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Opposing Roles of Interferon Gamma in Transplantation

Under Costimulation Blockade

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

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Under Costimulation Blockade

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B.S., Auburn University, 1998

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Abstract

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During the last fifty years, organ transplantation has become a favored treatment option for patients with otherwise incurable conditions. Nevertheless, the immune response to donor tissue remains the major obstacle to long-term graft survival. Prolongation of allograft survival through T cell costimulation blockade (CoB) is based on the principle that T cell receptor stimulation in the absence of costimulation leads to abortive activation or deletion of T cells. However, resistance to CoB-induced allograft survival is seen in some mouse models. CD8 T cells are necessary for this resistance, but the mechanism by which CD8 T cells mediate rejection in the absence of major costimulatory signals is poorly understood. Interferon-gamma (IFNγ) promotes CD8 T cell responses, but IFNγ-deficient mice show early graft loss despite CoB. In contrast, we found that IFNγ receptor-deficient mice show dramatically prolonged graft survival under CoB. Investigating this paradox, we addressed the effects of IFNγ on T cell alloresponses *in vivo* independent of the effects of IFNγ on graft survival. We found that neither IFNγ receptor-deficient recipients nor IFNγ-deficient recipients mount anti-graft CD8 T cell responses. To explain graft loss despite undetectable T cell responses in IFNγ-deficient recipients, we found that direct action of IFNγ on the graft was necessary for graft survival, as either IFNγ neutralization in IFNγ receptor-deficient recipients or the lack of the IFNγ receptor on the graft precipitated early graft loss.

Furthermore, IFNγ decreased cellular infiltrates and hemorrhage and increased transcripts for tolerance-associated molecules within grafts at early time points. In contrast to other models, NK cells were dispensable but CD4+ cells were necessary for graft maintenance. At late time points, IFNγ held in check a dormant immune response, as IFNγ neutralization not only precipitated graft loss but also led to increased transcripts for cell-mediated response genes within graft-draining lymph nodes. Importantly, late-term control via IFNγ was by direct action on graft-derived cells and by indirect action on the recipient immune system. Thus, IFNγ is required both for the recipient to mount a donor-specific CD8 T cell response under CoB as well as for allografts to initiate and maintain survival after transplantation.

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http://www.jimmunol.org/cgi/content/full/174/3/1269

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Wo aber Gefahr ist, waechst das Rettende auch.

But where Danger is, there the Rescuing grows as well.

-- Friedrich Hölderlin, “Patmos,” 1802.

Introduction

Nearly 30,000 Americans received transplanted organs last year, bringing the ten-year total number of transplantations in this country to over 440 thousand, based on Organ Procurement and Transplantation Network (OPTN) data as of October 2008. Since the first successful human solid organ transplant in December 1954, transplantation has become a realistic, life-saving option for patients with otherwise incurable conditions. Though improved surgical techniques, innovations in donor organ care, and novel immunosuppressive, antibiotic and antiviral drugs have improved the success rate of transplanted organs, the five-year survival of kidney grafts, from deceased donors, for example, remains wanting at 70%. The major obstacle to transplantation remains the recipient's immune response to grafted tissue.

Activation of an Immune Response

To initiate an immune response, T cells and professional antigen presenting cells (APC) interact in the specialized microenvironment of the lymph node (1). Professional APCs are important in the initiation of an immune response because they can be activated to express high levels of MHC molecules, to cross-present exogenous antigens to activate CD8+ T cells, and to express high levels of costimulatory B7 molecules (CD80 and CD86) necessary to activate naïve T cells (2, 3). The activation of naïve T cells by APC is broken down into two or three parts; Signal One, Signal Two, and sometimes Signal Three are considered necessary for full T cell activation. Signal One consists of TCR signals received when the T cell encounters its cognate MHC-peptide complex and initiates the first rounds of cell division and gene transcription. Signal Two consists of costimulatory signals sent

when cell surface molecules on the T cell are ligated by molecules on the surface of the APC and affords the T cell various survival mechanisms such as increased expression of growth factor receptors and transporters as well as increased expression of anti-apoptotic molecules (4, 5). Signal Two interactions are many, but the best-studied are those between CD28 on the T cell and B7 molecules on the APC, CD154/CD40, LFA-1/ICAM, CD2/LFA-3 as well as others of increasing importance, sometimes called "alternative costimulatory molecules," such as 4-1BB, ICOS and OX40 (6). Signal Three is sometimes considered as separate from Signal Two in terms of the activation of naïve CD8+ T cells and consists of soluble factors, such as IL-2, which act on the T cell during activation and which are necessary for acquisition of full effector function (7).

In many cases, the activation of CD4+ T cells is necessary for the full activation and differentiation of naïve CD8+ T cells to cytotoxic effector CD8+ T cells. The concept of CD4+ T cell "licensing" of APC to activate naïve CD8+ T cells has been proposed in which CD154 on the CD4+ T cell which has bound its cognate peptide-MHC Class II complex on an APC also binds CD40 on the APC, inducing its maturation, so that the presenting cell is now fully potent to activate naïve CD8+ T cells which interact with their cognate peptide-MHC Class I complexes on the surface of the newly "conditioned" APC (8-10). This conditioning was shown to be dependent on CD40 signaling in the APC, as blocking CD154/CD40 interaction ablated the effect and stimulation of CD40 with agonist antibodies in the absence of CD4+ T cells was sufficient to recapitulate the effect (8-10). In transplantation, the initial generation of effector CD8+ T cells is the key step to target in the attempt to prevent acute graft rejection.

Clinical Methods to Prevent Graft Rejection: Immunosuppression

In the 1950s, early attempts to avoid immune rejection of transplanted organs relied on generalized immunosuppression by way of sublethal doses of whole body irradiation (WBI). By the 1960s, anti-cancer drugs such as Methotrexate, Azathioaprine, Cyclophosphamide and Actinomycin C were used in place of WBI, still with the idea of inhibiting proliferation of donor-reactive cells but with the added benefit that the effects of the drugs could be reversible with drug withdrawal as opposed to the irreversible effects of WBI (11). However, toxicity due to non-specific inhibition of cell division of all cell types was a major concern, and bone marrow suppression after treatment with the anti-neoplastic drugs led to serious increases in infection (12). Also in the 1960s, the adjunct use of corticosteroids with anti-cancer drugs became popular, with higher doses of corticosteroids used immediately after transplant then reduced with the option to increase the dose again in the event of a rejection crisis (11). The side effects of high dose corticosteroids are serious, but because of their relative efficacy, their use in clinical prevention of graft rejection reigned until the late 1970s (11). In 1976, Borel *et al* published the first report of the effects of Cyclosporine A (CSA) in suppressing rejection of skin grafts in mice (13). CSA had the advantage that it could inhibit T cell responses at low doses and with a decreased risk of subsequent infection as compared to azathioprine, although higher doses of CSA led to bone marrow suppression (11). By the 1980s, CSA was accepted as a standard clinical therapy for preventing rejection of organ allografts. A decade later, another drug was accepted into clinical practice, FK506 (Tacrolimus), which was found to work by the same general mechanism as CSA in that it could inhibit early events in T cell activation through calcineurin inhibition, but FK506 showed better clinical efficacy than CSA. Rapamycin was soon to be added to this growing list of drugs which target activation of T cells, and these drugs were recognized for their advantage of specificity of action relative to the other

drugs developed around the same time which target all lymphocytes (mycophenylate mofetil) or all T cells, naïve or activated (OKT3). However, rapamycin and the calcineurin inhibitors, like CSA and FK506, show potent toxicities (such as systemic hypertension, neurotoxicity, nephrotoxicity, myocardial hypertrophy, hypercholesterolemia, hyperlipidemia and delayed wound healing), and all of the immunosuppressive drugs have drawbacks. As they are immunosuppressive by design, they increase the patient's risk for infection. Furthermore, lifelong use is not only expensive but can lead to organ damage with chronic use. Most importantly, however, is the fact that none of these drugs allow for acceptance of the graft after cessation of drug delivery. In other words, they do not induce donor-specific immune tolerance. There is a need for more-specific, rationally designed drugs to prevent allograft rejection – drugs with minimal toxicity and without life-long immunosuppression.

Costimulation Blockade to Induce Graft-specific Peripheral Tolerance

With the drawbacks of current immunosuppressive regimens to prevent transplant rejection in mind, physicians and scientists searched for more refined ways to target specifically the rejection response while sparing the competence of the host immune system to fight infection as well as preserving the integrity of other organ systems. In the mid-1990's, scientists demonstrated in mice that the rejection response could be controlled with biomolecular drugs designed to block the costimulation of T cells (14). This approach is advantageous because only the T cells activated by TCR stimulation at the time of costimulation blockade are affected – specifically targeting for anergy or deletion the graft-responsive T cells while sparing the remainder of the T cell repertoire.

