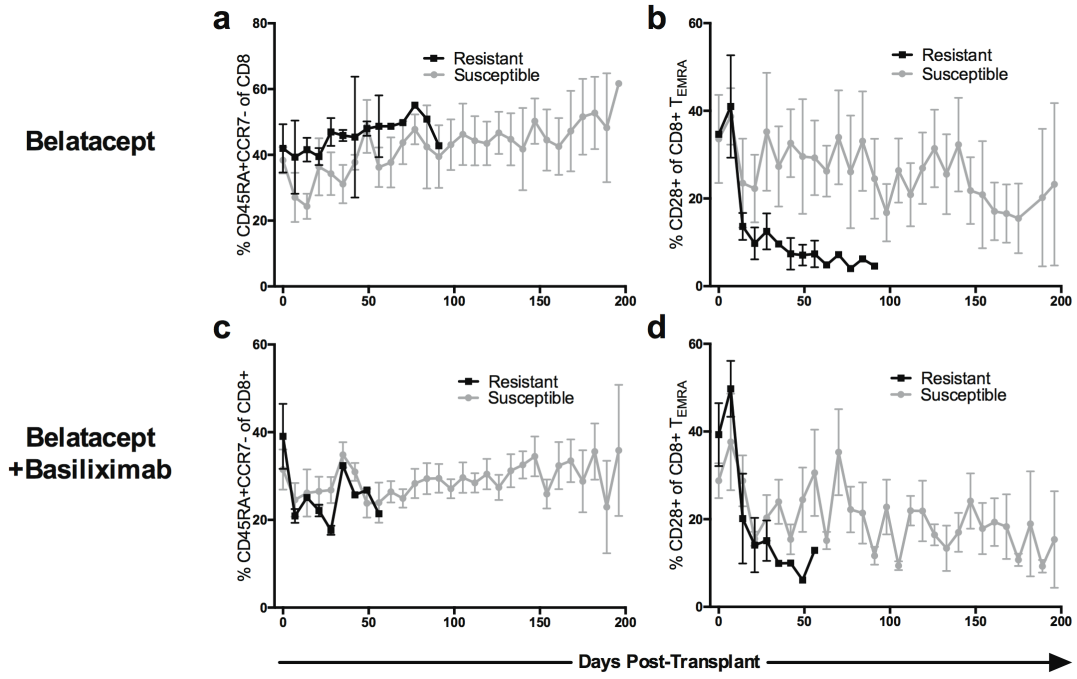


Supplementary Figure 2.1 Graft Function and Survival. Animals were assessed weekly for renal function utilizing serum creatinine. Depressed renal function pursuant to allograft rejection was determined by two consecutive Cr > 5. Resistant animals (black lines) and susceptible animals (grey lines). Weekly serum creatinine and corresponding survival curves

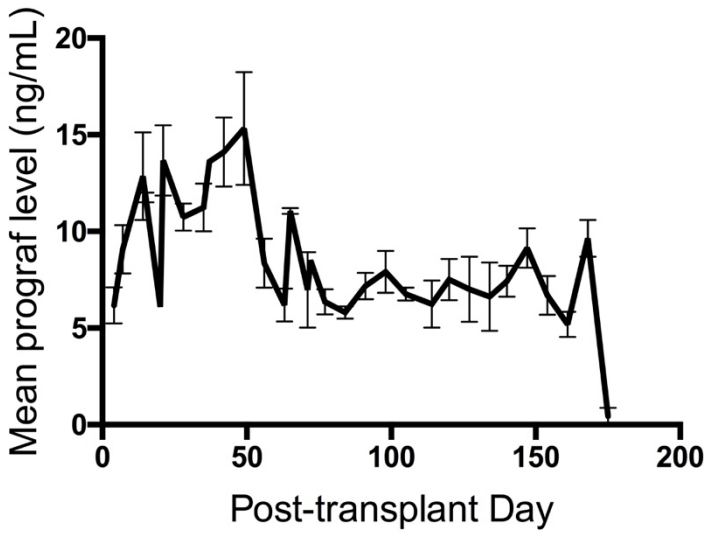
of belatacept treated animals (a-b), belatacept treated animals who received basiliximab induction (c-d) and tacrolimus treated animals (e-f). Survival analysis revealed no statistically significant differences between treatment groups, belatacept vs. basiliximab+belatacept (b, P1 =0 .1122) or belatacept vs. basiliximab+tacrolimus (b, P2 = 0.1175), or basiliximab+belatacept vs. basiliximab+tacrolimus (P = 0.6678). Belatacept and basiliximab+belatacept treated animals demonstrated an identical rate of therapeutic resistance (n=3/grp).



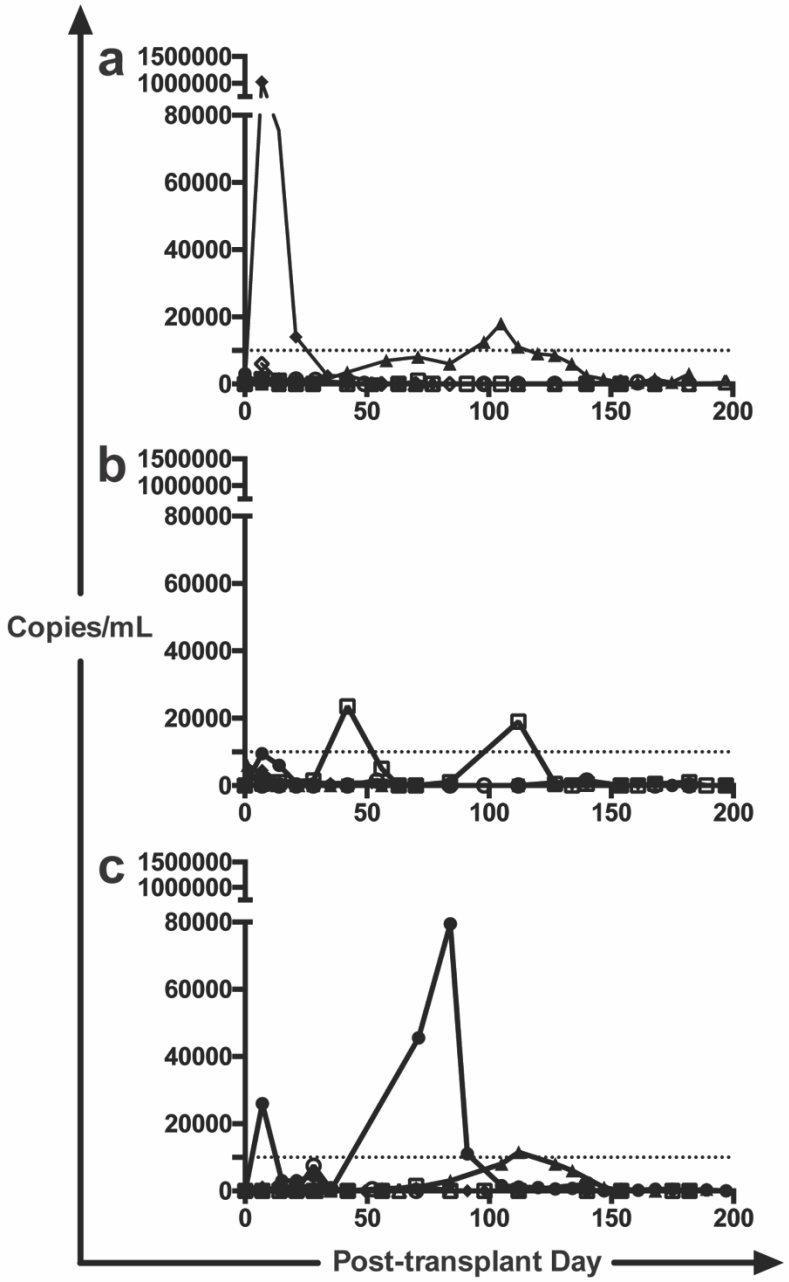
Supplementary Figure 2.2 Pre-transplant immunophenotype Segregates Belatacept Resistance. Pre-transplant immunophenotyping of therapy resistant (black circles) versus therapy susceptible animals (grey circles), with corresponding survival curves. Belatacept and basiliximab+belatacept treated animals demonstrate similarly elevated pre-transplant CD28+CD95+CD45RA+CCR7- cells as a frequency of total CD8+ T cells (c and e), as well as identical rates of therapy resistance (d and f). These differences were not observed in tacrolimus resistance (a and b).



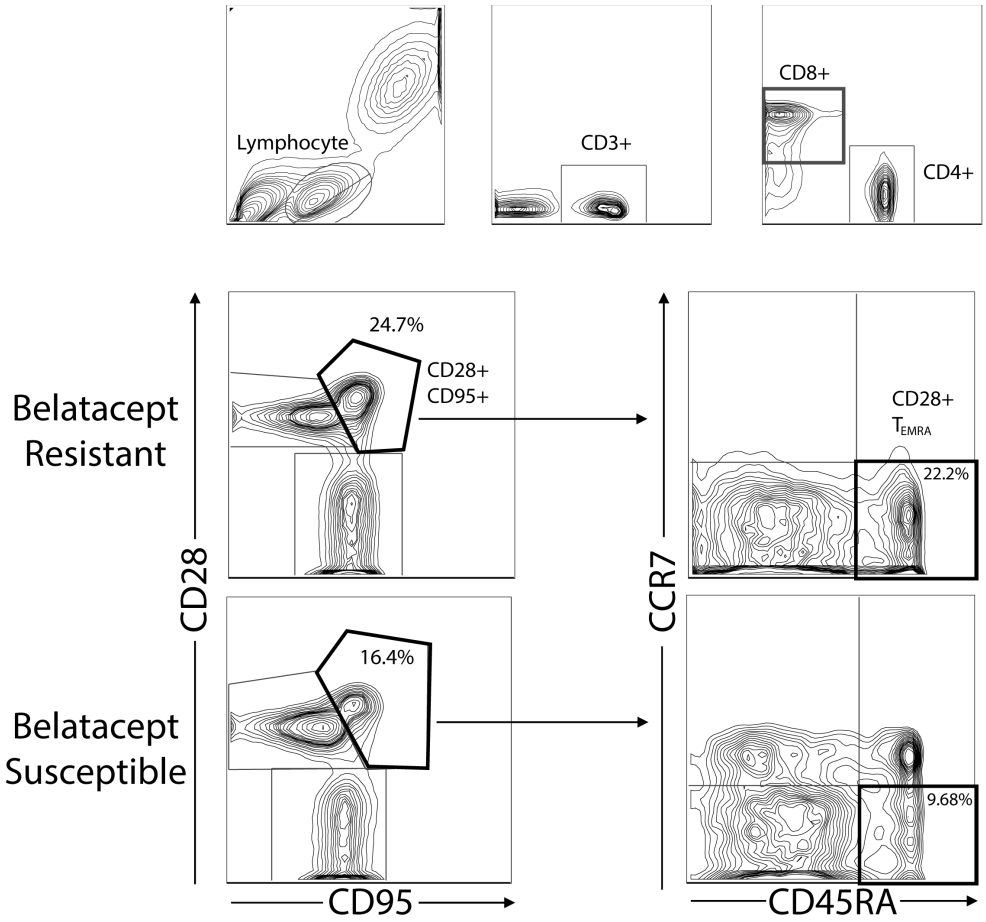
Supplementary Figure 2.3 Kinetics of CD8+ TEMRA in Belatacept Therapy. Overall percentage of CD45RA+CCR7- (CD8+ TEMRA, all CD95+) as a subset of CD8 T cells remained stable over time in (a) belatacept and (c) belatacept+basiliximab treated animals while the fraction of CD8+ TEMRA which were CD28+ decreased rapidly post-transplantation and remained low in belatacept resistant animals (black lines), compared to animals susceptible to therapy (grey lines), in both (b) belatacept and (d) basiliximab+belatacept treated animals. Belatacept and basiliximab+belatacept treated animals demonstrate similar kinetics of CD28+CD8+ TEMRA.



Supplementary figure 2.4 Mean Prograf Levels. Tacrolimus treated animals were monitored weekly for prograf levels, and doses were adjusted in order to maintain blood levels from (8-12ng/ml up to day 56, and 5-8ng/ml until day 140).



Supplementary Figure 2.5 CMV Reactivation. Animals in all treatment groups (a) belatacept, (b) belatacept + basiliximab, (c) tacrolimus + basiliximab experienced infrequent (n=1/group) and equivalent rates of CMV reactivation, defined as greater than 10,000 copies of viral DNA/ul of blood, as measured by quantitative-PCR.



Supplementary Figure 2.6 Gating Strategy for pre-transplant Immunophenotyping. Gating strategy involved gating first on lymphocytes, then CD3+ cells, then CD8+ (vs. CD4+). Gates were then set on CD28+CD95+ cells as pictured. CD28+CD95+ cells were then further analyzed by CD45RA and CCR7, as pictured. Sample plots show pre-transplant flow cytometry from one therapy resistant animal (top) and therapy susceptible animal (bottom).

Chapter 3. CD122 Signaling in CD8$^+$ Memory T cells Drives Costimulation Independent Rejection

Introduction

Blockade of key T cell costimulatory pathways represents a more targeted strategy to prevent unwanted immune responses such as rejection in transplant recipients. Recently, belatacept, a high affinity variant of the CTLA-4-Ig fusion protein, became the first approved alternative to conventional non-specific immunosuppression for renal transplant recipients (68, 69). Compared to patients receiving cyclosporine, transplant patients treated with belatacept enjoyed superior function of their transplanted kidney with fewer off-target toxicities and a 43% risk reduction of death or graft loss in seven-year follow-up (73, 181, 183). Despite these improvements a subset of patients experienced elevated rates and grades of acute allograft rejection during belatacept therapy (71). Belatacept specifically interrupts T cell costimulatory signals mediated by CD28-CD80/CD86 interactions. Memory CD8+ T cells are capable of mounting alloimmune responses despite blockade of CD28 and CD154 costimulatory molecules (74, 87, 200, 201). We have recently demonstrated that a critical threshold of T cell memory can effectively predict belatacept resistance in patients and non-human primates and that belatacept resistant rejection is uniquely characterized by allograft infiltrate that is more fully differentiated, with a unique pro-inflammatory cytokine signature (99, 191). There are subsets of memory CD8+ T cells in humans and non-human primates that lack CD28 expression altogether and rely on alternative signals for activation (78, 79). One such signal is provided by the shared IL-2 and IL-15 cytokine signaling complex.