Costimulation blockade as a way to induce peripheral tolerance was designed along the principle that TCR stimulation without costimulation can lead to anergy, death or deletion of the T cell (15, 16). CD28 and CD40 are two major costimulatory receptors important to the initiation of effective T cell responses, and blocking these pathways can induce profound suppression of primary immune responses. To block the CD28 and CD40 pathways, two biomolecular drugs are typically used. CTLA-4:Ig is a fusion protein of a soluble form of human CTLA-4 and the human IgG_1 F_c region, which confers an increased serum half-life over that of the soluble CTLA-4 molecule alone. CTLA-4:Ig binds to B7 molecules (B7.1 and B7.2, CD80 and CD86, respectively), blocking their potential interaction with CD28 molecules on T cells. MR1 is a monoclonal antibody (Armenian Hamster IgG) with specificity for CD154 such that the interaction of CD154 on T cells and CD40 on APC is prevented. The lack of CD40 ligation on the APC works to keep the APC in the immature state, as CD40 signaling in the APC leads to maturation, which leads to more efficient activation of naïve T cells.

Blockade of CD28 and CD154 via CTLA-4:Ig and MR1, respectively, can effectively lead to long-term survival of fully allogeneic skin and heart grafts in some murine models (14). However, the C57BL/6 mouse receiving a BALB/c skin graft is particularly resistant to the effects of combined blockade of CD28 and CD154, and the rejection phenotype observed in this setting is termed “costimulation blockade-resistant rejection” (CoBRR). In this model of CoBRR, CD8+ T cells are necessary for mediating graft rejection, as depletion of CD8+ T cells alone but not depletion of CD4+ T cells alone leads to prolonged allograft survival (17-19).

Some possible reasons why combined blockade of CD28 and CD40 costimulatory pathways fails in different models of transplantation are the presence of cross-reactive memory T cells which may be present at an increased precursor frequency or have a decreased requirement for CD28 signals for activation, the

strength of the TCR signal to CD8+ T cells during stimulation with allogeneic MHC (direct presentation) as well as the generally-observed phenomenon that barrier tissues like skin show increased immunogenicity compared to vascular, solid organ grafts (20). In addition, alternative costimulatory molecules can provide survival or proliferative signals in the absence of CD28 signaling which aid expansion of effector T cell populations, as additional blockade of some of these molecules (ICOS, OX40, 4-1BB) has been shown to prolong allograft survival (21-23).

Interestingly, in some cases, CD8 T cells have been shown to be able to mount an immune response in the absence of CD4 T cells. Studies in viral infection models have shown that CD8+ T cells activated without CD4+ T cell help ("helpless CD8+ T cells") are competent to clear the infection (24). Later studies showed these helpless CD8+ T cells to lack the normally-seen robust recall responses from the ensuing CD8+ memory T cell population (25). In the transplant setting, however, where the initial CD8+ T cell effector response is sufficient to mediate acute graft destruction, the lack of robust recall responses from newly-generated allospecific CD8+ memory cells may be irrelevant.

Furthermore, as we discovered through preliminary experiments and explored throughout the work that follows, interferon gamma (IFNγ) may be an important factor contributing to rejection of allografts seen despite blockade of CD28 and CD40 costimulatory pathways. In the following section, we summarize what is known about IFNγ in terms of its effects on T cell responses, both CD4 and CD8, and its roles in transplantation outcomes and in peripheral tolerance mechanisms.

Interferon Gamma

IFNγ is member of a family of cytokines first described for their ability to confer anti-viral protection to cells (26). This cytokine is expressed by T cells, NK cells and

DC and acts on most cells of the body with pleiotropic effects (27, 28). Functioning as a homodimer (29), IFNγ binds to a heterodimeric receptor expressed ubiquitously and composed of a ligand-binding chain, IFNγR1, and a second chain, IFNγR2, necessary for signal transduction (27). A plethora of genes are known to be upregulated by IFNγ stimulation, and the classical effects attributed to IFNγ are increased efficiency of antigen processing and presentation, upregulation of MHC expression, induction of expression of nitric oxide and microbicidal molecules in macrophages, regulating the production of other cytokines both immunomodulatory and proinflammatory, antibody class switching, polarization of T helper cell responses and regulating leukocyte-endothelium interactions (27, 30). The major signaling pathway used by the IFNγR is the JAK-STAT pathway, with JAK1 and signals JAK2 activating STAT-1 by tyrosine phosphorylation, leading to formation of STAT-1 homodimers which initiate gene transcription at GAS (IFNγ-activated site) sequences (31). Other pathways are involved as well, centering around serine phosphorylation of STAT-1, such as the phosphotidylinositol-3-kinase (PI3K) pathway, and downstream pathways such as those involving Akt, mammalian target of Rapamycin (mTOR) and protein kinase C (PKC) isoforms, and it has been postulated that the selective use of pathways downstream of PI3K may explain the pleiotropic effects of IFNγ (31, 32).

Influence of IFN gamma on T cell responses

The action of IFNγ on T cells has been traditionally thought of as anti-proliferative. Part of this thought was based on the study of Th1/Th2 polarization and the role of IFNγ in this process. Recently, IFNγ was shown to have a pro-apoptotic effect on CD4+ T cells. Experiments focusing on the action of IFNγ on CD8+ T cells are

fewer and show some different results from the experiments looking at the impact of IFNγ on CD4+ T cells.

IFNγ inhibits CD4+ T cell responses

In 1988, Gajewski and Fitch found that Th2 clones showed decreased proliferation *in vitro* when IFNγ was present (33). On the other hand, proliferation of Th1 clones *in vitro* was not affected by the presence of IFNγ (33). Almost ten years later, Pernis *et al* showed that the ligand-binding chain (IFNγR1) and the signaling chain (IFNγR2) are both expressed by Th2 cells, but the signaling chain of the IFNγR is not expressed by Th1 cells (34). Furthermore, Th1 cells are not responsive to IFNγ as shown by lack of STAT-1 activation or expression of genes activated by IFNγ stimulation (34). Later, Tau *et al* showed that expression of the signaling chain of the IFNγR determines responsiveness of cells to IFNγ (35). They created IFNγR2 transgenic mice where expression of the signaling chain of the IFNγR is under the control of the CD2 promoter and enhancer so that all T cells in this mouse constitutively express IFNγR2. *In vitro* and *in vivo*, Th1 cell differentiation and Th1 type responses were impaired. The authors concluded that naïve CD4+ T cells must become unresponsive to IFNγ in order to develop into Th1 cells capable of generating Th1 polarized immune responses.

Later, Refaeli *et al* showed not an anti-proliferative effect but a pro-apoptotic effect of IFNγ on CD4+ T cells (36). In their experiments, $IFN\gamma^{-/-}$ CD4+ T cells stimulated *in vitro* show more expansion, less apoptosis and the same amount of proliferation when compared to wild-type (WT) CD4+ T cells. The authors concluded that IFNγ has a pro-apoptotic effect on activated CD4+ T cells (36).

Actions of Interferon Gamma on CD8+ T cell responses

Conflicting reports on the effects of IFNγ on CD8+ T cells also exist. Several studies focusing on the role of IFNγ in generation of effector CD8+ T cell populations have found different results in different systems, results ranging from a negative effect, to no effect, to a clearly positive effect of IFNγ on cytotoxic T lymphocyte (CTL) generation. Bucy *et al* showed that in an *in vitro* mixed leukocyte reaction, IFNγ is not necessary for generation of CTL (37). They confirm the necessity of IL-2 for CTL activation and proliferation, but reject the idea that IFNγ is important for these functions (37). More recently, Hildago *et al* showed that IFNγ acts indirectly to decrease production of CD8+ effector cells, as seen through *in vitro* experiments using mixed leukocyte cultures (MLC) of stimulators and responders differing in their ability to produce or respond to IFNγ (38). Interestingly, in an experiment where IFNγR1$^{-/-}$ B6 splenocytes were stimulated with WT BALB/c splenocytes, they saw increased CTL activity relative to the same type of stimulation of WT B6 splenocytes. When purified T cells were used in the culture system instead of total splenocytes, their results were not as extreme, though the same trend was seen. The authors concluded that IFNγ suppresses generation of CTL by a negative feedback loop where IFNγ acts on directly on CD4+ T cells to limit their production of IL-2, the addition of which was shown to increase the generation of effector CD8+ T cells (38). They also note that while IFNγ is necessary for activation induced cell death (AICD) of CD4+ T cells (36), little is known about how the life and death of CTL are affected by the lack of IFNγ (38).