IL-2 and IL-15 signals depend on the assembly of high-affinity heterotrimeric receptors which share a β-chain (CD122) and common γ-chain (γc) (130). The unique

contributions of these cytokines to host protection and alloimmunity while described are not fully elucidated. Interestingly, IL-2/IL-2Rα knockout animals, exhibit autoimmunity, while IL-15/IL-15Rα knockout animals have diminished CD8, IELS, NK and NKT cells, suggesting that these two cytokines have divergent phenotypes and unique biological roles despite a shared signaling complex (115, 116, 119, 120). Blocking the shared IL-2/IL-15Rβ has ameliorated disease in a murine model of IL-15 dependent autoimmunity (202). Additionally we know that IL-15 signaling is critical for memory CD8+ T cell homeostasis and survival (203-205). Exogenous IL-15 has been shown to induce expansion of memory CD8+ T cells in rhesus monkeys (24). Inflammation may drive IL-15 production leading to enhanced trafficking and proliferation of memory T cells following viral infection but it is unclear what role it has in transplant rejection (206). We and others have demonstrated that IL-2 and IL-15 signaling induces the loss of CD28 while providing other activation signals (108, 141). Thus, signaling through the IL-2/IL-15 receptor complex may activate alloreactive T cells while making them increasingly resistant to belatacept due to loss of CD28. In support of this, recent studies by Traitanon et al demonstrate that IL-15 uniquely drives the proliferation of human alloreactive memory CD8+ T cells, despite costimulatory blockade with CTLA- 4-Ig (143). Here we demonstrate that blockade of the shared IL-2 and IL-15 receptor β-chain, CD122, synergizes with costimulatory blockade to abrogate both primary and memory CD8+ T cell responses to transplanted tissue and results in prolonged transplant survival in mice and non-human primates. Distinctively, blockade of the high-affinity IL-2 receptor failed to inhibit T cell re-call and graft rejection, whereas blockade of CD122 controlled CD8+ T cell re-call, suggesting re-call responses uniquely require IL-15, but can dispense with IL-2. In contrast, the high-affinity IL-2 receptor in combination with costimulatory blockade is sufficient to prevent primary allo-specific T cell responses. CD122

directed therapy allowed for blockade of two pathways for T cell activation, IL-2 and IL-15, which play distinct roles as signal 3 cytokines in primary and recall responses respectively.

We translated these findings into a pre-clinical, non-human primate renal transplant model where we characterized the expression of CD122 as a marker of antigen experienced, memory CD8+ T cells, and found that IL-15 augments effector function of memory T cells, more so than IL-2. Belatacept resistant allograft infiltrate was characterized by high expression of CD122, but not CD25. The addition of a novel humanized, Fc-silent, CD122 blocking antibody synergized with belatacept to abrogate alloreactivity and significantly prolong survival of NHP renal transplant recipients. These data offer a novel strategy for the optimization of costimulation blockade in transplantation and define a critical role for CD122 in both primary and secondary immune responses, as part of the IL-2 and IL- 15 receptor systems respectively. Our studies suggest that signaling through the IL-2/IL-15R (CD122) directly contributes to costimulation independence. The translation of CD122 directed therapy for transplantation may be superior to current therapies targeting CD25, which may also deplete regulatory T cells (29). Further, CD122 directed therapy has the benefit of interrupting the IL-2 receptor and the IL-15 receptor, inhibiting both primary and secondary alloreactive T cell responses. These data improve our understanding of the basic signaling requirements of T cells, and highlight the distinctive role of IL-15R in graft-specific memory responses.

Materials and Methods

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (207) transgenic mice, purchased from Taconic Farms, were bred to Thy1.1+ background at Emory University. mOVA mice (C57BL/6 background, H-2^b; (208)) were purchased from The Jackson Laboratory. All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

Viral Infection and Kinetic Analysis. To induce acute infection, mice were inoculated with 2×10^5 PFUs of the lymphocytic choriomeningitis virus (LCMV) Acute Armstrong strain (i.p. injection). Virus specific CD8 T cells were monitored with APC conjugated gp33-41 tetramer.

Donor-reactive T cell adoptive transfers & Memory Generation. To generate ova-specific memory T cells, splenocytes from Thy1.1+ OT-I mice were resuspended in PBS and 1.0×10^4 of Thy1.1+ OT-I T cells were injected i.v. 24–48 h prior to inoculation with 10^4 CFU Listeria monocytogenes–OVA (Lm.Ova) (209) by i.p. injection. After 30 days, peripheral frequencies of Thy1.1+ OT-I T cells were assessed and mice were given recall challenge with Ova expressing skin graft from mOva donors.

Skin transplantation and antibody treatment. Full-thickness tail or ear skin was transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages. Where indicated, mice were treated with 250 μg CTLA4-Ig (Bristol Myers Squibb, Princeton,

NJ), 250 μg hamster monoclonal anti-mouse CD154 (MR-1, BioXcell, West Lebanon, NH), 200 μg anti-CD122 (ChMBC7, JN Biosciences, Mountain View, CA) or 200 μg anti- CD25 (PC61, Bio X cell, West Lebanon, NH) intraperitoneally on days 0, 2, 4 and 6 post-transplantation.

Acute Graft-versus-Host Disease model. C57BL/6 splenocytes were labeled with 10 μM Cell Trace Violet (CTV, C34571, Invitrogen, Carlsbad, CA). 3 x 10^7 C57BL/6 CTV labeled splenocytes were transferred i.v. into sublethally irradiated (800 rads) BALB/c recipients, and selected groups received therapy on day 0 and 2 as described above. Splenocytes were harvested on day 3, and analyzed by flow cytometry to assess CTV labeled cell division.

Donor-recipient pair selection and kidney transplantation. All experiments described herein were performed in compliance with the principles set forth in The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, DHHS). Outbred rhesus monkeys (Macaca mulatta) ranging between 3 and 5 years old were obtained from AlphaGenesis, Inc. (Yemassee, SC) and Yerkes National Primate Research Center (Lawrenceville, GA). Donor–recipient pairs were chosen to maximize genetic disparity at both MHC class I and class II alleles based on 454 deep sequencing analysis (University of Wisconsin, Madison, WI). Animals were heparinized (100 Units/kg) during organ procurement and implantation. Left native nephrectomy was performed at least 3 weeks prior to transplantation, and a completion right native nephrectomy was performed at the time of transplantation. All transplanted animals were monitored with daily clinical assessment and serial laboratory evaluations, including complete blood count and serum chemistry. Animals demonstrated excellent graft function post-

operatively. Depressed renal function pursuant to allograft rejection was determined by two consecutive Cr>5.

NHP experimental groups and immunomodulation. Rhesus macaques underwent bilateral nephrectomy and life-sustaining renal allograft transplantation. All Donor Recipient pairs were MHC defined and maximally mismatched. Five Animals received belatacept (Bristol Myers Squibb, Princeton, NJ) d0: 10mg/kg, d4: 15 mg/kg, d14-d28: 20mg/kg weekly, d42: 20 mg/kg, d56: 20mg/kg, d84: 20mg/kg, d112: 20mg/kg, d140:20mg/kg. Two animals received anti-CD122 alone (HuABC2, JN Biosciences, Mountain View, CA) 5mg/kg: d0, d4, d7, d14, d21, d28, d42, d56, d70. Five animals received combination belatacept and anti-CD122 as described (Figure 3.6C).

Antibodies and flow cytometric analysis *Non-human Primate*: Flow cytometric analysis was performed up to 3 times pre-transplant and serially post-transplant to characterize peripheral blood immune cell phenotypes. Total T cells and T cell subsets were quantified by complete blood cell count and flow cytometry. Fresh PBMCs were isolated by Ficoll density gradient centrifugation (BD Biosciences, Franklin Lakes, NJ). PBMCs were stained with the following mAbs: CD3 PacBlue, CD95 V450, CD3 Alexa 700, CD4 PerCP-Cy5.5, CD8 V500, CD28 PE-Cy7, CD25 PE-Cy7, IFNy PE-Cy7, CD28 APC, TNF APC, CD122 (both clones Mikβ2 and Mikβ3) (all BD Biosciences). PBMCs (1.5×10^6) were incubated with appropriately titered antibodies for 15 min at 4°C and washed twice. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular staining was performed with FoxP3 V450 (Biolegend, San Diego, CA) to detect regulatory T cells (Treg cells). Samples were acquired

immediately on a BD LSR II multicolor flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA). For mixed lymphocyte reactions, 1×10^6 PBMCs were labeled with CFSE labeling dye, and incubated with CTV labeled and irradiated MHC mismatched responder PBMCs (1×10^6). For the stimulation assay, 1.5×10^6 PBMCs were cultured in RPMI 1640 (Corning cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and stimulated with 10 μM phorbol 12-myristate 13- acetate (PMA) and 200 nM ionomycin (Sigma-Aldrich, St. Louis, MO) for 5hr. IL-2 (100ng/ML) and/or IL-15 (10ng/mL) were utilized in both 4-5hr stim (Figure 5.2) and in MLR (Figure 6) as described (Peprotech, Rocky Hill, NJ). PBMCs were washed twice prior to antibody staining and data acquisition.

Murine Surface stains and flow cytometry

Spleens or draining axillary and brachial LN were stained for CD4, CD8, Thy1.1, CTLA4, PD-1, KLRG1, CD127, CD62L, CD122 (clone 5H4, different regional binding site than ChMBC7), and CD44 (Biolegend, San Diego, CA). Samples were analyzed using an LSRII FACS machine (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Intracellular cytokine staining

Where indicated, responder lymphocytes or splenocytes were stimulated with PMA/Ionomycin, or 10 nM OVA257–264 (Genscript, Inc., Piscataway, NJ) in the presence of 10 μg/mL Brefeldin A for 4– 5 h. Intracellular staining kit was used to detect TNF, IFN-γ (Biolegend) and IL-2 (BD Biosciences) according to manufacturer's instructions.

Statistics

Survival statistics were calculated using a log-rank test. T cell frequencies, absolute numbers and MFI were compared using 1-way ANOVA with Tukey's multiple comparisons test, or unpaired t-test (comparison between two groups). Data were analyzed using Prism 6 (GraphPad Software, La Jolla, CA). A P value of <0.05 was considered statistically significant.

Study Approval

Both murine and nonhuman primate experimental subjects received humane care and treatment in accordance with Emory University IACUC guidelines, and all experimental protocols utilizing animals were conducted with approval by this institutional review board.

Results

CD122 is highly expressed on Antigen Specific Memory CD8+ T cells.

High levels of CD122 expression distinguish memory CD8+ T cells and NK cells (210). Consistent with previous reports, we observed elevated CD122 expression on nearly all activated (CD44+) CD8+ T cells following viral infection (211). In a well-described model of acute viral infection with the Armstrong Strain of lymphocytic choriomeningitis virus (LCMV) we found CD122 to be expressed on greater than 95% of viral antigen specific cells (Figure 3.1A). Virus-specific CD8+ T cells not only maintained CD122 expression over time but the level of expression increased as the population of antigen-specific T cells matured to a memory phenotype (CD122 MFI = 1157 on day 108 compared to the peak of infection CD122 MFI = 588 on day 8, P=0.0002, Figure 3.1B). We observed that IL-2/IL-15Rβ (CD122) is expressed on both short-lived effector cells (SLECs) at the peak of infection (KLRG1hiCD127lo CD8+ T cells) as well as stable memory T cells (KLRG1loCD127hi CD8+ T cells, Figure 3.1C). Antigen specific central memory CD44+CD62L+ CD8+ T cells exhibited the highest expression of CD122 when compared to effector memory CD44+CD62L- CD8+ T cells at 3 months post-infection (Figure 3.1D). Phenotypically, the density of CD122 expression on antigen specific cells suggests an important role for CD122 during acute responses and in memory.

Virus Specific and Alloreactive CD8+ T cells demonstrate similar expression of CD122.

We translated these findings to a model of transplantation to characterize CD122 expression on alloreactive CD8+ T cells during a primary challenge. We characterized

CD122 expression on alloreactive CD44+ CD8+ T cells (Figure 3.1E-H). The expansion, contraction and homeostasis of alloreactive CD8+ T cells in a BALB/c (H-2^d) to C57BL/6 (H-2^b) skin transplant model was similar to LCMV acute infection as previously described (87). CD122 expression on alloreactive CD8+ T cells was comparable to the LCMV-specific response and was similarly highest on central memory CD8+ T cells (CD122 MFI TCM = 1545 vs. TEM = 564, P=0.0016, Figure 3.1H), These findings suggest an important role for CD122 signaling in alloimmunity and potentially a distinctive role in alloreactive CD8+ T cell memory.

CD122 signaling underlies Costimulation Independent Rejection

Immunosuppressive strategies employing costimulation blockade (CoB) have already shown promise in kidney transplant recipients but wider adoption has been limited in part due to elevated rates of T cell mediated acute rejection (69, 71, 75, 184). We sought to investigate the role of CD122 in costimulation independent rejection. C57BL/6 (H-2^b) recipients of BALB/c (H-2^d) skin allografts demonstrate vigorous costimulation blockade resistant rejection during primary challenges (MST=21 days with CoB, vs. MST=10 days without treatment, Figure 3.2A). We developed a chimeric, Fc-silent murine monoclonal antibody specific for CD122, ChMBC7, to investigate the role of the shared IL- 2/IL-15Rβ in costimulation independent rejection. Mice receiving anti-CD122 alone rejected with similar kinetics to untreated mice (MST=10, Figure 3.2A). Costimulatory blockade extended graft survival modestly compared to control animals, but combined CD122 and costimulatory blockade prolonged allograft survival significantly (MST>80 days, P<0.0001, Figure 3.2A). These data demonstrate signaling through CD122 as part of either the IL-2 and/or the IL-15 receptor supports costimulation independent rejection. We investigated the

mechanisms underlying the survival benefit observed in animals treated with CoB + anti-CD122. CoB alone fails to completely suppress alloreactive CD8+ T cells, but the addition of CD122 blockade efficiently mitigates the generation of an alloimmune response (Figure 3.2B-C). Combination therapy reduced both the expansion and effector function of alloreactive T cells by nearly 100-fold compared to CoB, and 200 fold compared to unmodified rejection. (absolute number of dLN CD8+CD44+IFNγ+ = $2.54x10^5$ in no treatment (No Rx) vs. $1.23x10^5$ in CoB vs. $1.27x10^4$ in CoB + anti-CD122 , $P<0.0001$, Figure 3.2B). In a model of graft versus host disease we found similar effects of combined CoB and anti-CD122 on alloreactive T cell proliferation and effector function (Figure 3.2C). These data suggest that in the absence of traditional costimulatory signals such as CD28 and CD154, signaling through CD122 supports the expansion, activation and effector function of naïve alloreactive T cells through the effects of IL-2 and/or IL-15.

CD122 Signaling Supports Costimulation Independent Memory Responses.

Immune memory can significantly contribute to transplant rejection (62, 95). Memory T cells can readily function without traditional costimulatory signals resulting in allograft rejection despite costimulatory blockade (76, 87, 97, 212). Previous studies outlined an important role for IL-15 in T cell homeostasis but there is still debate regarding the relative contributions of IL-2 and IL-15 in memory responses (126, 203, 206, 213, 214). We investigated the role of IL-2R and IL-15R signaling in CD8+ T cell recall, in the setting of costimulation blockade. Ova specific CD8+ T cells (OT-I) were transferred into naïve C57BL/6 recipients and immunized with Listeria monocytogenes engineered to express chicken ovalbumin (Lm-Ova). After 30 days, mice were re-challenged with Ova expressing skin grafts (Figure 3.3A). In the context of memory CD8+ T cell mediated transplant

rejection, anti-CD122 synergized with costimulatory blockade to prolong graft survival indefinitely (MST= >100 days, P<0.0001, Figure 3..3B). Costimulation blockade alone fails to significantly prolong graft survival (MST=16 days). Animals treated with anti-CD122 alone (data not shown) rejected with similar kinetics to animals who received no therapy (MST=11 days). We investigated the phenotypic and functional effects underlying prolonged graft survival in a model of memory CD8+ T cell mediated acute graft rejection. CoB alone did not significantly reduce the frequency of graft reactive CD8+ memory T cells compared to untreated mice (Figure 3.3C-D). The addition of anti-CD122 to CoB dramatically constrained the expansion and effector function of graft reactive cells (Figure 3.3C-D). Further, we observed a change in phenotype, where the combination of anti-CD122 and CoB induced a PD-1 high, CTLA-4 high exhausted phenotype (Figure 3.3E). CoB+anti-CD122 reduced Ki67 expression in graft reactive memory CD8+ T cells as well, suggesting that the difference in numbers was due to decreased expansion/proliferation and not only increased cell death (Figure 3.3E). These data suggest that signaling through the shared IL-2/IL-15Rβ chain during recall responses is critical for memory CD8+ T cell proliferation and function, however the relative importance of IL-2R versus the IL-15R remained undetermined.

The high affinity IL-2 Receptor is Dispensable for Costimulation Independent Memory Re-call

Current immunosuppressive strategies for transplant recipients include the use of anti-CD25 reagents (145, 194). Additionally, the role of IL-2 and IL-15 in the generation of re-call responses is the subject of great interest for vaccine development, cancer immunotherapy and transplantation (210). We investigated the impact of the addition of a

short course of anti-CD25 mAb as an adjuvant therapy to costimulation blockade in this memory T cell mediated model of graft rejection. The addition of anti-CD25 failed to prolong graft survival (MST= 22, Figure 3.4A), whereas adjuvant anti-CD122 therapy prolonged graft survival indefinitely and controlled the expansion of graft reactive memory T cells, relative to CoB or CoB+anti-CD25 (Figure 3.4B-C). Previous studies underscored the importance of the high-affinity IL-2 receptor in costimulation independent rejection during a primary allo-immune response (142). Our studies confirmed these results, demonstrating that both anti-CD25 and anti-CD122 synergize with costimulatory blockade to prolong graft survival during a primary response (Figure 3.4D). These data suggest that the IL-15 receptor is necessary for costimulation independent re-call responses, whereas the high-affinity IL-2R receptor is dispensable. In the setting of a primary allo-immune challenge, costimulation independent cells rely on the high-affinity IL-2 receptor. Targeting CD122 interrupts both the IL-2 and IL-15 receptor.

CD122 phenotype and function in Rhesus Macaques

In an effort to evaluate whether these findings were translatable we sought to characterize the phenotype of CD122 expression on CD8+ T cells in preclinical model using rhesus monkeys. CD122 is highly expressed on central (TCM, CD28+CD95+) and effector memory (TEM, CD28-CD95+) but not naïve (CD28+CD95-) CD8+ T cells (Figure 3.5A-J). We assessed the effector function of memory T cell subsets and the relative effects of exogenous IL-2 and IL-15. The addition of IL-15 for 5 hours dramatically increased effector cytokine production by CD8+ T cells, more so than IL-2 (Figure 3.5K-L). We have previously reported that CD28+ memory T cells in rhesus monkeys and humans predict costimulation independent rejection (99, 191). Interestingly, we found CD28+ memory T

cells demonstrated more potent cytokine potential in response to exogenous IL-15, than CD28- memory T cell subsets (Figure 3.5K-L). To better understand the mechanism by which CD122 signaling contributes to alloreactivity, we utilized an ex-vivo mixed lymphocyte reaction with non-human primate PBMCs. We found that IL-15 augmented alloreactivity, specifically by increasing proliferation and effector function. Further, IL-15 induced a loss of CD28 expression in CD8 T cells (Figure 3.6A-D). In the context of exogenous IL-15, belatacept fails to inhibit these alloreactive responses in-vitro, but the addition of a humanized, Fc-Silent CD122 specific monoclonal antibody (HuABC2) synergistically inhibits proliferation, effector function and CD28 loss of CD8+ T cells during MLR (Figure 3.6A-B). These data demonstrate that signaling through the shared IL-2/IL-15R augments memory T cell effector function, and in particular IL-15 augments CD28+ memory T cell effector function. These data suggest signaling through CD122 in primate CD8+ T cells is sufficient to support costimulation independent responses, and in fact signaling through CD122 may drive loss of the costimulatory molecule CD28, potentiating therapeutic resistance to CD28 directed therapies, such as belatacept.

A humanized Fc-silent Anti-CD122 mAb synergizes with Belatacept to significantly prolong allograft survival in non-human primates.

Given the promising data observed in-vitro we next tested whether treatment with a humanized anti-CD122 antibody would impact costimulation blockade resistant rejection in a non-human primate kidney transplant model (Figure 3.6C). This rigorous model gives rise to rapid allograft rejection that is resistant to costimualtion blockade with belatacept monotherapy (68). We observed no survival benefit in animals treated with anti-CD122 monotherapy (n=2, MST=7 days, Figure 3.6C). The combination of costimulation blockade

using belatacept and anti-CD122 significantly prolonged kidney transplant survival in non-human primate (n=5, MST=138, P<0.0001, Figure 3.6C). We did not observe a marked increase in viral reactivation (Supplemental Figure 3.1). These data indicate that belatacept independent rejection relies on CD122 signaling. Flow cytometric characterization of belatacept resistant rejection revealed uniform up-regulation of CD122, but not CD25, on all graft infiltrating T cells at the time of rejection (Figure 3.7A). We investigated the impact of CD122 directed therapy on T cell subset frequencies including those of a regulatory phenotype and found that anti-CD122 did not decrease the frequency of T cell subsets, including CD4+CD25+Foxp3+ Tregs following transplantation (Figure 3.7B-E).

Discussion

The introduction of potent, non-specific immunosuppression with calcineurin inhibitors dramatically improved short-term outcomes in solid organ transplantation. Unfortunately most transplant patients eventually lose their allograft from rejection or die as the result of increased cardiovascular complications or infections. Despite excellent improvements early after transplant, the late outcomes remain essentially unchanged and represent the greatest challenge for transplant recipients (28). The advent of costimulation blockade as a more targeted strategy for transplant immunosuppression has demonstrated the first evidence of improved long-term outcomes and graft function in the setting of clinical trials and in post-trial use (73, 75). Despite these promising improvements patients treated with belatacept experienced elevated rates and grades of acute rejection within the first six months of transplantation (71, 75). Studies that investigate T cell reactivity in the setting of costimulation blockade improve our understanding of the dynamic signaling requirements of T cells while addressing a pressing clinical need for safer, targeted transplant immunosuppression. We find that Signal 2, costimulation, and Signal 3, provided by cytokine, are synergistic and in some respects redundant – in the absence of Signal 2, Signal 3 cytokines can support robust responses. Here we focused our investigation on the ability of Signal 3 cytokines, particularly IL-2 and IL-15, to support costimulation independent responses. In a primary immune response, the high-affinity IL-2R provided sufficient signaling to support T cell reactivity. Interestingly, despite sharing 2 of 3 receptor subunits, our data suggests the IL-2R and IL-15R play distinct roles in primary and recall responses by CD8+ T cells: IL-15R is critical for CD8+ T cell recall, whereas the high affinity-IL-2R is not.

IL-2 and IL-15 are structurally and genetically distinct cytokines, sharing little sequence similarity. Though these cytokines share two signaling subunits, CD122, the shared IL-2 and IL-15 receptor β-chain, and CD132 the common γ-chain (γc), they have distinct contact residues with CD122 and CD132 (112). Their non-redundant roles are highlighted by the divergent phenotypes of IL- 2/IL-2Rα -/- mice, which suffer from autoimmunity due to CD4+CD25+ Treg deficiency and IL-15/IL-15Rα knockout mice, which have decreased CD8, IELs, NK and NKT cells (115, 116, 119, 120, 215). Saturating doses of IL-2 and IL-15 give rise to near identical CD8+ T cell transcriptomes (112). Thus, the temporal and spatial differences in receptor subunit expression (CD25 vs.CD122) expression may underlie the unique roles of IL-2 and IL-15 in the generation and maintenance of adequate adaptive immune responses as opposed to unique intrinsic signaling properties of IL-2/IL-2Rα and IL-15/IL15Rα (112, 216). IL-2 is readily taken up by CD4+CD25+ Tregs, which rapidly express the high-affinity IL-2R as predicted by high expression of CD25. On the other hand, CD122 expression is highest on CD8+ memory T cells and NK cells, allowing for formation of the high-affinity IL-15R. Notably, IL-15Rα is expressed in trans by antigen presenting cells, as well as a number of peripheral tissue cell types – notably in renal epithelium (131). IL-15Rα presents IL-15 in complex and binds with 150 times greater affinity than circulating IL-15 to cells expressing CD122 and the common γ-chain (112, 129).

Given the extensive characterization of these receptor systems it is somewhat surprising that the relative importance of IL-2 compared to IL-15 in primary and recall responses is still debated. Some groups have identified a critical role for IL-2 in the primary response, development of effective CD8+ T cell memory and recall, whereas other groups have highlighted the role of IL-15 in optimal memory T cell development and recall (125, 126, 206, 217-219). The type of immune challenge used in these studies, the chronicity and

antigen load, as well as the strength and duration of signaling through the IL-2R and IL-15R influences the quality of CD8+ T cell memory development and recall responses (123, 124, 128, 220). It is likely that costimulation further confounds studies of the individual contributions of either IL-2 or IL-15 at specific moments in the immune response (127). Costimulation provides a set of redundant activation signals that may obscure the unique contribution of either IL-2 or IL-15 in recall in studies utilizing IL-2/IL-2Rα or IL-15/IL-15Rα mice (214, 221).

In our studies, the use of costimulation blockade revealed distinct roles for the IL-2R compared to the IL-15R. The high-affinity IL-2R was dispensable for effective memory CD8+ T cell recall responses, whereas blockade of CD122 which interrupts both the IL-2R and IL-15R abrogated memory CD8+ T cell dependent graft rejection. Previous studies in models of infection have outlined the importance of inflammation and type I interferon dependent IL-15 signaling as a driver of memory T cell responses (206). The source and sequence of events leading to IL-15R mediated costimulation independent recall responses in graft rejection requires further investigation, including the role of type I interferons. Targeting CD122 provides the opportunity to block two distinct pathways that support T cell responses with a single reagent. Both allograft rejection and autoimmune disease are characterized by naive and memory T cell recruitment into a pathogenic response, thus there is a need to address both primary and recall responses in order to ameliorate disease. Previous studies have underscored the potential of the IL-15 pathway to mediate autoimmune disease and allograft rejection. Interruption of IL-15 signaling alleviated autoimmunity and prevented islet allograft rejection (136-140, 202). Current clinically approved therapeutics in transplantation are designed to solely interrupt the high-affinity IL-

2R in order to promote allograft acceptance by ablating T cell mediated graft reactivity (145, 194).

Our data suggests targeting both the IL-2 and IL-15 pathways with a single agent, anti-CD122, may be a superior strategy for limiting pathogenic T cell responses. The requirement of high-affinity IL-15R remains to be directly tested, and furthermore, the source of IL-15 in supporting memory T cell recall responses requires further investigation. Beyond its role as a signaling receptor subunit for both IL-2 and IL-15, recent studies have identified CD122 expression as a marker of stem cell memory T cells, or TSCM (222-224). These cells have superior proliferative capacity compared to conventional TCM or TEM and in a model of Graft- versus-Host Disease, these cells required CD28 and IL-15 signaling (225). Studies aimed at assessing the role of TSCM in alloimmunity and autoimmunity, and the role of CD122 as a phenotypic or functional marker may aid in the development of therapeutic strategies. Others have defined role for CD122+ CD8+ T cells as potent regulators of the immune response (226, 227). In both mice and non- human primates, we found that CD122 blockade prolonged allograft survival, and in NHPs we observed that the frequency of CD4+CD25+FoxP3+ Tregs was not impacted. Current clinically approved CD25 directed therapy is thought to have a detrimental impact on these tolerogenic cells. Adoption of a strategy that targets CD122 rather than CD25 may spare CD4+CD25+ Tregs. The physiologic impact of CD122+CD8+ regulatory T cells, and any potential detrimental impact of CD122 directed therapy on this tolerogenic subset warrant further study. In this report we outline a new strategy for the optimization of clinical costimulation blockade, built on a finer mechanistic understanding of the role of the IL-2R and IL-15R, respectively. Costimulation independent responses highlight the unique role of these cytokines and together with emerging data regarding the capacity of IL-15 to uniquely support

costimulation independent responses of human memory CD8+ T cells, these studies provide the basis to explore potential future clinical translation (143).

Figures

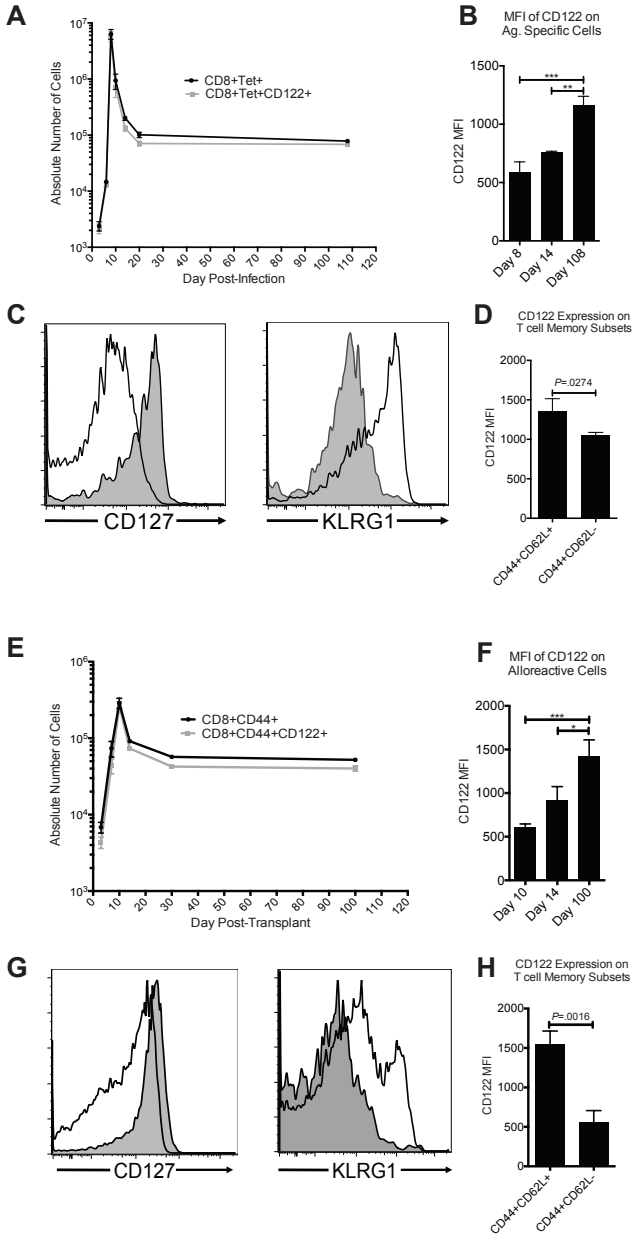


Figure 3.1 Kinetics of CD122 Expression on CD8 T cells in Acute Viral Infection and Allograft Rejection. (A) C57BL/6 Mice were infected with LCMV Armstrong strain and the frequency and phenotype of antigen specific (gp33 tetramer+) splenic CD8+ T cells were evaluated over the course or infection (CD8+Tet+, black circles). Greater than 95% of

tetramer+ CD8+ T cells expressed CD122 (CD8+Tet+CD122+, grey squares). (B) The MFI of CD122 on tetramer+CD8+ T cells was highest at later time-points once memory was formed (day 108 compared to day 8, P=0.0002). (C) Representative histograms of CD122+tetramer+ CD8+ T cells demonstrate a CD127lo KLRG1hi phenotype at day 8 p.i. (black line, no fill) compared to a memory time-point at day 108 where tetramer+ CD8+ T cells were CD127hi KLRG1lo (black line, grey fill). (D) CD122 is more highly expressed on tetramer+ TCM (CD44+CD62L+) CD8+ T cells, compared to tetramer+ TEM (CD44+CD62L-) CD8+ T cells (P=0.0274). (E) CD8+ T cells in acute allograft rejection (black circles) in C57BL/6 ($H2^b$) mice receiving BALB/c ($H2^d$) skin grafts demonstrate a similar kinetics to LCMV infection (A), with high levels of CD122 expression (grey squares) on alloreactive CD8+ T cells over the course of rejection. (F) CD122 expression was highest at a memory time-point day 100 post-transplant (P=0.0011). (G) CD122+ CD8+ T cells in acute allograft rejection demonstrate similar phenotypic changes in CD127 and KLRG1 at the peak of rejection (black line, no fill) and in memory (black line, grey fill). (H) Alloreactive TCM CD8+ T cells express higher levels of CD122 compared to T CD8+ T cells (P=0.0016). (B and F) P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; (D and H) Student's t test. Bars represent the mean ± SEM of 3 mice/group. Results are representative of 3 independent experiments. *P<0.05; **P<0.01; *** P<0.001.