Recent studies have shown that IFNγ signals in T cells activate PKC theta (PKC θ), a kinase also activated downstream of TCR signals and culminating in

activation of Jnk, Jun, and AP-1 transcription factors (32, 39). PKC θ also has been shown to synergize with calcineurin, activate NFκB, and importantly, to recapitulate CD28 signals by providing an alternate way to activate AP-1 (39). As activation of PKC θ in CD8+ T cells was shown to be more important for cell survival than for proliferation(25), activation of PKC θ via IFNγ signaling could be one way that donor-reactive CD8+ T cells can generate active AP-1 to continue their programmed response after TCR stimulation in the absence of CD28 signals, resulting in CoBRR.

To try to understand the seemingly opposing results surrounding the effects of IFNγ, Bernebei *et al* investigated the mechanism behind which IFNγ induces either apoptosis or proliferation in T cells (40). The authors looked at levels of expression of IFNγR2 on surface of human T cells and how this correlated with the kinetics of STAT-1 activation and the final cellular outcome of either apoptosis or proliferation. They detected IFNγR1 expression on normal T cells from human peripheral blood mononuclear cells, but found very little expression of IFNγR2 on these cells. Through *in vitro* experiments looking at the relationship between IFNγR2 expression levels on human T cell lines and the action of IFNγ on these cells, the authors concluded that higher levels of IFNγR2 expression result in swift activation of STAT-1 and trigger apoptosis, whereas low levels of IFNγR2 expression result in delayed activation of STAT-1 and trigger proliferation (40). Interestingly, they also found that IFNγR2 expression is upregulated on human malignant T cell lines after serum starvation and that in this setting IFNγ stimulation leads to apoptosis. However, when the same T cell lines were cultured with serum and stimulated with IFNγ, proliferation was seen (40).

Interferon gamma can both augment and suppress T cell responses in vivo

Using a novel technique of dual adoptive transfer of WT and IFNγR$^{-/-}$ cells into WT mice subsequently infected with lymphocytic choriomeningitis virus (LCMV), Whitmire *et al* studied the direct effects of IFNγ on responding CD8+ T cell populations (41). In their model, CD8+ T cells lacking a functional IFNγR do not accumulate to the extent that WT CD8+ T cells do. Although the number of IFNγR$^{-/-}$ CD8+ T cells was less than the number of WT CD8+ T cells at the peak of infection, the same percentages of each CD8+ T cell population displayed activated and effector phenotypes (41). Lohman and Welsh had already found that IFNγR$^{-/-}$ mice are immunocompetent to clear LCMV infection and, in fact, display hyperproliferation in the T cell compartment due to a decreased susceptibility to apoptosis after activation (42). However, delayed viral clearance was seen along with delayed contraction of the T cell pool (42). he results from Lohman's experiments might be reconciled with those of Whitmire by a situation of uncontrolled CD4+ T cell expansion alongside very little increase in CD8+ population size, with the delayed clearance of virus possibly due to a slow rate of expansion of IFNγR$^{-/-}$ effector CD8+ T cell population perhaps due to defects in survival of proliferating CD8+ T cells unable to respond to IFNγ.

On the other hand, while WT mice exhibit a transient lymphopenia immediately after acute LCMV infection, IFNγR$^{-/-}$ mice infected with LCMV show no transient lymphopenia, suggesting that IFNγ may be acting to suppress lymphocytes in the early stages of acute infection though this result could also be brought about by differences in redistribution of cells under the influence of IFNγ (42). Rodriguez *et al* found that IFNγ$^{-/-}$ mice do not show immunodominance of CD8+ T cell clones normally seen after LCMV infection (43). Without IFNγ,

subdominant epitopes appear alongside dominant epitopes, and a single dominant CD8+ T cell response which normally can suppress other CD8+ T cell responses to LCMV cannot do so in the absence of IFNγ expression (43). The authors postulate that IFNγ produced from the antigen-responding CD8+ T cell acts on the APC to suppress other responses (43).

Looking at the impact of the lack of IFNγ on CD8+ T cell expansion in a transplant model, Sayegh's group studied rejection kinetics of MHC-mismatched and minor-mismatched grafts on IFNγ$^{-/-}$ recipients treated with CD154 blockade (44). They found that IFNγ was necessary for tolerance to fully-allogeneic transplants, but is dispensible for induction of tolerance to grafts expressing only multiple minor antigenic differences in the presence of CD154 blockade (44). MHC mismatched hearts into IFNγ$^{-/-}$ mice either left untreated or treated with CD154 blockade rejected at the same early time point as untreated WT recipients. On the other hand, when minor-mismatched hearts were transplanted into IFNγ$^{-/-}$ mice, 50% of recipients receiving CD154 blockade showed long-term surviving grafts (>150d) (44). Their work suggested that the CoBRR seen in IFNγ$^{-/-}$ recipients of MHC-mismatched grafts was due to an increased CD8+ T cell burst size after direct presentation as compared to that occurring with indirectly presented donor antigen in the case of minor-mismatched grafts which were easily accepted following CD154 blockade in IFNγ$^{-/-}$ recipients (44).

Influence of Interferon Gamma on Transplant Outcomes

Koniezsky *et al* observed that allografts placed on recipients deficient in IFNγ are swiftly lost, and this loss could not be prevented with costimulation blockade (45). Furthermore, IFNγ neutralization in mice attenuates the graft survival induced by

combined blockade of CD28 and CD154 (45). These results led to the belief that IFNγ may have tolerogenic properties in some situations.

In line with the proposed protective role for IFNγ in tissue transplantation is the observation that vascularized allografts undergo early necrosis in the absence of IFNγ (46). The authors postulated that large amounts of IFNγ produced within the allograft acts on graft tissue to prevent necrosis during the initial stages of immune rejection. Furthermore, spontaneous acceptance of liver allografts is dependent on the action of IFNγ on the graft, as IFNγR-/- livers showed also showed early necrosis and could not survive in WT hosts while WT livers were spontaneously accepted (47). The same early necrosis was seen in kidney allografts in the absence of IFNγ (48). Kidney grafts in the presence of IFNγ in untreated recipients showed no necrosis even up to day 21, though rejection of grafts eventually occurred. These experiments highlighted the importance of IFNγ in preventing early necrosis of transplanted tissue in untreated recipients.

Mechanisms of Tolerance involving Interferon Gamma

Several tolerance mechanisms are dependent on or are associated with the presence of IFNγ. The timing of IFNγ expression within allografts is an important predictor of survival or eventual rejection of the graft (49-51). IFNγ production from recipient cells, such as natural killer cells (NK), regulatory T cells (Treg), and even CD8+ T cells have been implicated in donor-specific acceptance of allografts (52-56). In order to induce functional regulatory T cells, tolerogenic dendritic cells (DC) must be able to respond to IFNγ, and IFNγ is involved in upregulation in DC the expression of indoleamine-2,3-dioxygenase (IDO), an enzyme which functions in suppression of T effector cell responses (57-60).

To begin our study of the mechanisms of costimulation blockade-induced prolongation of graft survival and the pathways of costimulation blockade-resistant rejection, we sought to recreate in our hands the basic findings from the field of transplantation under costimulation blockade. Through these experiments, we uncovered an unusual finding, a paradoxical result leading to conclusions in stark contrast to ideas in the published literature. Near the end of Chapter One, we show that recipients lacking the ability to respond to interferon gamma and those lacking the ability to produce IFNγ show dramatically different kinetics of graft rejection under costimulation blockade. This apparent contradiction led us to ask many more questions about the role of IFNγ in transplantation under costimulation blockade, and so became the seed that grew the subsequent studies published in this work.

In Chapter Two, we explore the anti-donor immune response mounted within WT, IFNγ-deficient and IFNγR-deficient recipients under CoB. We found find that recipients treated with CoB and lacking IFNγ signals, whether by lack of the cytokine or lack of the cytokine receptor, show decreased CD8 effector T cell responses relative to WT recipients under CoB. We go on to show that graft loss on IFNγ-deficient recipients is due to the lack of IFNγ action on the graft itself.

In Chapter Three we investigate further the mechanism of direct IFNγ action on the graft to preserve and prolong its survival. We found that IFNγ prevents early damage to the graft evident even under costimulation blockade and that the action of the cytokine on the graft-derived cells increases transcripts for tolerogenic molecules within the graft tissue. Furthermore, we show that at late time points, IFNγ is still required for graft maintenance, as well as CD4+ cells.

When IFNγ is removed from the system, long-survived grafts are lost and transcripts for cell-mediated response genes are upregulated in graft-draining lymph nodes. These results indicate that IFNγ not only provides important integrity-sustaining signals to tissue at early time points after transplantation when the inflammatory milieu is thick but also provides necessary signals to graft-derived cells allowing them to hold at bay a capable recipient immune response once the graft bed is quiescent and costimulation blockade is no longer present.