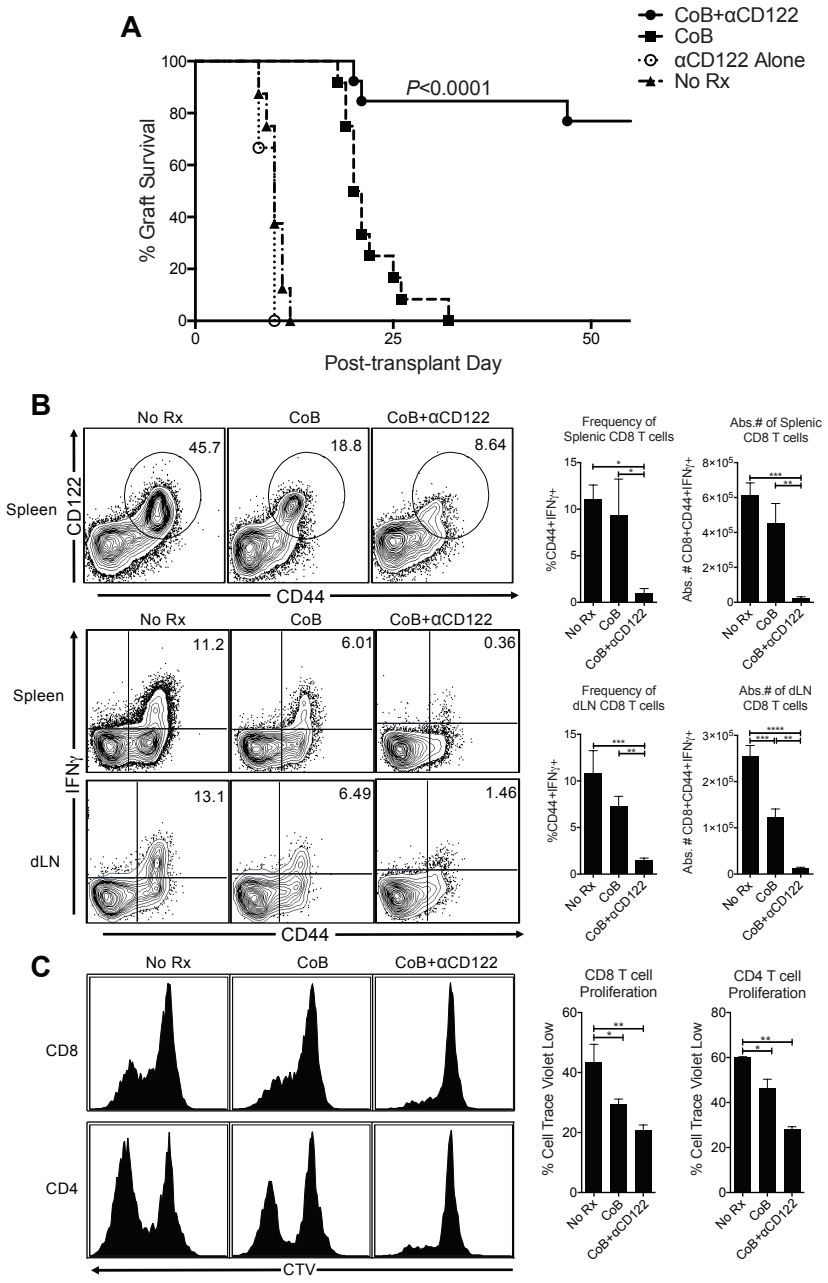


Figure 3.2 CD122 Signaling Underlies Costimulation Independent Rejection (A) Median survival time (MST)of BALB/c skin allografts on C57BL/6 recipients without treatment was 10 days (black triangles, No Rx). anti-CD122 alone failed to improve graft survival (open circles, MST=10). Mice treated with costimulation blockade (CoB, CTLA4-Ig+αCD40L) succumb to costimulation independent rejection (black squares, MST=21

days). Combination CoB+αCD122 prolongs survival to >80 days, preventing costimulation independent rejection in the majority of recipients (n=6-13/group, representative of 3 independent experiments, P<0.0001, Mantel-Cox log-rank test). (B) Mice were sacrificed at day 10 post-transplant. Representative FACS plots of splenocytes from untreated (No Rx), CoB, and CoB+αCD122 treated animals. CoB+αCD122 resulted in reduced frequency of alloreactive CD44+CD122+ CD8+ T cells. Correspondingly, there is marked decrease in frequency of CD44+IFNγ+ CD8+ T cells in both the spleen (P=0.0048) and dLN (P=0.0009), as well as a reduction in absolute numbers of alloreactive CD44+IFNγ+ CD8+ T cells in the spleen (P=0.0002) and dLN (P<0.0001). (C) In a model of acute Graft-versus-Host Disease, C57BL/6 splenocytes were labeled with Cell-Trace Violet (CTV) and transferred into sublethally irradiated BALB/c recipients which were either untreated (No Rx), treated with CoB, or treated with CoB+αCD122. After 72 hours splenocytes were harvested and assessed for CTV-labeled cell division, as depicted in representative histograms. Both CD8 (P=0.0032) and CD4 (P<0.0001) alloproliferation was significantly constrained with combination CoB+αCD122 treatment. (B-C) P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; bars represent the mean ± SEM of 3 mice/group. Results are representative of 2-3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

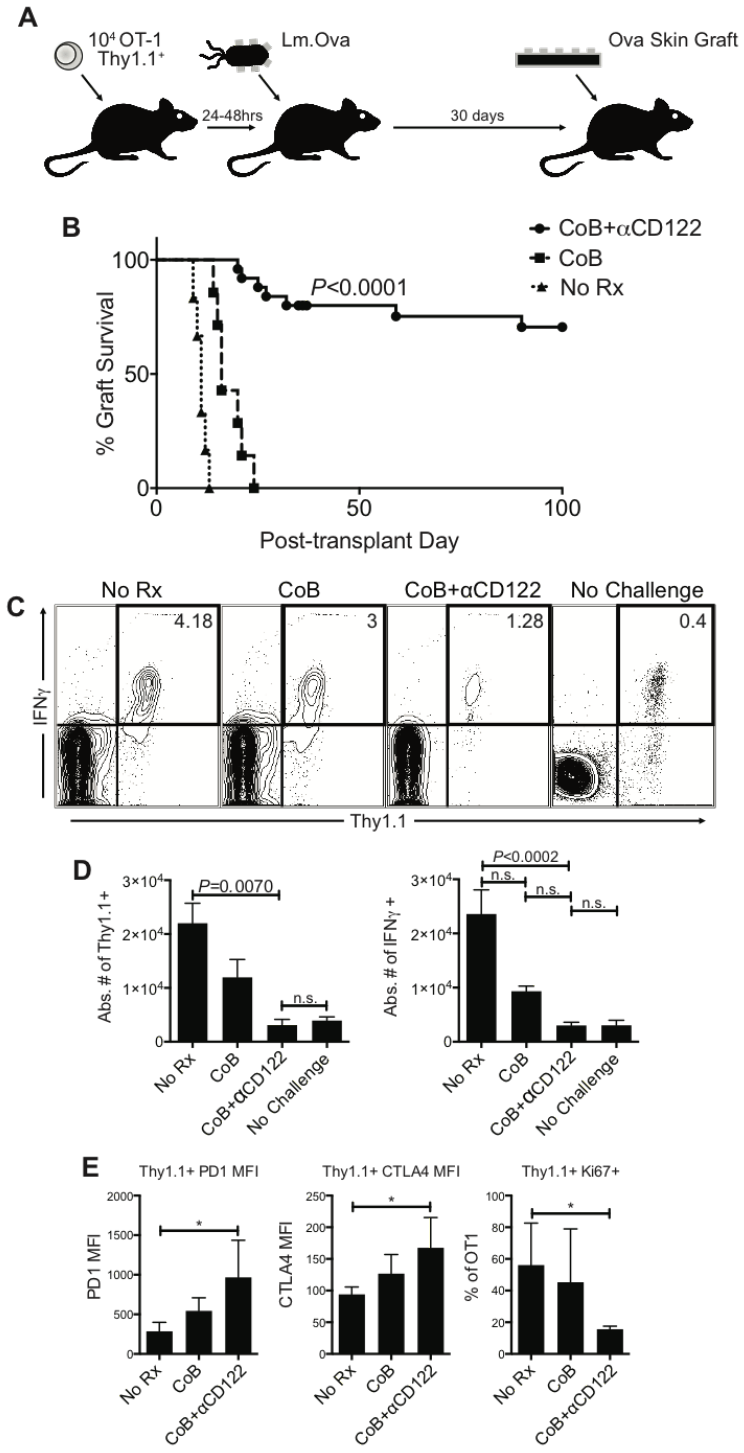


Figure 3.3 CD122 Signaling Supports Costimulation Independent Recall Responses

(A) In a model of memory CD8+ T cell mediated graft rejection, Ova-specific CD8 T cells

(Thy1.1+ OT-1) were transferred into naïve C57BL/6 mice. Mice were immunized 24-48hrs later with Listeria monocytogenes expressing Ovalbumin (Lm.Ova). After 30 days, mice were challenged with an Ova- expressing skin graft. (B) Untreated mice experienced rapid rejection (black triangles, No Rx, MST=11). CoB treated mice experienced memory CD8+ T cell mediated costimulation independent rejection shortly after (black squares, CoB, MST=16). Addition of αCD122 synergized with CoB to prolong graft survival indefinitely (black circles, CoB+αCD122, MST>100 days, n=6-13/group, P<0.0001 Mantel-Cox log-rank test). (C) We investigated the impact of CoB+αCD122 by examining the frequency and function of graft specific CD8 T cells in the draining lymph nodes 5 days after transplantation. Addition of αCD122 constrains the expansion of graft specific CD8+ T cells as demonstrated in representative FACS plots. (D) Both absolute number of graft specific (Thy1.1+) cells (P=0.0070) and IFNγ+ cells (P=0.0002) were diminished with the addition of αCD122. (E) CoB+αCD122 therapy resulted in increased coinhibitory receptor expression on remaining graft specific CD8+ T cells. Thy1.1+ cells demonstrated increased PD-1 (P=0.0303) and CTLA4 (P=0.0065) expression. Additionally, decreased Ki67 expression (P=0.0250) indicated less cell-cycle entry. (C-E) P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; bars represent the mean ± SEM of 3 mice/group. Results are representative of 2-3 independent experiments. *P<0.05.

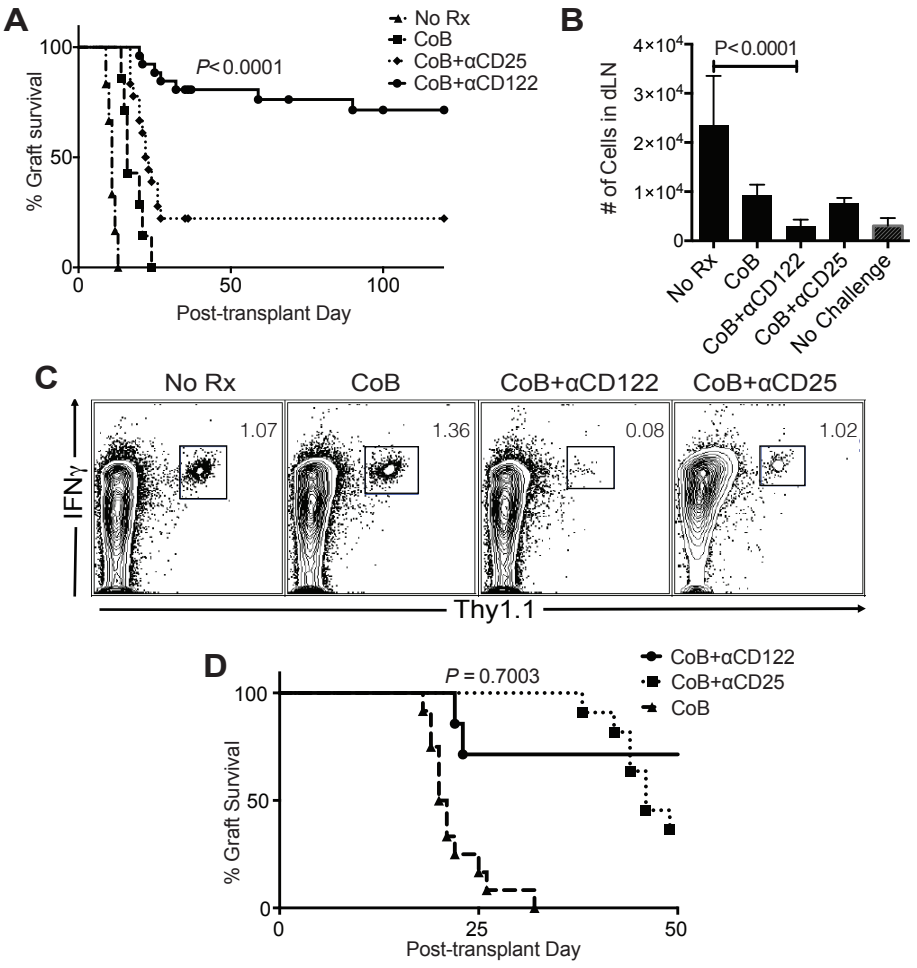


Figure 3.4 The High Affinity IL-2R is Dispensable for Recall Responses. (A) Utilizing the same model of memory CD8+ T cell mediated graft rejection, we evaluated the relative importance of the high affinity IL-2 receptor by blocking CD25, compared to blocking CD122, which interrupts both IL-2R and IL-15R. Untreated mice rejected rapidly (black triangle, MST=11 days). Combined CoB+αCD25 (black diamonds, MST= 22.5 days) failed to prevent memory CD8+ T cell costimulation independent rejection in mice treated with CoB (black squares, MST=16 days), while combined CoB+αCD122 led to indefinite graft survival (black circles, MST>100 days, P<0.0001, n=6-13/group Mantel-Cox log-rank test). (B) CoB+αCD122 synergistically controlled the expansion of the absolute numbers of graft specific CD8+ T cells during re-call responses more effectively than CoB alone or

CoB+αCD25 (P<0.0001). (C) Representative FACS plot demonstrate a reduced frequency of graft specific cells in combination CoB+αCD122 treated animals relative to No Rx, CoB or CoB+αCD25. (D) In the BALB/c to C57BL/6 skin transplant model, a primary alloimmune challenge, the addition of αCD25 therapy (black squares), which interrupts the high affinity IL2R, demonstrated similar efficacy in prolonging allograft survival as the addition of αCD122 (black circles), which blocks both the high affinity IL-2 and IL15 receptor complexes. P values generated though 1-way ANOVA followed by Tukey's multiple comparisons test; bars represent the mean ± SEM of 3 mice/group. Results are representative of 2-3 independent experiments.