Chapter One

Costimulation Blockade: Successes & Shortcomings in Prolonging Graft Survival

Figure 1.1 *Graft survival is prolonged by blockade of CD28 & CD40 signals* 27

Figure 1.2 *CD28 / CD40 blockade impairs the immune response to allografts* 29

Figure 1.3 *Synergy is found through blockade of additional costimulatory signals* 31

Figure 1.4 *IFN-gamma Receptor-deficient mice are similar to wild-type mice* 33

Over the last half-century, organ transplantation has become a life-saving treatment option for patients with otherwise incurable conditions. Nevertheless, the immune response to donor tissue remains the major obstacle to long-term graft survival. Prolongation of allograft survival through T cell costimulation blockade (CoB) is based on the principle that T cell receptor stimulation in the absence of costimulation leads to abortive activation or deletion of T cells (15, 16). CD28 and CD40 are two major costimulatory receptors important to the initiation of effective T cell responses, and blocking the ligands for these receptors with CTLA4-Ig and monoclonal antibodies specific for CD154, respectively, induce profound suppression of primary immune responses (61, 62).

Graft survival is prolonged by blockade of CD28 & CD40 signals

Combined blockade of CD28 and CD40 signals with CTLA-4Ig and MR1 is effective in allowing for prolonged survival of grafts differing from the recipient in only minor histocompatibility antigens. Skin grafts from donor mice on the B6 background but expressing membrane bound chicken ovalbumin under the control of the actin promoter (mOVA) were placed on B6 recipients with or without treatment with CTLA-4Ig and MR1. As shown in figure 1.1A, mOVA skin graft survival was significantly prolonged on treated recipients (MST >250d) compared with untreated recipients (MST 14d, p=0.008). However, when grafts from donors differing from the recipient not only in minor antigens but also in major histocompatibility complex (MHC) antigens, combined CD28 and CD40 blockade was not as effective in prolonging graft survival (fig. 1.1B). Survival of skin grafts from BALB/c (H-2^d) donors was modestly but significantly prolonged on B6 recipients (H-2^b) treated with CTLA-4Ig and MR1 (MST 31d) compared with untreated B6 recipients (MST 13d, p=0.001). That combined treatment with CTLA-4Ig and MR1

can prolong survival of single-antigen mismatched skin grafts greater than 100 days and can double the survival time of fully-MHC mismatched skin grafts on B6 recipients suggests that this treatment may be dampening the immune response to the graft. Though the success of costimulation blockade in the minor-mismatch setting is impressive, this setting is not fully representative of the clinical transplantation setting where not only multiple minor antigens differ between donor and recipient but MHC are often different as well. Therefore, our subsequent studies focused on the model of the B6 response to fully-allogeneic grafts from BALB/c recipients.

CD28 / CD40 blockade impairs the immune response to allografts

As an initial look into how costimulation blockade affects the immune response to fully-allogeneic tissue transplantation, we analyzed systemic levels of cytokines (IFNγ, IL-2, IL-4, IL-5 and TNF) in the serum of graft recipients with or without CTLA-4 and MR1 treatment (CoB). Importantly, CoB treatment was seen to suppress the initial burst of IFNγ and IL-2 seen at post-operative day (POD) 7 in untreated recipients (fig. 1.2A, p=0.006 for IFNγ, p=0.04 for IL-2). Over the course of the first month after BALB/c skin graft placement, serum cytokines in B6 recipients treated with CoB did not differ from levels found in naïve animals, and no differences were found between the treatment groups at any time point for IL-4, IL-5 or TNF.

To investigate the impact of CoB on T cell responses, we harvested splenocytes from recipients of BALB/c skin grafts at weekly time points over the course of the first month after transplantation and analyzed these cells by flow cytometry for total number of activated T cells. As illustrated by figure 1.2B, transplantation of BALB/c skin led to an increase in total number of activated

(CD44^{hi}) T cells in the spleen in the first two weeks after graft placement, with an increase in activated CD4 T cells seen on POD 7 and POD 14 before returning to naïve levels, and with an increase in total number of activated CD8 T cells evident by POD 14. CoB was able to prevent these increases in total number of activated T cells in the spleen, as CoB-treated recipients harbored a reduced total number of activated CD4 T cells (POD 7, p=0.02; POD 14, p=0.01) and activated CD8 T cells (POD 14, p=0.003) in the spleen compared with untreated recipients.

To address in another way the question of how CoB affects the immune response to transplanted tissue, we analyzed graft-draining lymph nodes at POD 7 for evidence of T cell priming and differentiation. By real-time PCR (RT-PCR), we found that the relative quantity of mRNA for the CD8 T cell effector molecule, granzyme B, and for the Th1-associated transcription factor, T-bet, were significantly reduced when the recipient was treated with CoB (fig. 1.2C, p=0.05 by one-tailed Mann Whitney test, comparison with untreated recipients).

Furthermore, as figure 1.2D shows, the total number of donor-specific effector CD4 T cells was reduced with CoB as compared to that found in untreated recipients. We defined donor-specific effector T cells as those capable of producing both IFNγ and TNF after short-term *ex vivo* restimulation with donor splenocytes (POD 7, p=0.01; POD 14, p<0.0001; POD 21, p=0.2; POD 25, p=0.002; POD 28, p=0.03; POD 35, p=0.06).

An important effector function of CD4 T cells is the ability to promote antibody responses in B cells. As an indirect measure of CD4 effector functionality after treatment with CoB, we assayed serum from recipients treated with or without CoB for anti-donor antibodies. Serum from individual recipients was incubated with donor splenocytes and secondarily labeled with FITC anti-mouse IgG1, PerCP anti-mouse IgG2a+2b, and PE anti-mouse IgM then analyzed by flow cytometry. We found that CoB treatment reliably suppressed the development of anti-donor

antibodies, as the levels in treated recipients did not differ from naïve levels throughout the first month and showed a significant reduction relative to levels in untreated recipients (fig. 1.2E).

Synergy is found through blockade of additional costimulatory signals

Though treatment with CTLA-4Ig and MR1 to block the CD28 and CD40 pathways can prolong graft survival, eventual rejection of BALB/c skin grafts does occur in B6 recipients. Blockade of additional costimulatory signals may lengthen graft survival times and might point to mechanisms of costimulation blockade-resistant rejection seen with blockade of simply CD28 and CD40 pathways. As OX40 / OX40L interactions have been shown to enhance the survival of T cells undergoing an immune response (63), we added anti-OX40L mAb (RM-134L) to the standard treatment regimen of CTLA-4Ig and MR1 or substituted OX40 knockout ($OX40^{-/-}$) recipients for B6 recipients. As shown in figure 1.3A, prolonged BALB/c skin graft survival was seen on CoB-treated B6 background recipients (median graft survival time, MST, 28 days, d) with further interruption of OX40/OX40L interactions either by mAb to OX40L (MST 73d) or by genetic deficiency in OX40 (MST 64d).

Blockade of CD40 with anti-CD154 mAb, MR1, is one strategy to prevent maturation of APC, so to prevent subsequent efficient costimulation of naïve T cells. However, IFNγ signaling in APC can also lead to a state of maturation with upregulation of costimulatory molecules (27, 64). As shown in figure 1.3B, when recipients are not only blocked from receiving signals from the CD28 and CD40 pathways of costimulation but also lacked the ability to receive IFNγ signals, grafts survive dramatically longer (MST 85d) than when the ability to receive IFNγ signals in the recipient remained intact (MST 31d, p=0.002).

IFN-gamma Receptor-deficient mice are similar to wild-type mice

As IFNγR-deficient animals might have a reduced capacity to develop cell-mediated responses due to the important role IFNγ is known to play in resolution of viral infections (27), we investigated whether or not IFNγR knockout (GRKO) animals are competent to reject BALB/c skin grafts without treatment. As shown in figure 1.4A, untreated GRKO recipients reject grafts with the same kinetics as normal B6 (WT) recipients (GRKO MST 14d; WT MST 13d, p=0.13).

In addition, GRKO mice may have altered cell populations relative to WT mice that might explain the permissiveness of GRKO recipients to long-term graft survival under CoB, for example fewer CD8 T cells which are known to mediate resistance to CoB, so we investigated differences in immune cell composition in naïve GRKO and WT animals. As shown in figure 1.4B, we found the CD8 T cell compartment to be similar between GRKO and WT naïve mice, as similar numbers of CD8 T cells in the spleen and similar percentages of CD8 T cells in the lymph nodes were found. A reduced number of CD4 T cells in the spleen was evident in naïve GRKO mice (p=0.01), but the percentage of CD4 T cells in the lymph nodes was not different between GRKO and WT. Furthermore, as regulatory T cells (Treg), NK cells and NKT cells have been shown in various models of transplantation to aid graft survival (52, 53, 55), we analyzed these cellular compartments and found no differences between naïve GRKO and WT mice in terms of total number of Treg (CD4+FoxP3+) or percentage of NK or of NKT cells in the spleen.