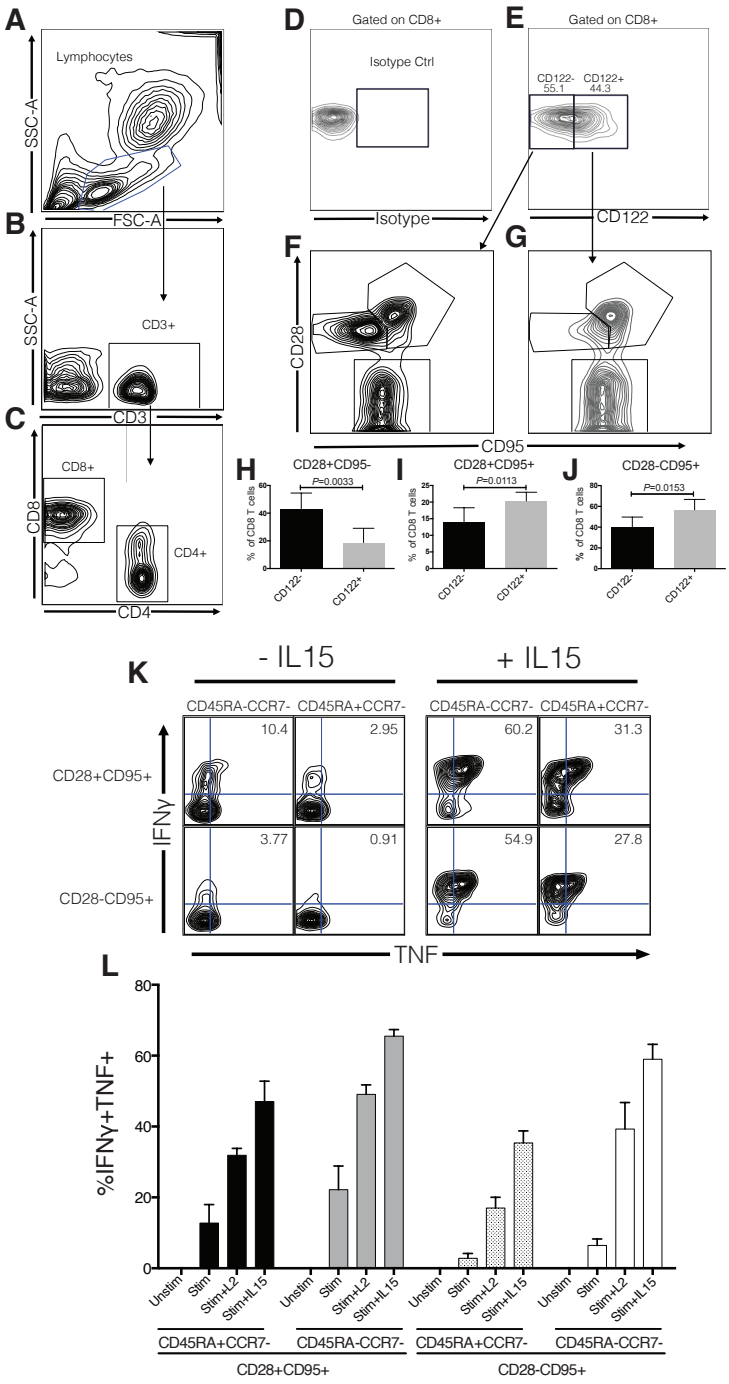


Figure 3.5 CD122 Phenotype and function on Rhesus macaque CD8 T cells. (A) Rhesus PBMCs were analyzed by FACS. Gates based on lymphocytes were defined by forward and side-scatter, (B) further gated on CD3+ T cells and then (C) CD8+ T cells and CD4+ T cells. (D) Gating on CD8+ T cells, an isotype control was utilized to define (E)

CD122- vs. CD122+ CD8+ T cells. (F) CD122- cells demonstrated higher frequencies of CD28+CD95- naïve CD8 T cells in contrast to (G) CD122+ cells which were predominantly TEM CD28-CD95+ or TCM CD28+CD95+ CD8+ T cells. (H-J) The increased memory phenotype of CD122+ (grey bars) CD8+ T cells vs. more naïve phenotype of CD122- (black bars) CD8+ T cells in is depicted graphically. (K) The addition of IL-15 in vitro increased frequencies of CD8+ T cells recruited into the effector response as measured by dual IFNγ and TNF production. IL-15 augments effector function across the spectrum of memory differentiation, as defined by CD28, CD95, CD45RA and CCR7 expression. (L) IL-15 was superior to IL-2 in recruiting CD8+ T cells into an effector response. IL-15 augments cytokine production by CD28+CD95+ cells compared to CD28-CD95+ cells ($P<0.0001$). P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; bars represent the mean ± SEM of 6 Rhesus macaques.

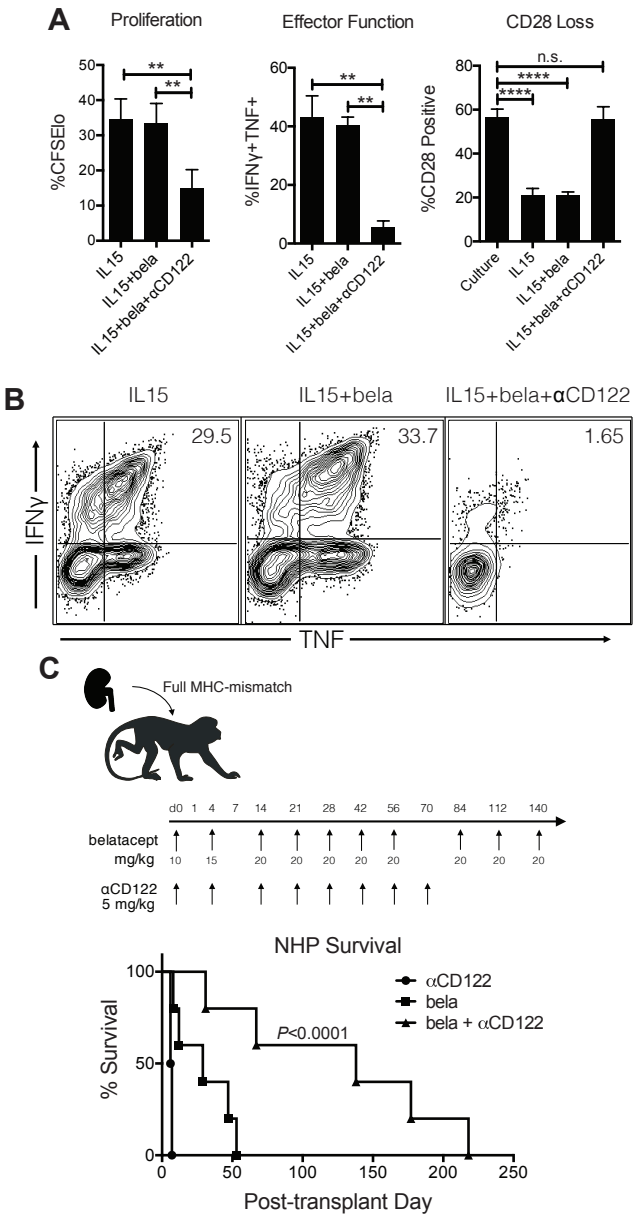


Figure 3.6 Humanized αCD122 Synergizes with Belatacept to inhibit Alloreactivity and Prolong NHP Survival (A) In vitro mixed lymphocyte reaction (MLR) of NHP PBMCs between fully MHC-mismatched pairs. CFSE-labeled responder lymphocytes were incubated for 96 hours with irradiated stimulators, with IL-15, IL=15+bela (belatacept), or IL-15+bela+αCD122. The combination of bela+αCD122 reduced allo-proliferation of NHP CD8+ T cells (P=0.0024). After 96hr MLR culture, cells were re-stimulated with PMA/Ionomycin to evaluate effector function of alloreactive (CFSElo) CD8+ T cells. The

combination of bela+αCD122 ablated effector function of alloreactive CD8+ T cells (P=0.0169). The addition of IL-15 to the culture resulted in loss of CD28 expression, addition of αCD122 restored CD28 expression on CD8+ T cells to similar levels as culture conditions without IL-15 (P=0.8011). (B) Representative FACS plots of CFSElo CD8+ T cell effector function as measured by dual IFNγ and TNF production (corresponds to graph in A). (C) NHPs underwent bilateral nephrectomy and life-sustaining renal transplantation from a fully MHC- mismatched NHP donor. Animals were treated with humanized αCD122 alone (5mg/kg, black circles, n=2, MST=6.5 days), belatacept alone (black squares, n=5, MST=29 days), or bela+αCD122 (black triangles, n=5, MST=138 days, P<0.0001, Mantel-Cox log-rank test). Combination bela+αCD122 synergized to prolong NHP survival compared to belatacept monotherapy, or αCD122 monotherapy. (A-B) P values were generated by 1-way ANOVA and Tukey's multiple comparisons test; bars represent the mean ± SEM of 6 NHPs per group. Results are representative of 2-3 independent experiments. *P<0.05; **P<0.01, ****P<0.0001.

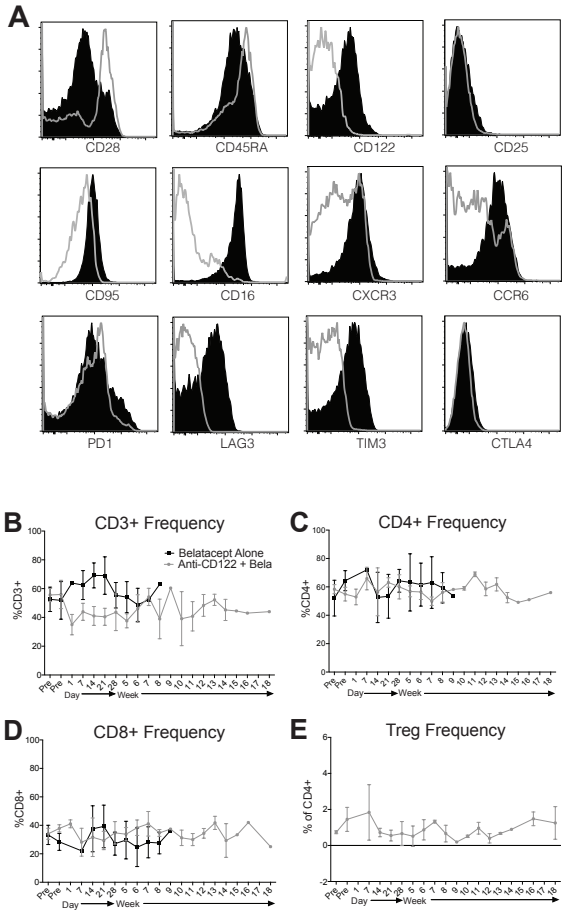
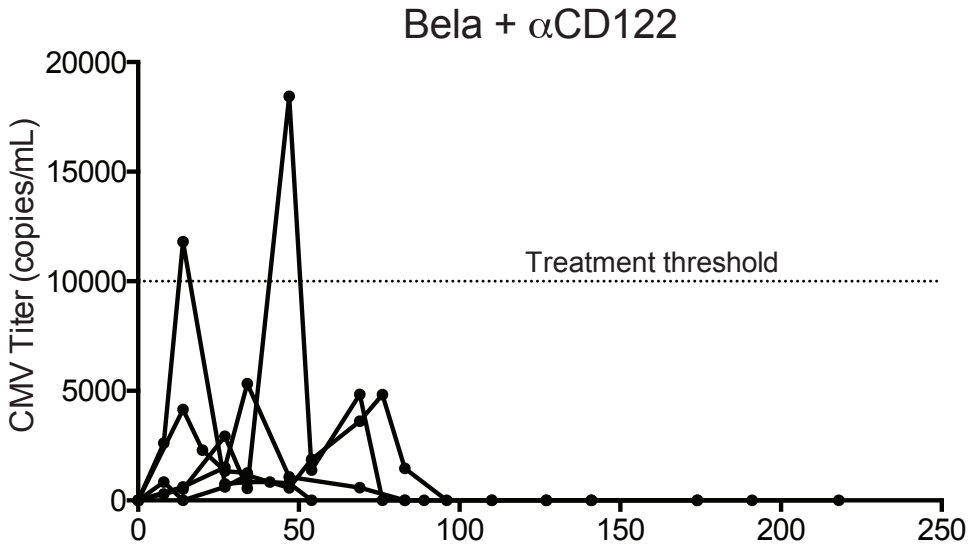


Figure 3.7 Belatacept + αCD122 Combination Therapy does not impact overall T cell frequencies including Tregs (A) Representative Histograms from FACS analysis of NHP Graft infiltrating CD8+ T cells from a belatacept monotherapy animal (solid black) compared to peripheral blood (grey line, no fill) at the time rejection reveals a unique immunophenotype, distinguished by uniformly high expression of CD122 but not CD25. Longitudinal immunophenotyping of peripheral lymphocytes demonstrates no significant reduction in frequencies of (B) CD3+, (C) CD4+, or (D) CD8+ in bela+αCD122 (grey circles and grey line) compared to belatacept monotherapy (black circles and black lines). (E) Similarly, CD4+CD25+Foxp3+ frequencies were preserved in animals receiving

belatacept+αCD122, and were similar to previously reported NHP transplant recipients receiving belatacept-based immunosuppression at our center.



Supplemental Figure 3.1 CMV Reactivation. Animals treated with Belatacept+αCD122 were monitored weekly for CMV reactivation, defined as greater than 10.000 copies of viral DNA/ul of blood, as measured by quantitative PCR. Two animals experienced CMV reactivation during therapy, which was well controlled with CMV anti-viral therapy (ganciclovir) and did not recur.

Chapter 4. The Role of IL-7Rα In Costimulation Independent Allograft Rejection

Introduction

For years since the introduction of calcineurin inhibitors (CNIs) - potent, non-specific immunosuppressive agents - twelve-month survival has been the benchmark of success in solid organ transplantation. Due to their non-specific mechanism of action and nearly ubiquitous target distribution, CNI therapies promote a number of comorbidities including cardiovascular disease, metabolic dysfunction, and vasculopathy which result in premature death or graft loss (29, 31, 180). The introduction of clinical costimulation blockade as the first alternative to CNIs has yielded the first significant improvement in long-term outcomes for transplant recipients in over 30 years, and yet, costimulation blockade based immunosuppression gives rise to increased rates of acute rejection in a subset of transplant recipients (71, 73, 75). Our group recently identified a subset of CD28+CD95+CD45RA+CCR7- CD8 T cells, so called CD28+ CD8 TEMRA, which were highly predictive of costimulation independent allograft rejection, and associated with a distinctive and related phenotype of graft infiltrating CD8 T cell (99). Phenotypic characterization of this memory T cell subset which we hypothesize may give rise to costimulation independent rejection, revealed relatively high levels of IL-7Rα expression.

Interleukin-7 is canonically thought to play a critical role in lymphopoesis, T cell development in the thymus and homeostasis (156, 157, 228). Mice who lacked IL-7 and the IL-7Rα suffer from similar forms of severe lymphopenia, and people with deleterious mutations in *IL7Ra* are diagnosed with a form of Severe Combined Immunodeficiency

(158, 159, 229, 230). Many studies have described the contributions of IL-7 in lymphocyte development and homeostasis, as well as the mechanisms by which IL-7/IL-7Rα interacts to support T cell renewal.

Activating polymorphisms in the *IL7Ra* locus are highly associated with the development of autoimmune disease, and those suffering from progressive multiple sclerosis demonstrate increased IL-7 and IL-7Rα mRNA in their CSF (174, 175). Blockade of IL-7Rα has been shown to ameliorate autoimmune disease but the precise mechanism is not well defined (179, 231). The stimulation of this pathway in order to augment effector T cell responses is being studied for its promise as an adjuvant in vaccine development and cancer immunotherapy, and as a target for blockade in autoimmune disease.

Our studies suggest that addition of anti-IL-7Rα prevents costimulation independent allograft rejection by controlling the expansion and effector function of graft specific CD8 T cells, promoting CD8 T cell exhaustion, and expanding regulatory T cells. Future studies will be aimed at better understanding the cell intrinsic signaling events that are the basis of these changes, the level of induced regulatory T cell stability at the genomic level, exploration of the early events that give rise to costimulation independence (cell intrinsic and extrinsic) and how these costimulation independent mechanisms are mitigated by therapeutic IL-7Rα blockade, and finally, translation of these findings into the non-human primate model of renal transplantation with a therapeutic humanized anti-CD127 monoclonal antibody.

Materials and Methods

Materials and Methods

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I and OT-II (72) transgenic mice, purchased from Taconic Farms, were bred to Thy1.1+ background at Emory University. mOVA mice (C57BL/6 background, H-2^b; (73)) were purchased from The Jackson Laboratory. All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in pathogen-free animal facilities at Emory University.

Donor-reactive T cell adoptive transfers. Splenocytes from Thy1.1+ OT-I mice were resuspended in PBS and 1.0×10^6 of Thy1.1+ CD8+ OT-I T cells and 1.0×10^6 of Thy1.1+ CD4+ OT-II T were injected i.v. Mice were given Ova expressing skin graft from mOva donors after 24 hours, and treated with therapies as indicated.

Skin transplantation and antibody treatment. Full-thickness tail or ear skin was transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages. Where indicated, mice were treated with 250 μg CTLA4-Ig (Bristol Myers Squibb, Princeton, NJ), 250 μg hamster monoclonal anti-mouse CD154 (MR-1, BioXcell, West Lebanon, NH), 200 μg anti-CD127 (A7R34, Bio X cell, West Lebanon, NH) intraperitoneally on days 0, 2, 4 and 6 post- transplantation.

Acute Graft-versus-Host Disease model. C57BL/6 splenocytes were labeled with 10 μM Cell Trace Violet (CTV, C34571, Invitrogen, Carlsbad, CA). 3 x 10^7 C57BL/6 CTV labeled splenocytes were transferred i.v. into sublethally irradiated (800 rads) BALB/c recipients, and selected groups received therapy on day 0 and 2 as described above. Splenocytes were harvested on day 3, and analyzed by flow cytometry to assess CTV labeled cell division.

Antibodies and flow cytometric analysis *Non-human Primate*: Fresh PBMCs were isolated by Ficoll density gradient centrifugation (BD Biosciences, Franklin Lakes, NJ). PBMCs were stained with the following mAbs: CD3 PacBlue, CD95 V450, CD3 Alexa 700, CD4 PerCP-Cy5.5, CD8 V500, CD28 PE-Cy7, CD25 PE-Cy7, IFNy PE-Cy7, CD28 APC, TNF APC (all BD Biosciences). PBMCs (1.5×10^6) were incubated with appropriately titered antibodies for 15 min at 4°C and washed twice. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Samples were acquired immediately on a BD LSR II multicolor flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA). For the stimulation assay, 1.5×10^6 PBMCs were cultured in RPMI 1640 (Corning cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and stimulated with 10 μM phorbol 12-myristate 13- acetate (PMA) and 200 nM ionomycin (Sigma-Aldrich, St. Louis, MO) for 5hr. IL-7 (100ng/ml) (Peprotech, Rocky Hill, NJ). PBMCs were washed twice prior to antibody staining and data acquisition.

Murine Surface stains and flow cytometry

Spleens or draining axillary and brachial LN were stained for CD4, CD8, Thy1.1, CTLA4, PD-1, KLRG1, CD127, CD62L, CD122 and CD44 (Biolegend, San Diego, CA). Samples

were analyzed using an LSRII FACS machine (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Intracellular cytokine staining

Where indicated, responder lymphocytes or splenocytes were stimulated with PMA/Ionomycin, or 10 nM OVA257–264 (Genscript, Inc., Piscataway, NJ) in the presence of 10 μg/mL Brefeldin A for 4– 5 h. Intracellular staining kit was used to detect TNF, IFN-γ (Biolegend) and IL-2 (BD Biosciences) according to manufacturer's instructions.

Statistics

Survival statistics were calculated using a log-rank test. T cell frequencies, absolute numbers and MFI were compared using 1-way ANOVA with Tukey's multiple comparisons test, or unpaired t-test (comparison between two groups). Data were analyzed using Prism 6 (GraphPad Software, La Jolla, CA). A P value of <0.05 was considered statistically significant.

Study Approval

All studies utilizing mice, non-human primates and human samples were carried out in accordance with institutional guidelines, including Emory University IACUC guidelines, and all experimental protocols utilizing animals were conducted with approval by this institutional review board.

Results

Phenotype and Function of IL-7 on NHP PBMCs

Previous studies demonstrated that increased frequencies (>3% of total CD8 T cells) CD28+ CD8 TEMRA were highly predictive of costimulation independent rejection in non-human primates (99). Extensive phenotypic analysis of CD8 T cell memory subsets revealed that CD28+ TEMRA were distinguished by high levels of IL-7Rα expression (Fig 4.1A), relative to CD28- TEMRA (Fig 4.1B, MFI=606.6 on CD28+ TEMRA compared to 99.24 for CD28- TEMRA, n=5 NHPs, P<0.0001). Surface staining also revealed increased expression of CD122 (data not shown), but no increased expression of a number of adhesion molecules such as VLA-4, LFA-1, and CD2. CD28+ CD8 TEMRA cells did not exhibit increased levels of PD-1, TIM3, LAG3, or CTLA4. Next, we investigated the impact of IL-7 on T cell activation. The addition of IL-7 augmented CD28+ and CD28- effector function (Fig4.1C), and specifically, augmented the effector function of CD28+ CD8 TEMRA by as much as 6-fold in some animals (Fig1.3D, P<0.0001).

Addition of Anti-IL7Rα Therapy Synergizes with Costimulatory Blockade to Prolong Allograft Survival

Given the role of IL-7 as a potential Signal 3 cytokine, we asked if IL-7Rα signaling could support costimulation independent allograft rejection. In a stringent model of fully MHC-mismatched skin transplantation between Balb/C donor mice and C57BL/6 recipients, mice uniformly reject their transplanted tissue on costimulation blockade therapy. The addition of anti-IL7Rα therapy synergized with CoB to significantly prolong allograft survival, and gave rise to indefinite survival in some mice (MST > 80 days. P<0.0001). To investigate the

mechanism of this survival benefit we first asked if the addition of IL-7Rα could control the allostimulated proliferation of CD8 T cells. In a model of acute Graft-versus-Host Disease, we tracked the rate of proliferation of CFSE labeled C57BL/6 CD8 T cells in sublethally irradiated Balb/C hosts, and found that the combination of CoB+anti-IL7Rα synergized to constrain the expansion of allostimulated T cells (Figure 4.3, 41.8% reduction compared to unmodified GvHD, and 12.77% reduction compared to CoB alone, P=0.0002).

Tracking the Fate of Graft Specific CD4 and CD8 T cells During CoB+anti-IL7Rα Therapy

To further understand the mechanisms underlying the survival benefit seen in mice receiving combined CoB+anti-IL7Rα therapy, we utilized a model antigen system, where Ova specific OT-1 and OT-2 T cells participate in Ova expressing skin graft rejection, a system which has been well characterized and developed for transplant studies, and utilized in the study of costimulation independence (100, 208). First, we investigated the efficacy of combined CoB+anti-IL7Rα in this model, and found that combination therapy, similar to the fully allogeneic model, gave rise to indefinite graft survival, greater than 100 days post-transplant (Fig 4.4, P<0.0001). Next, we tracked the fate of graft specific cells. At day 10 post-transplant, we interrogated the absolute number of graft specific CD4 and CD8 T cells (Figure 4.4), and found that combination CoB+anti-IL7Rα constrained the expansion of CD4 T cells (mean cell number: No Rx = $1.57x10^5$, anti-IL7Rα = $3.90x10^4$, CoB = $5.3 x10^3$, CoB+ anti-IL7Rα = $1.8 x10^3$, P = 0.0113) and CD8 T cells (mean cell number: No Rx = $2.41x10^5$, anti-IL7Rα = $7.08x10^4$, CoB = $1.96 x10^4$, CoB+ anti-IL7Rα = $7.47 x10^3$, P = 0.0022). Next, we characterized the effector function of the CD8 T cells that remained after

dual therapy, and our analysis revealed that combination CoB+anti-IL7Rα ablates effector function of graft specific CD8 T cells (Figure 4.5, mean freq. of IFNγ+TNF+ OT-1: No Rx = 10.37%, anti-IL7Rα = 14.13% , CoB = 3.16%, CoB+ anti-IL7Rα = .93%, P = 0.0004).

CoB+anti-IL7Rα Durably Expands Graft Specific Tregs

We interrogated the fate of graft specific CD4 T cells after transplantation in the model antigen system, and found that over the course of transplantation the frequency of graft specific Tregs increased starting at day 10 post-transplant (Figure 4.6A, mean freq. of CD4+CD25+Foxp3+ OT-2: No Rx = 2.07%, anti-IL7Rα = 2.61% , CoB = 3.26%, CoB+anti-IL7Rα = 9.84%, P = 0.0369). This trend continued to day 20 (Figure 4.6B, mean freq. of CD4+CD25+Foxp3+ OT-2: No Rx = 5.18%, CoB = 11.07%, CoB+anti-IL7Rα = 18.10%, P = 0.0369), and persisted at day 30 (data not shown). Further analysis of these Tregs to assess stable epigenetic program of bona fide Tregs is pending.

Phenotypic Changes of Graft Specific CD8 T cells

Tracking graft specific CD8 T cells in this model, we interrogated the phenotype of these cells over the course of transplantation. We found that CD8 T cells downregulated CD28 during therapy with CoB, while increasing expression of IL-7Rα (Figure 4.7A). Furthermore, at day 20 post-transplant, CD8 T cells in animals treated with CoB+anti-IL7Rα coordinately increased expression of coinhibitory receptors PD1 and TIGIT (Figure 4.7B, mean freq. of PD1+TIGIT+ OT-21 No Rx = 6.41%, CoB = 25.77%, CoB+anti-IL7Rα = 50.00%, P <0.0001). Both CoB and CoB+anti-IL7Rα demonstrated similarly low levels of Ki67 expression as well (data not shown).

Discussion

The role of the IL-7/IL-7Rα pathway in lymphopoesis, thymopoesis, and homeostasis is well established. Additionally, signaling through IL-7Rα is critical for the development of high quality memory T cells. Seminal studies described IL-7Rα expression early in an effector response as a marker which segregates memory precursor T cell subsets which gives rise to highly functional central memory T cells, apart from effectors which lack IL-7Rα expression and are dubbed "short lived effector cells" (232, 233). IL-7Rα expression on antigen experienced cells denote a highly functional memory T cell, as opposed to antigen experienced cells which lack IL-7Rα and are characterized by increased coinhibitory receptor expression and reduced functionality (234-237).

In our studies we identified a CD28+ TEMRA subset which are highly associated with costimulation independent rejection, and extensive immunophenotypic analysis revealed that these cells highly express IL-7Rα. Many questions remain as to why these cells express the highest levels of IL-7Rα among T cell subsets (equal to but not less than others, including CD28+ TCM). Do these cells represent a qualitatively superior heterologous memory populations with augmented capacity to expand in the face of alloantigen? Do they act to sequester IL-7 from other cell types? Do they rely on IL-7 for renewal? Here we found that these cells respond to IL-7 as a signal 3 cytokine, and augment their effector function.

Stromal cells of the lymphoid organs stably secrete IL-7, and are not thought to dynamically regulate their secretion of IL-7 (154). Binding of IL-7 to IL-7Rα induces the expression of pro-survival molecules, B cell lymphoma-2 family members (BCL-2 and MCL-

1) and also result in the downregulation of IL-7Rα (155, 238, 239). These suggest that IL-7 is regulated not by the supply of cytokine, but by consumption. In a revealing experiment, mice who received retrovirally transfected IL-7Rα over-expressing myeloid cells demonstrated a loss of peripheral T cells, giving evidence to the hypothesis that perhaps IL-7Rα based limitation of IL-7 supply regulates T cell numbers in the organism (240). The idea that IL-7Rα bearing T cells dynamically regulate expression of IL-7Rα in order to allow other cells to receive IL-7 signaling has been termed the altruistic hypothesis. If all effector memory cells maintained high levels of IL-7Rα, they would likely decrease the clonality of the T cell repertoire, crowding out naïve T cells from critical pro-survival signals, an idea which several groups have explored by demonstrating that transfer of large numbers of IL-7Rα bearing cells into lymphopaenic hosts reduced the number of naïve T cells (213). In our studies we found that in the context of allograft rejection, costimulation blockade elicited a phenotypic change in graft specific CD8 T cells, where cells decreased their expression of CD28, and increased their expression of IL-7Rα. The question remains, can IL-7Rα act as a redundant signaling molecule for T cell activation? And how does costimulation blockade induce increased expression of IL-7Rα? We found that indeed blockade of IL-7Rα in addition ot costimulation blockade led to T cell exhaustion and a complete mitigation of the alloreactive immune response, suggesting that IL-7Rα signaling is an alternative activation pathway for graft specific T cells, in lieu of costimulation.

Previous studies had described the phenotype of regulatory T cells as distinct from other T cells, based on the nearly dichotomous expression of the IL-7Rα (on memory and activated T cells) as opposed to the IL-2Rα which is expressed on regulatory T cells, as well as a subset of activated T cells (241). In practice, IL-7Rα could be used to distinguish non-

Tregs from Tregs which express high levels of IL-2Rα (242). The administration of IL-7 led to relative decrease in the frequency of regulatory T cells in humans, which would be reasonable to accept if one assumes IL-7Rα expressing CD4 T cells are being selectively expanded while IL-2Rα cells are at a relative disadvantage (243). Some groups have reported IL-7Rα signaling may augment regulatory activity, and in a sophisticated model of skin allotransplantation utilizing the bm12 mouse model and titrated alloreactive CD4 and regulatory phenotype cells on a Rag KO background, researchers demonstrate a potential role for IL-7Rα signaling in supporting regulatory T cell dependent graft protection (244).