Summary

Despite the efficacy of CoB in suppressing CD4 T cell responses in terms of expansion of activated T cells and donor-specific cytokine production, in dampening expression of genes associated with cell-mediated immune responses as well as in prevention of anti-donor antibody formation, BALB/c grafts cannot survive long-term on B6 animals. If additional costimulatory pathways are blocked, such as those allowing for increased survival of recently stimulated T cells (i.e. the OX40/OX40L pathway or direct action of IFNγ on T cells (25, 41, 65)), graft survival can be further prolonged with CoB. We found that IFNγR-deficient recipients of BALB/c skin grafts show dramatically prolonged survival under CoB and that this effect cannot be attributed to an underlying immunocompetence to reject tissue allografts or to gross deficit or overcompensation of any relevant cellular compartment in the immune system. Others have also had the idea that IFNγ might contribute to CoB-resistant rejection in B6 recipients, but when they looked at kinetics of graft rejection on IFNγ-deficient recipients they found not increased but drastically decreased graft survival times even under CoB. These contradictory results in graft outcome depending on whether the recipient is ligand-deficient or receptor-deficient spurred us onward. We hypothesized that the immune responses within these recipients must be the same whether the ligand or the receptor are missing. We next designed experiments using a novel system where either the recipient or the graft is unable to respond to IFNγ, but leaving intact the presence of the cytokine, to dissect the requirements for IFNγ signals in each party independently.

Materials & Methods

Mice: Male mice [C57BL/6J (WT), B6.129S7-*Ifngr1*^{tm1Agt}/J (GRKO) aged 6-12 weeks were purchased from The Jackson Laboratories and used as recipients. Mice expressing membrane-bound ovalbumin under the control of the actin promoter (mOVA) were originally obtained from The Jackson Laboratory, then bred in-house and used as donors. BALB/cJ (BALB/c) female mice aged 6-12 weeks, also purchased from The Jackson Laboratories, were used as donors. OX40$^{-/-}$ mice were obtained from Dr. Rafi Ahmed, Emory University, and used with permission by Dr. Nigel Killeen, University of California, San Francisco. Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

Skin Grafting: Full-thickness ear and tail skin grafts were placed bilaterally on the dorsal thorax and secured with an adhesive bandage for six days. Grafts were scored by visual inspection for signs of necrosis, and rejection was declared when less than 10% of the original graft remained viable.

Costimulation Blockade: 500 μg CTLA-4:Ig (abatacept, Bristol-Myers Squibb) and 500μg anti-CD154 monoclonal antibody (mAb), clone MR1 (BioXCell, New Hampshire), were delivered intra-peritoneally (i.p.) on post-operative day (POD) 0, 2, 4, and 6. Graft recipients not treated with CTLA-4Ig and MR1 were given isotype control reagents, human Fc IgG1 and hamster IgG (BioXCell, New Hampshire), at the same dose and schedule. OX40L blockade was via mAb (clone RM-134L, BioXCell, New Hampshire) with 500μg delivered i.p. on POD 0, 2, 4, 6.

Serum cytokine measurement: Graft recipients were bled at selected times after graft placement, and blood was allowed to clot at 4° C. Fifty μL of neat serum from each recipient was assayed by Cytometric Bead Array, Th1/Th2 kit (BD), according to kit instructions. Samples were run on a FACS Calibur, and data were acquired and analyzed using CBA software (BD).

Measuring in vivo-*generated T cell alloresponses:* At indicated time points after graft placement, recipients were euthanized, and splenocytes were isolated. CD45+, CD4+ or CD8+ T cells were enumerated by TruCount bead analysis according to manufacturer's instructions (BD Biosciences). To assess for donor-reactive T cells, 10^6 recipient splenocytes were incubated with 2×10^6 BALB/c splenocytes per well in flat-bottom 96-well plates in the presence of 1 mg/mL Brefeldin A for 5 hours at 37° C. Subsequently, cells were stained with anti-CD4 Pacific Blue, anti-CD8 Pacific Orange and anti-K^d FITC (to exclude stimulator cells), then fixed, permeabilized and stained with anti-IFNγ PE and anti-TNF APC (BD Pharmingen) according to kit instructions (eBioscience). All cells were acquired on an LSR-II flow cytometer (BD Coulter), and flow data were analyzed using FlowJo software (TreeStar).

RT-PCR. Tissues were harvested and immediately placed into 1.5 mL tubes of RNA-Later (Ambion), left for 24 hrs at RT, then placed at –80° C until RNA isolation. RNA was isolated by modified Trizol method, and cDNA were prepared with First Strand cDNA kit (Roche) using 2.5 μg RNA per sample according to kit instructions. Real time PCR was performed using inventoried TaqMan assays (ABI) for granzyme B (Assay ID: Mm00442834_m1) and T-bet (Assay ID: Mm00450960_m1) along with 100 μg "RNA" from cDNA samples. Assays were run on a 7900 thermalcycler (Applied Biosystems), and data were analyzed using RQ Manager software (SDS).

Anti-donor antibody detection. Serum from individual graft recipients was incubated with donor splenocytes and secondarily with FITC anti-mouse IgG1, PerCP anti-mouse IgG2a+2b, PE anti-mouse IgM, and APC anti-mouse CD3. Cells were acquired on a LSR-II flow cytometer (BD Coulter), and acquisition data were analyzed with FlowJo software (TreeStar) by gating first on total leukocytes by forward scatter / side scatter, then on CD3+ cells to limit background staining due to non-specific binding to Fc receptors before analysis of anti-mouse antibody staining.

Statistical Analyses: Skin graft survival data were plotted using Prism (Graph Pad), and significance was determined using the Mann-Whitney U test. T cell response data were plotted as the geometric mean ± SEM at each time point using Prism (Graph Pad), and significance was determined using the unpaired, two-tailed t-test. Transcript data from RT-PCR assays was analyzed for significant increase in the mean by one-tailed Mann Whitney test, and for significant differences in the means by two-tailed Mann Whitney test.

Figure 1.1

Graft survival is prolonged by blockade of CD28 & CD40 signals

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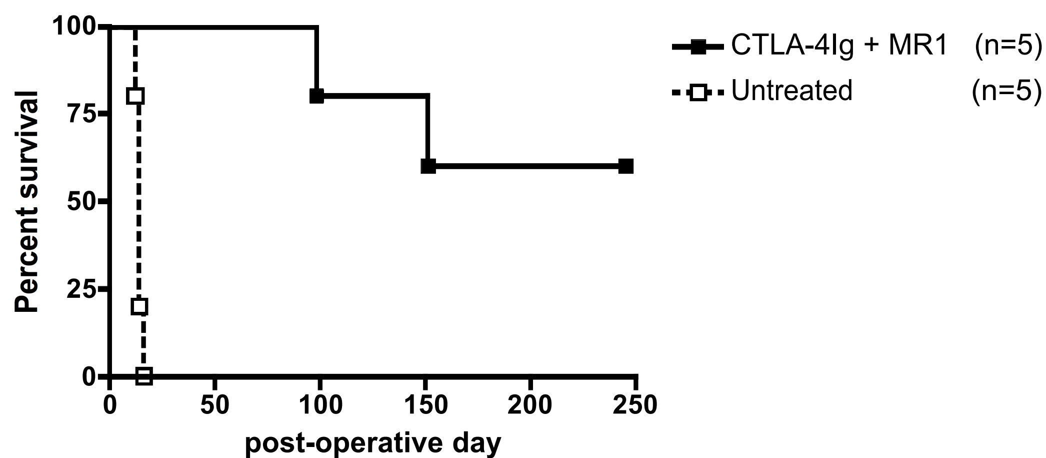

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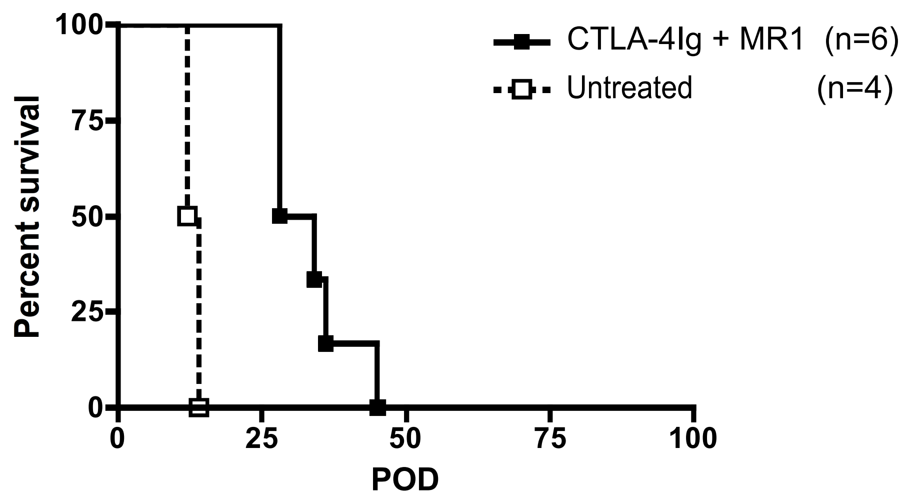

Figure 1.1

Graft survival is prolonged by blockade of CD28 & CD40 signals

A) Skin grafts from mOVA donors were placed on B6 recipients with or without treatment with CTLA-4Ig and MR1. mOVA graft survival was significantly prolonged on treated recipients (MST >250d) compared with untreated recipients (MST 14d, p=0.008). B) Skin grafts from BALB/c donors were placed on B6 recipients treated with CTLA-4Ig and MR1 or with isotype control reagents. BALB/c graft survival was modestly but significantly prolonged on recipients treated with CTLA-4Ig and MR1 (MST 31d) compared with untreated recipients (MST 13d, p=0.001).

Figure 1.2

CD28 / CD40 blockade impairs the immune response to allografts

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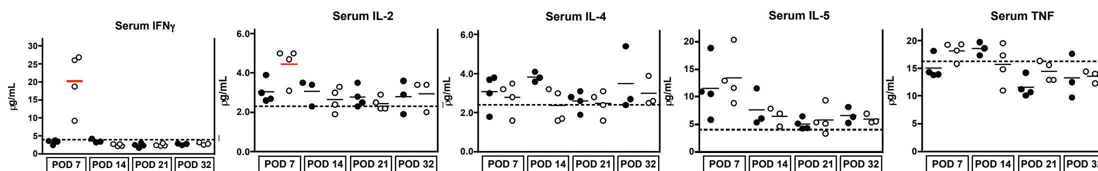

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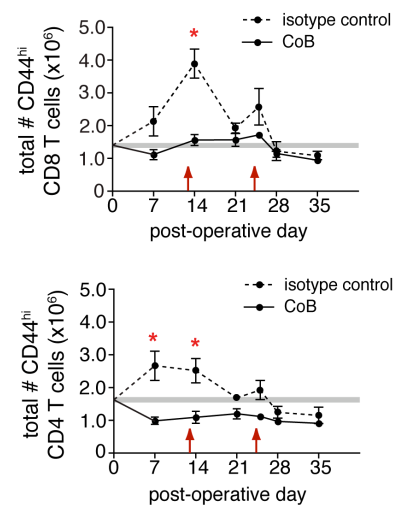

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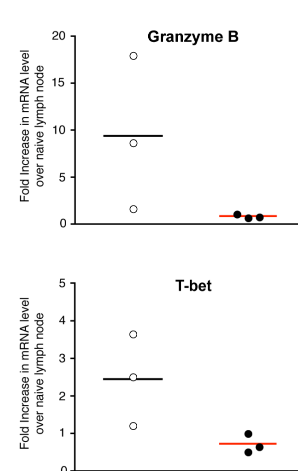

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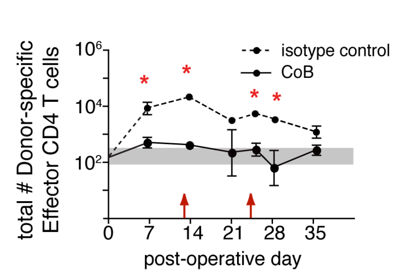

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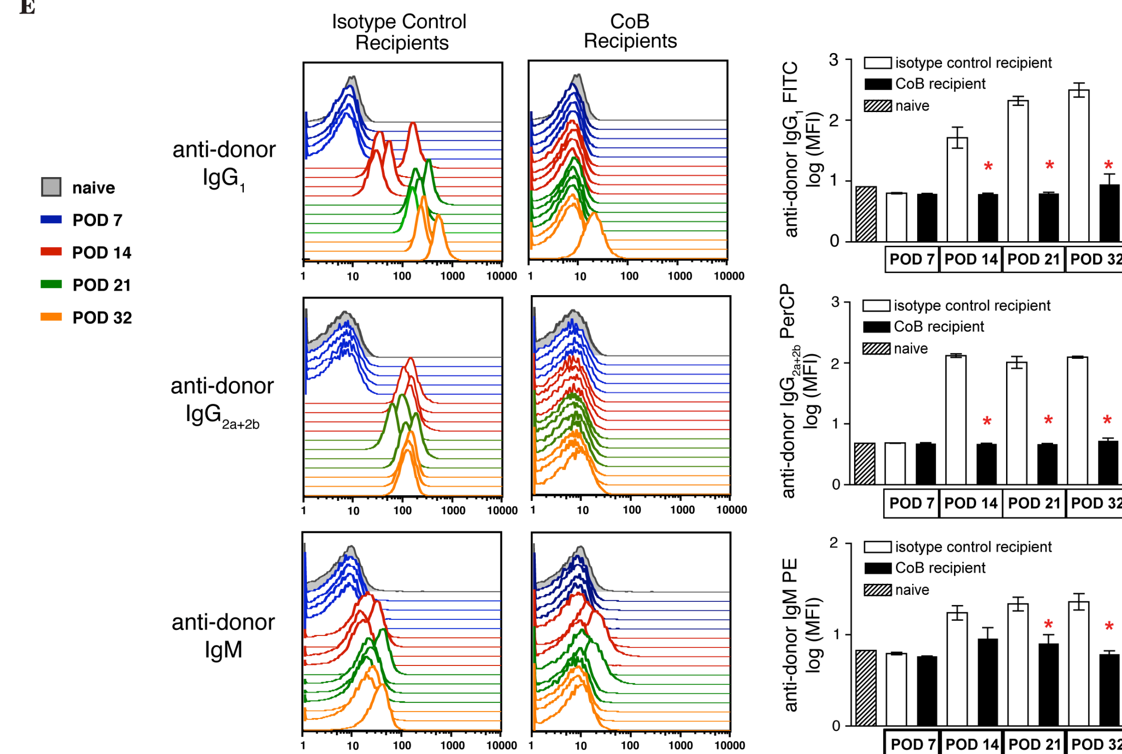

Figure 1.2

CD28 / CD40 blockade impairs the immune response to allografts

A) Serum cytokine levels in B6 recipients of BALB/c skin were lower when recipients were treated with CTLA-4Ig and MR1 (filled circles) than when left untreated (open circles) at POD 7 (p=0.006 for IFNγ, p=0.04 for IL-2). No differences were seen between treatment groups at any time point for IL-4, IL-5 or TNF. Naïve levels of each cytokine are represented by a horizontal dashed line, and data shown are of 3-4 mice per group per time point. (B & D) Splenocytes were harvested from recipients of BALB/c skin grafts (n=3-4 per group per time point) and analyzed by flow cytometry. Data from naïve, ungrafted, mice (n=24) are represented as a shaded horizontal bar indicating the mean +/- SEM. Similar results were found in four independent experiments with CoB-treated recipients. (B) Total number activated ($CD44^{hi}$) CD8 T cells (POD 14, p=0.003) and total number activated ($CD44^{hi}$) CD4 T cells (POD 7, p=0.02; POD 14, p=0.01). (C) mRNA level by TaqMan RT-PCR in skin graft-draining lymph nodes at POD 7 relative to level in lymph nodes of naïve animals (p=0.05 by one-tailed Mann Whitney U test for reduction in granzyme B and T-bet mRNA levels in recipients treated with CoB compared to untreated recipients). (D) Total number donor-specific dual cytokine-producing effector CD4 T cells (POD 7, p=0.01; POD 14, p<0.0001; POD 21, p=0.2; POD 25, p=0.002; POD 28, p=0.03; POD 35, p=0.06). (E) Anti-donor antibodies. Serum from individual recipients (n=3-4 per group per time point) was incubated with donor splenocytes and then with FITC anti-mouse IgG_1, PerCP anti-mouse IgG_{2a+2b}, and PE anti-mouse IgM. Serum antibody levels in naive, ungrafted B6 mice are indicated by gray shaded histograms. * $p<0.05$ isotype control versus CoB treatment.