We observed that graft specific CD4+ Tregs were augmented following blockade of both costimulatory signals and IL-7Rα. Further studies are required to fully understand the mechanisms underlying this relative expansion of regulatory T cells. Here we demonstrate combined blockade of costimulatory signals and the IL-7Rα in the setting of transplantation result in a relative expansion of regulatory T cells. We hypothesize that in the context of costimulatory blockade, Signal 3 cytokines play a distinct and non-redundant role in supporting T cell activation. In the absence of inflammation, costimulation and IL-7 signaling, but with persistent presence of antigen in the form of skin allograft we find induction or perhaps maintenance of graft specific regulatory T cells. However, the relative expansion of regulatory T cells may be due to a number of possible factors and requires further exploration.

Figures

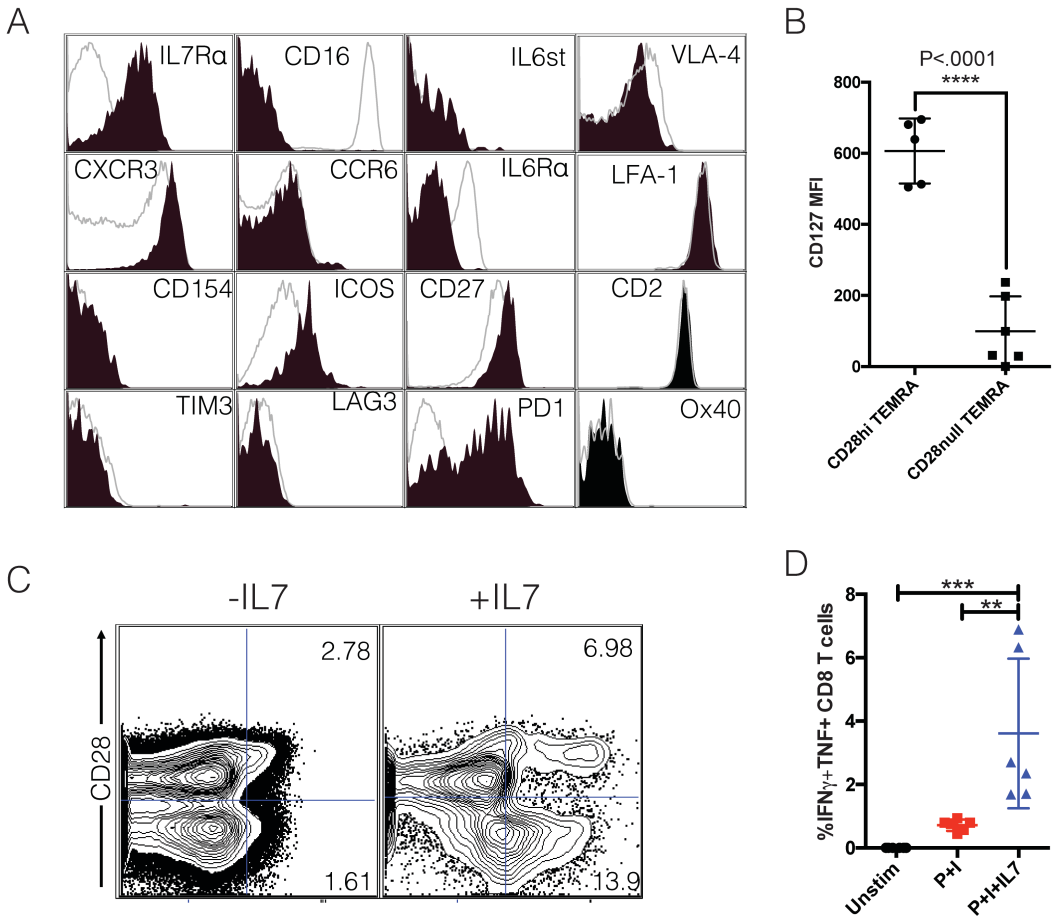


Figure 4.1 NHP CD8 T cell Phenotype and Function (A) Histograms depict surface expression of a panel of phenotypic markers on CD28+CD95+CD45RA+CCR7- CD8 T cells (CD28+ CD8 TEMRA, black fill) compared to CD28-CD95+CD45RA+CCR7- CD8 T cell subsets (CD28- CD8 TEMRA, grey line, no fill). Representative FACS plot of n=5 rhesus macaques, peripheral blood. (B) MFI of CD127 expression is significantly increased on CD28+ CD8 TEMRA (black circles) compared to CD28- CD8 TEMRA (black squares). (C) Addition of IL-7 augments effector function of CD28+ and CD28- CD8 T cells as

depicted in representative FACS plot of CD3+ CD8+CD4- T cells (n=5). (D) The Addition of IL-7 augments double IFNy and TNF producing CD8+ T cells *in vitro*.

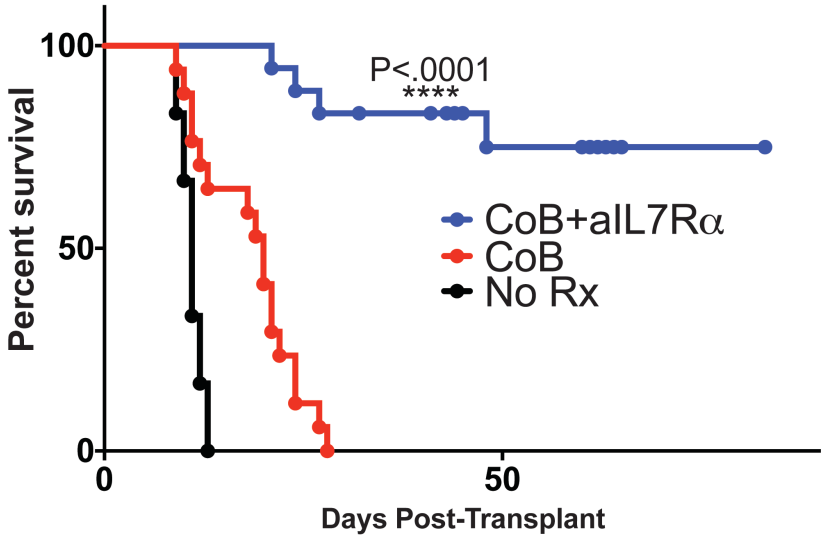


Figure 4.2 Blocking IL-7Rα Prevents Costimulation Independent Rejection In a stringent model of fully allogeneic Balb/C to C57BL/6 skin transplantation, the addition of anti-IL7Rα monoclonal antibody therapy (200 ug anti-IL7Rα day 0, 2, 4, 6) results in prolonged survival (blue line), demonstrating that costimulation independent rejection is supported by IL-7Rα signaling. Costimulation Blockade alone (red line, 250ug of CTLA4-Ig and 250ug of anti-CD40L, given i.p. day 0, 2, 4, 6) gives rise to therapy resistant rejection MST=25 days). Untreated mice (black line) reject rapidly (MST=10 days).

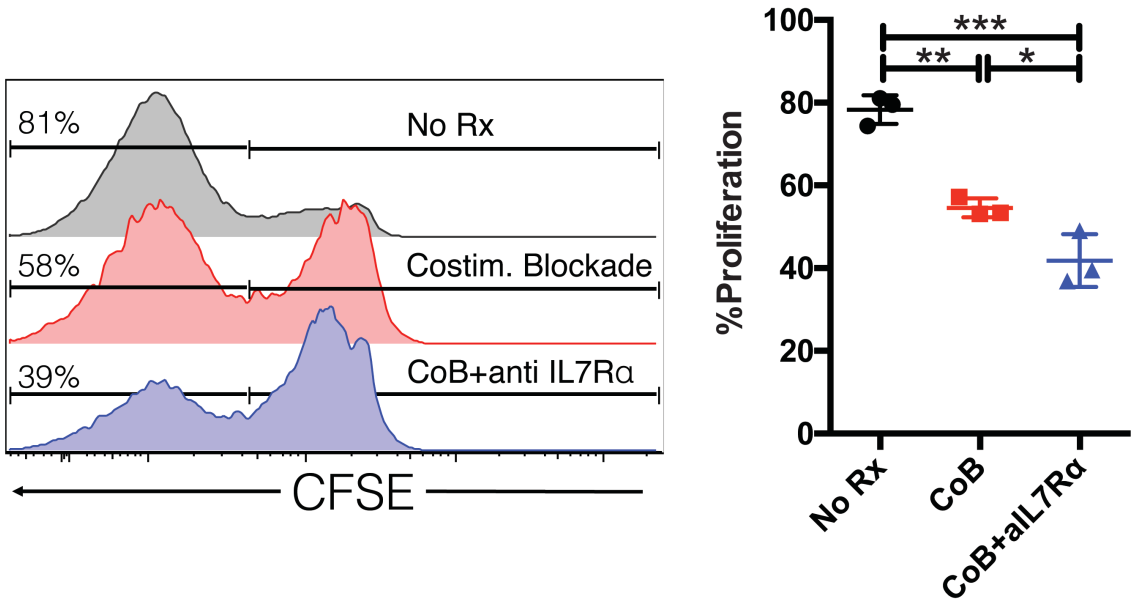


Figure 4.3 Blocking IL-7Rα Prevents Costimulation Independent Proliferation In a model of Graft-versus-Host Disease (GvHD), fully allogeneic Balb/C mice receive CFSE labeled lymphocyte adoptive transfer from C57BL/6 mice, after sublethal irradiation. Untreated mice (black histogram, black circles) show rapid proliferation marked by CFSE dilution as seen in the layered histograms and corresponding graphical depiction. Costimulation blockade alone (red histogram, red squares) controls expansion to a degree, but the addition of anti-IL7Rα (blue histogram, blue triangles) significantly abrogates the allostimulated proliferation of CD8 T cells. ***P<0.001, **P<0.01, *P<0.05

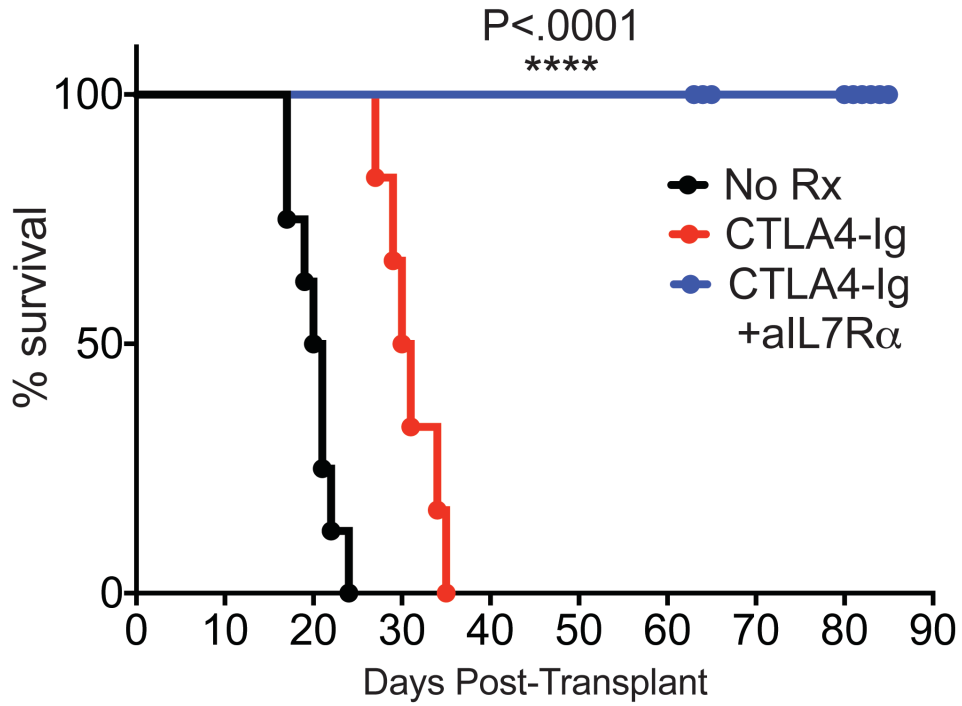


Figure 4.4 Addition of Anti-IL7Rα Synergistically Prevents Costimulation Independent Rejection In a model developed to track graft specific T cell responses to transplanted tissue, 10^6 OT-1 (CD8+) and OT-II (CD4+) Ova specific T cells were adoptively transferred into naïve C57BL/6 mice who subsequently received mOva expressing skin grafts. Untreated mice reject rapidly (black line, MST=21 days), and costimulatory blockade with CTLA4-Ig prolongs survival, but gives rise to costimulation independent rejection (red line, MST=32 days). The addition of anti-IL7Rα to costimulatory blockade (blue line) results in indefinite survival (MST>80 Days, Mantle-Cox log-rank test, P<0.0001).

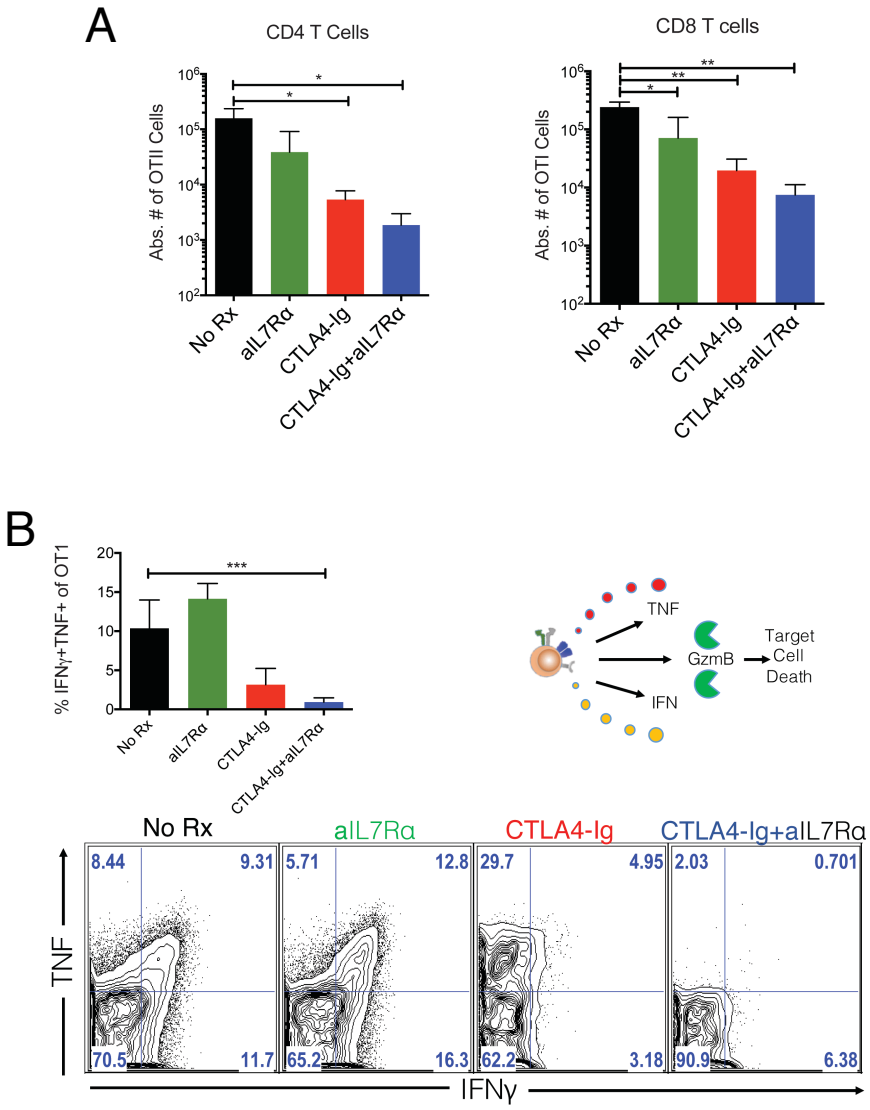


Figure 4.5 Anti-IL7Rα + CoB Results in Decreased Expansion and Effector Function of Graft Reactive T cells (A) In the model of Ova-specific CD4 and CD8 T cell mediated mOva graft rejection, treatment with combination anti-IL7Rα + CoB (CTLA4-Ig) results in a markedly reduced expansion of graft specific CD4 and CD8 T cells. (B) The cells that remained in anti-IL7Rα + CoB treated animals were ineffective, and failed to mount effector responses as demonstrated by a lack of effector cytokine production. P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; Bars represent

the mean ± SEM of 3 mice/group. Results are representative of 3 independent experiments. *P<0.05; **P<0.01; *** P<0.001.