Figure 1.3

Synergy is found through blockade of additional costimulatory signals

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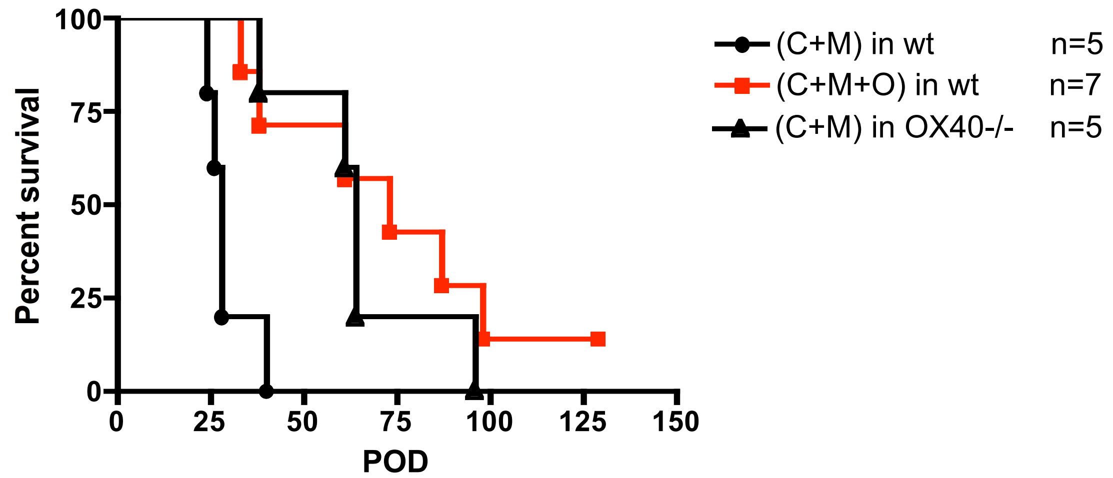

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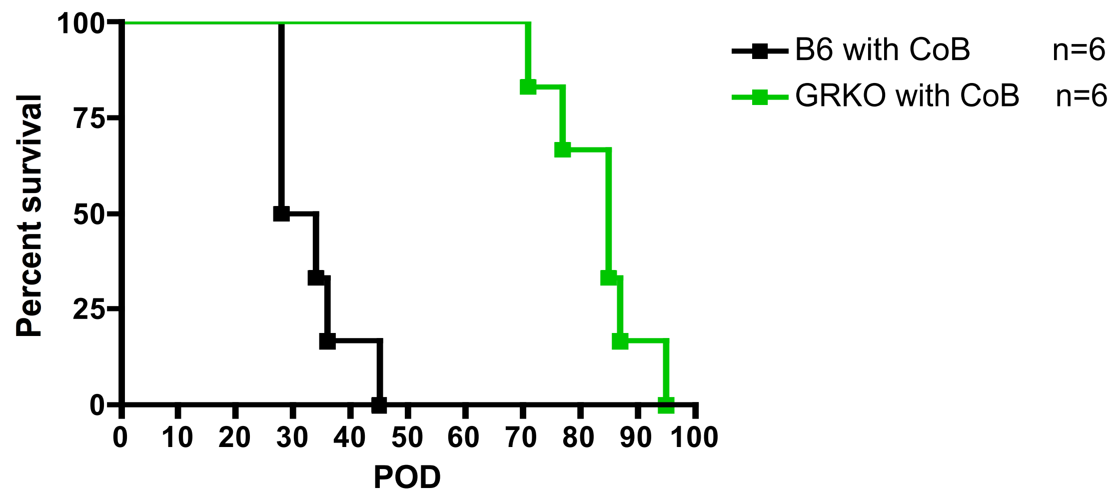

Figure 1.3

Synergy is found through blockade of additional costimulatory signals

A) B6 background mice were grafted with BALB/c skin and treated with CTLA-4Ig and MR1. Graft survival was prolonged over CTLA-4Ig + MR1 alone treated recipients (MST 28d) via the addition of antiOX40L mAb treatment (MST 73d, p=0.01) or the use of OX40$^{-/-}$ mice as recipients (MST 64d, p=0.02). B) B6 background mice were grafted with BALB/c skin and treated with CTLA-4Ig and MR1. Graft survival was prolonged on GRKO recipients (MST 85d) compared with WT recipients (MST 31d, p=0.002).

Figure 1.4

IFN-gamma Receptor-deficient mice are similar to wild-type mice

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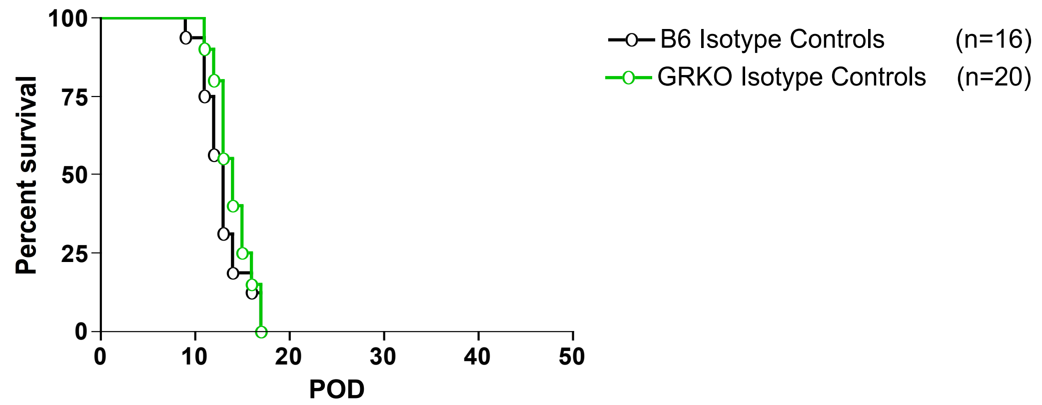

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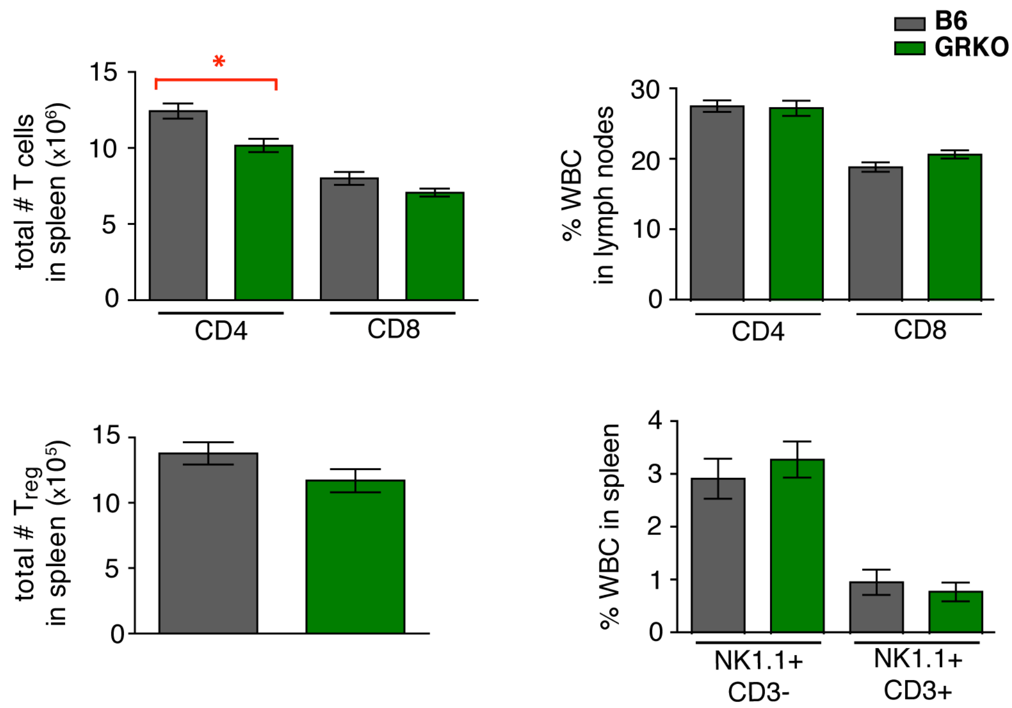

Figure 1.4

IFN-gamma Receptor-deficient mice are similar to wild-type mice.

A) B6 or GRKO mice were grafted with BALB/c skin and treated with isotype control reagents (human IgG1 Fc and Hamster IgG). Graft survival was not significantly different between B6 recipients (MST 13d) and GRKO recipients (MST 14d, p=0.13). Data are cumulative from >4 experiments. B) Naïve animals, either B6 or GRKO, were analyzed for absolute number and percentage of WBC of various cell types in spleen or dorsal skin-draining lymph nodes. Total CD4 T cell number but not CD8 T cells number in the spleen was significantly different between the two groups (n=21 per group, p=0.01 for CD4), but neither CD4 T cells nor CD8 T cells as percentage of total WBC in the lymph nodes were significantly different between the groups (n=5 per group, p=0.8 for CD4, p=0.07 for CD8). Absolute numbers of Treg (n=16 per group), of NK or of NKT cells (n=8 per group) in the spleen were not significantly different between GRKO and B6 naïve mice (p>0.05 for all cell types).