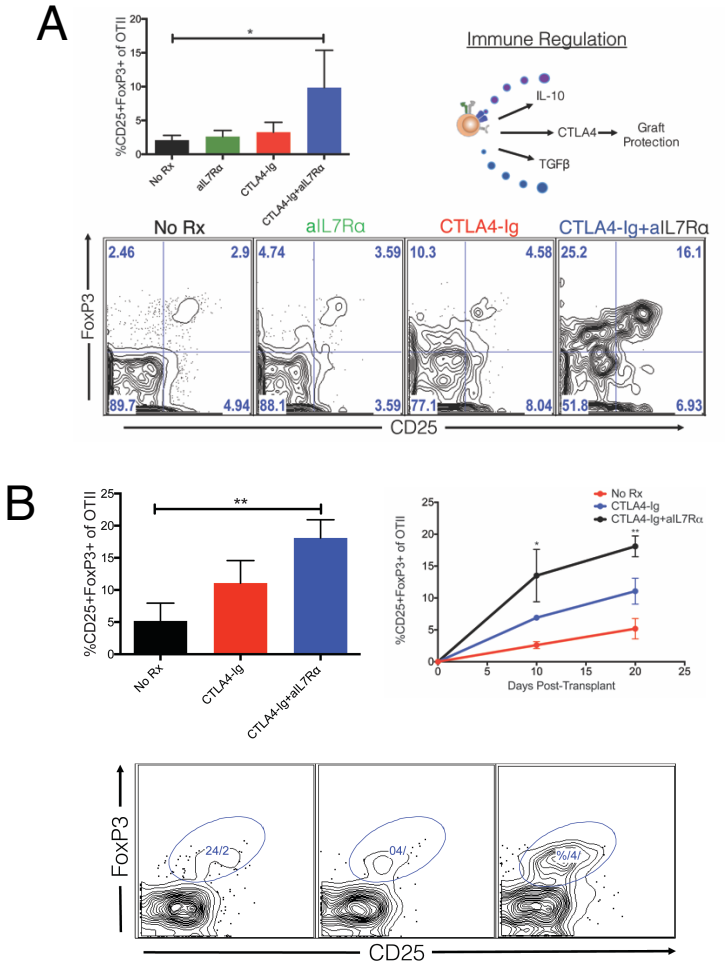


Figure 4.6 Anti-IL7Rα + CoB Augments the Frequency of Graft Specific iTregs (A) At Day 10 post-transplant, mice treated with combined anti-IL7Rα + CoB demonstrate a relative expansion of graft specific CD4 regulatory T cells. (B) at Day 20, this relative expansion of iTregs was sustained and even somewhat augmented. P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; Bars represent the mean ± SEM of 3 mice/group. Results are representative of 3 independent experiments. *P<0.05; **P<0.01

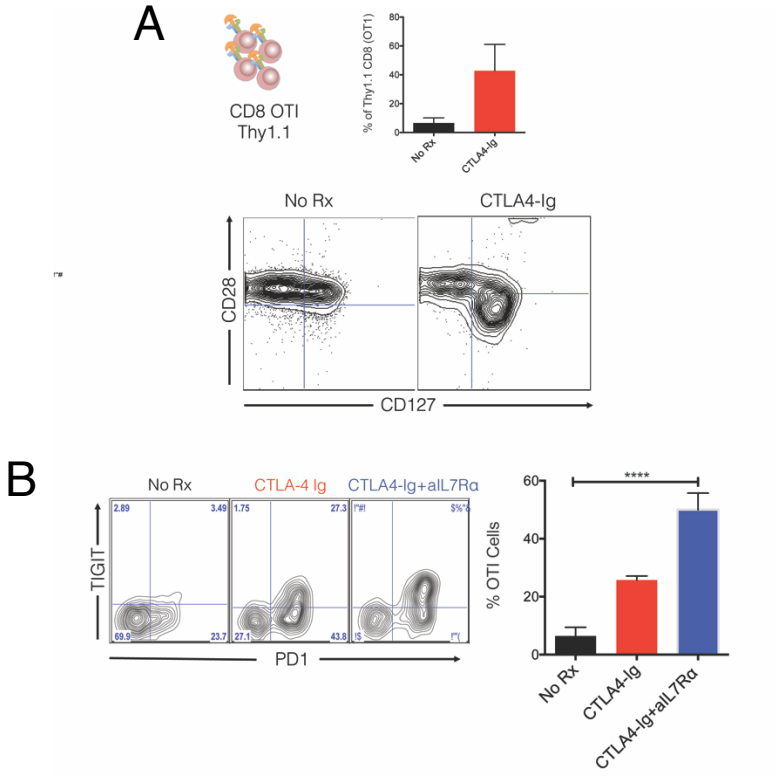


Figure 4.7 Phenotypic Changes in Graft Specific CD8 T cells (A) CTLA4-Ig therapy induces a phenotypic change in graft specific CD8 T cells, whereby cells decrease their expression of CD28 and concomitantly increase expression of IL-7Rα (CD127). (B) Combination costimulatory blockade with CTLA4-Ig + anti-IL7Rα therapy results in augmented coinhibitory receptor expression (PD1+TIGIT+) on graft specific CD8 T cells representative FACS plots, with corresponding graphical data (black bar = Untreated; red bar = CTLA4-Ig; blue bar = CTLA4-Ig+anti-IL7Rα). P values were generated by 1-way ANOVA followed by Tukey's multiple comparisons test; Bars represent the mean ± SEM of 3 mice/group. Results are representative of 3 independent experiments. *P<0.05; **** P<0.0001.

Chapter 5

Discussion

This body of work describes Signal 3 cytokine signaling in costimulation independent rejection as an alternative pathway for T cell activation, expansion, and gain of effector function to enable graft destruction. Not all cytokines are created equal, despite shared signaling subunits and even similar signal transduction pathways. The distribution of cytokines, the spatial and temporal differences in the expression of receptor subunits, the kinetics of receptor complex formation, and the cell-intrinsic differences (i.e. naïve vs. memory) all influence the unique contributions of the γ_c cytokines we focused on in these experiments. One major distinction, for example, was the distinct role of the IL-2R versus the IL-15R in CD8 memory T cell dependent graft rejection, where we observed that the IL-2R is dispensable for re-call responses, but the IL-15R was critical (Figure 3.4). This illustrates just one instance of how these shared receptor systems give rise to unique signaling events.

The arrival at this focused study of Signal 3 cytokines, and the promising results of these experiments (promising for translation, and potential clinical relevance in transplantation) originate from the first set of studies in a translational model of non-human primate renal transplantation. One of the many benefits of utilizing this system is the ability to study immune responses in otherwise difficult to access anatomical compartments, and obviously the increased immunologic memory, and therefore a closer resemblance to the human system. There are of course "dirty mice" but the institutional hurdles to gain access to these models are significant – and the primate model offers additional benefits (245). The ability to interrogate the rejecting kidney in these primates provided a singular opportunity to interrogate the character of costimulation independent rejection. At the site of rejection, at

the time of acute graft injury we were able to gain a fascinating insight into exactly the kind of immune cell subset that posed a material threat to belatacept based therapies for transplant success. The data provided a striking picture of the difference between therapy resistant rejectors and therapy susceptible animals in which "rejection" was held at bay with belatacept therapy, but once therapy was withdrawn at 140 days, these animals too rejected their allografts. Many important observations can be made here:

(1) Belatacept most likely did not eradicate the alloreactive cells in therapy susceptible animals, but did effectively suppress them (although there is the possible of new thymic emigrants after withdrawal of therapy)

(2) belatacept therapy exerted a selective pressure, which resulted in a T cell subset with signaling sensitivities that allowed this subset to mount an effective immune response in the absence of CD28 signaling

(3) not all therapy resistant rejection was exactly alike and

(4) the inciting event for rejection versus the end result of rejection described by our experiments on the kidney may be – and we hypothesize most likely are – not mediated by a static cellular threat, but rather are mediated by a potently proliferative CD28+ subset which can lose CD28 expression in the course of allograft rejection.

As for number one, it is interesting to think that the cells that could give rise to rejection are likely either always with patients, or are emerging rapidly in the absence of therapy. Arguments for tolerance through chimerism might follow here. I would rather argue for a novel solution, which is to engineer responsive cellular therapies, with circuits that are able to sense rejection (perhaps through a constellation of cytokine and inflammatory sensors and receptors), which are then capable of secreting either therapeutic biologics or immunosuppressive cytokines, or trafficking to the graft to protect it. This is not wholly

novel, as synthetic biologists have been creating customizable cell circuits, and tools to engineer these are gaining more traction and wider application (246). It is a somewhat outlandish idea, but this seems like the most appropriate place to discuss something imaginative based on the work we have done.

To the second point, of belatacept therapy exerting a selective pressure which revealed T cell subsets with unique signaling sensitivities which were capable of mounting effective immune responses, well, this gave rise to the entire body of this thesis work. We hypothesized that one of those key signals might be Signal 3 cytokines, in this work we focused on γc cytokines, but this does not exclude the role of other cytokine families. Due to the finding that increased frequencies (>3% of total CD8 T cells) of CD28+ CD8 TEMRA cells in the peripheral blood was highly predictive of costimulation independent rejection, and the subsequent studies which demonstrated this subsets potent alloreactivity and costimulation independence (despite expression of CD28), we interrogated this subset for clues as to what signals they might rely on for activation in the absence of costimulation. CD28+ TEMRA cells ad increased expression of both CD122 and CD127 relative to most other subsets, although CD28- TEMRA were the primary comparison group. In the mOva model, where mice receive OT-1 (CD8) and OT-2 (CD4) T cells, we observed that the use of CTLA4-Ig gave rise to a unique phenotypic change, not dissimilar from the selective pressure seen in the primate studies. We observed that graft reactive cells downregulated CD28, and upregulated CD122 and CD127 (findings summarized in chapter 4, figure 4.7, CD122 data not shown) in the context of CTLA4-Ig therapy. We will investigate the exact mechanism of this shift but it nonetheless seems to fit with our hypothesis. To be clear, transferred OT-1 and OT-2 cells are not memory T cells (which are the subset we concern ourselves with in the non-human primate). In either case, the findings were promising and

instructive, and suggested some compensatory trade-off in activation pathways, as if loss of CD28 increased T cell sensitivity to Signal 3 cytokines.

Addition of CD122 blocking antibody has a number of benefits: it interrupts IL-2 and IL-15, as it is one of the shared signaling subunits of both, whereas current clinically approved therapies only block the high affinity IL-2R, by targeting CD25. CD25 blockade is falling out of favor because of its potential deleterious effects on Tregs (147). Translation of these findings back into the NHP model of renal transplantation demonstrated significant improvement in survival (Figure 3.6C, bela alone MST = 29 vs bela+anti-CD122 MST= 138). It is interesting to note that even in the context of adjuvant anti-CD122, we observed two primates that rejected on dual therapy, one who rejected after anti-CD122 was withdrawn at day 70, and two who rejected after belatacept was withdrawn. This argues for unique mechanisms of rejection, and interrogation of the graft infiltrating cells is pending, but likely to be revelatory.

To the third point, and most instructive, not all belatacept resistant rejection was identical in immunophenotype. In fact, one therapy resistant primate had a very distinct phenotype compared to its counterparts. This animal had the highest levels of CD28+CD95+ memory, and also happened to have high levels of CD28+ TEMRA prior to transplantation, but it was this animal's level of CD28+CD95+CD45RA-CCR7- cells that were highest. Interestingly, this primate rejected at day 7 post-transplant. This is the same speed of rejection we observe in this model when therapy is withheld completely. The graft infiltrate boasted an identical phenotype of high levels of CD28+CD95+CD45RA-CCR7- CD8 T cells. These data argue for multiple potential mechanisms of belatacept resistance: perhaps this animal had a subacute infection, perhaps this primate had a highly heterologous immune memory subset, perhaps the pre-transplant and rejection phenotype are unrelated

and represent different T cell clones altogether. It is interesting that our predictive test still captured this animal, because of its increased level of CD28+ TEMRA, but its graft infiltrate did not follow the pattern of its cohort.

The final point of our proposed hypothesis regarding the development of belatacept resistance, being mediated by a subset of CD28+ memory CD8 TEMRA cells, but giving rise to CD28- CD8 TEMRA graft infiltrate. Ofcourse this is an attempt to reconcile two distinct findings, with some rationale connection, but it may not the case at all. In order to study this phenomenon and make stronger claims, we may attempt to bring several technologies together. We may try to relate peripheral immunophenotyping and clonality by memory subset, with the immunophenotype and clones represented in the graft with Multiparametric Immunophenotyping and TCR sequencing. We may attempt to use whole body T cell imaging and tracking technologies (i.e. Immuno-PET) and barcoding techniques to better understand the fate of T cells, and whether costimulation independent rejection is marked by dynamic loss of CD28 in NHPs (247, 248).

Signal 3 cytokines may drive the loss of CD28, and provide the necessary activation for costimulation independent cell subsets that allow for allograft rejection. Our studies indicate that interrupting Signal 3, either by blocking CD122 or CD127, mitigates costimulation independent responses. Future studies utilizing inducible knockout systems will allow us to better understand when and in which cells these signals act to promote rejection. Tools such as tamoxifen inducible knockout systems will give us increased temporal specificity which may help define the role of these cytokine systems more clearly. The issue with knockouts, especially with cytokine systems such as IL-7, but likely others, is that we cannot be so sure of the developmental issues. Inducible knockouts do not solve that worry entirely, but provide another piece of evidence and an added level of

sophistication that may help in defining the precise role of Signal 3 cytokines. In addition to the added temporal control these studies will offer, we will pursue non-invasive intravital imaging techniques, such as Immuno-PET to define the trafficking pattern of these cells. Together we can build better spatial and temporal maps that can help us define costimulation independence, and the role of Signal 3 cytokines. The focus of this project has been on the IL-2/IL-15 and IL-7 cytokine systems. Much work is still to be done to define the contributions of these cytokines (where, when, in which cells, and how). Yet, recent data in our lab has indicated that other cytokine families may be instrumental or at least influential in supporting allograft rejection, and in particular costimulation independent rejection. The scope of this project continues to grow to include investigation of alternative inflammatory cytokines, and their contributions as Signal 3 cytokines.

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