Chapter Two

Interferon-gamma Dictates Allograft Fate via Opposing Effects on the Graft and on Recipient CD8 T-cell Responses

Figure 2.1 *Donor-specific dual-cytokine producing effector CD8 T cells expand during costimulation blockade-resistant rejection* 54

Figure 2.2 *Disruption of IFNγ or IFNγR in the recipient results in divergent effects on graft survival under costimulation blockade* 56

Figure 2.3 *IFNγR-deficient recipients show no CD8 breakthrough response under costimulation blockade* 58

Figure 2.4 *Paradoxical graft loss with no CD8 breakthrough response in IFNγ-deficient recipients under costimulation blockade* 60

Figure 2.5 *IFNγ is required for prolonged graft survival on IFNγR-deficient recipients under costimulation blockade* 62

Introduction

Long-term survival of skin allografts, a very stringent model for transplantation, can be achieved in some strains of mice by interrupting CD28/B7 and CD40/CD154 interactions with CTLA-4Ig and the anti-CD154 mAb, MR1 (66). However, the C57BL/6 strain (B6) is less susceptible to this mode of immunomodulation and displays costimulation blockade-resistant rejection (67). Studies using B6 mice deficient in CD8 T cells, either by CD8 gene deletion or by CD8-depleting monoclonal antibody treatment, showed that costimulation blockade-resistant rejection depends on the presence of CD8 T cells, though the mechanisms by which these CD8 T cells are able to mediate graft rejection in the absence of major costimulatory signals have not been completely defined (17-19). Furthermore, in B6 recipients but not in C3H recipients, which accept BALB/c grafts long-term under CoB, the frequency of IFNγ-secreting cells in the spleen increases around the time of graft loss (67). However, the nature of the IFNγ-secreting cell-type could not be discerned by the ELISPOT assay used in the aforementioned study.

IFNγ, produced by T cells, NK cells, macrophages and dendritic cells, was initially described as an anti-viral cytokine and potent inducer of cellular immunity and has the potential to act on virtually all hematopoietic and non-hematopoietic cell types (26, 27). As a homodimer, IFNγ binds to a heterodimeric receptor composed of two molecules of a ligand-binding subunit, IFNγR1, which then recruits two molecules of a second receptor subunit, IFNγR2, necessary for signal transduction (29). A plethora of genes are up-regulated after IFNγ stimulation, and some of the classical effects attributed to IFNγ are increased efficiency of antigen processing, upregulated MHC

expression, induced expression of microbicidal molecules in macrophages, and activation of endothelium (27).

Because of the multitude of IFNγ actions, the effects ascribed to this cytokine have been controversial and often contradictory. In particular, the results from studies describing the influence of IFNγ on CD8 T cell responses are often confounded by the possibility of indirect actions of IFNγ via other cell types within the system (36-38). Indeed, IFNγ is known to skew CD4 T helper cell responses toward the Th1 phenotype, thereby indirectly boosting CD8-mediated immune responses (68). More recently, IFNγ was shown in several infection models to promote CD8 T cell responses directly (41, 69, 70). In transplantation, however, IFNγ knockout (GKO) or IFNγ-neutralized mice treated with CoB unexpectedly show not increased but decreased survival of allografts as compared to wild type (WT) recipients, and it was suggested that accelerated graft loss on CoB-treated recipients lacking IFNγ might be due to uncontrolled proliferation of activated T cell populations in the absence of IFNγ (71-74). However, results from previous studies of graft survival in IFNγ-deficient recipients treated with CoB may have been confounded by the lack of available IFNγ to act on the graft to promote its survival. As has been shown in several untreated vascularized allograft models, the lack of IFNγ in the recipient or the lack of the IFNγR on the graft itself leads to early graft necrosis associated with microvascular thromboses, hemorrhage, and excessive neutrophil invasion (75-78). Also, IFNγ has been shown to be necessary for regulatory T cells to exert their suppressive effects on T cell alloresponses, and IFNγ is known to upregulate molecules associated with counterregulation of immune responses, molecules such as PD-L1 and IDO that are important in maintaining allograft tolerance (53, 79-82).

From these studies, IFNγ could be said to promote tolerance, though this statement would be in stark contrast to the classical understanding of the role of IFNγ as well as the conclusions from infection models where IFNγ was shown to promote CD8 T cell-mediated immunity.

In this chapter, we provide data that help to reconcile the seemingly contradictory effects of IFNγ in models of transplantation. We illustrate the impact of CoB on *in vivo* T cell responses to skin allografts as the immune response unfolds. Using polychromatic flow cytometry, intracellular cytokine staining and refined cell-counting techniques, we identified a population of donor-specific effector CD8 T cells and found that this population expanded after graft placement and peaked around the time of graft loss, whether or not CoB was present. As costimulation blockade-resistant rejection is dependent on CD8 T cells, and as IFNγ is known to promote CD8 T cell responses, we hypothesized that IFNγ may be supporting rejection in the absence of major costimulatory signals. While previous studies observed the impact of IFNγ in transplantation under CoB where the cytokine was lacking completely, we investigated the role of IFNγ in transplantation under CoB where the cytokine is present yet the recipient is unable to respond to it. Through this approach, we found that IFNγR expression in the recipient was necessary for population expansion of donor-specific effector CD8 T cells in the absence of costimulatory signals, as IFNγ receptor-knockout (GRKO) recipients treated with CoB showed no expansion of this population and exhibited dramatically prolonged graft survival. *In vivo*, CoB consistently impaired donor-specific T cell responses, and the impairment in population expansion of donor-specific CD8 T cells under CoB was more extensive in the absence of IFNγ or the IFNγR in the recipient, providing further evidence that the IFNγ pathway plays an important role in

costimulation blockade-resistant rejection. Because CD8 responses were equally impaired in GKO or GRKO recipients treated with CoB despite early graft loss in GKO recipients, we hypothesized that IFNγ may be acting on the graft to promote its survival. We show here that skin graft survival under CoB depended on direct action of IFNγ on the skin graft itself, as either IFNγ neutralization or lack of IFNγR1 on the graft precipitated early graft loss on GRKO recipients treated with CoB. Thus, while IFNγ was required by the recipient to expand a population of donor-specific effector CD8 T cells in the absence of costimulation, IFNγ was also required by the graft to sustain its survival after allotransplantation.

Results

Donor-specific dual cytokine-producing effector CD8 T cells preferentially expand during costimulation blockade-resistant rejection

First, we sought to characterize the cellular events underlying the process of costimulation blockade-resistant rejection. Consistent with an earlier report (67), B6 mice display costimulation blockade-resistant rejection when grafted with BALB/c skin, as CoB only modestly delayed graft rejection with an MST of 24d compared with 13d for isotype control-treated recipients ($p<0.0001$, fig. 1A). To delineate recipient T cell responses during the course of graft survival and rejection in normal mice under CoB, we analyzed splenocytes from WT recipients at post-operative days (POD) 7, 14, 21, 25, 28, and 35, with particular interest in time points around the MST of grafts in each treatment group. Graft placement on isotype control-treated recipients led to a significant increase in the total number of activated ($CD44^{hi}$) CD4 and CD8 T cells in the spleen at the time of graft loss, and though most grafts were lost by POD 24 under CoB, at no time point did CoB-treated recipients show a significant increase in the absolute number of activated T cells (data not shown). Furthermore, CoB prevented the development of anti-donor antibodies, which were evident as early as POD 14 in isotype control-treated recipients (data not shown). These results indicated that CoB significantly impaired the normal patterns of T cell activation but did not reveal insight into the mechanism of costimulation blockade-resistant rejection. Therefore, we sought to track more precisely the anti-donor T cell response by looking at the total number of donor-specific CD4 and CD8 effector T cells generated during the period of breakthrough rejection.

To identify donor-specific effector T cells in graft recipients, we analyzed recipient splenocytes for T cells capable of producing cytokines in response to donor cells in an *ex vivo* rapid recall assay using intracellular cytokine staining for IFNγ and

TNF. Single-producers of TNF in this type of assay have been shown to include naïve T cells stimulated by the short term culture conditions, so we did not consider these in our definition of effector cells generated during the graft response (83). Single-producers of IFNγ have been described as being in a state of partial exhaustion in chronic viral infection models, and in at least one transplant model under costimulation blockade CD8 T cells producing IFNγ have been shown to be tolerogenic (56, 84). Because of these findings and as dual IFNγ & TNF producers have been identified as fully-functional effector T cells (84), we restricted our definition of "donor-specific effector T cells" in our study to T cells producing both IFNγ and TNF. Though analysis of all IFNγ-producers (dual and single) yielded greater cell numbers overall than assessment of strictly dual-cytokine producers, all trends and significance of the differences between groups were the same whether the analysis is performed for all IFNγ producers or restricted to dual cytokine producers (data not shown).

Donor-specific dual cytokine producing CD4 effector T cells were evident only at POD 7 in isotype control-treated recipients, and CoB-treated recipients showed no discernable expansion of donor-specific CD4 T cells at this or any other time point during the first five weeks after graft placement (data not shown). This data is consistent with our prior observations in infection models that CD4 T cells are reliant on costimulation for acquisition of effector function (85). As shown in figure 1B, at POD 14, when grafts on isotype control-treated recipients were failing, a substantial percentage of CD8 T cells in the spleen of isotype control-treated recipients produced both IFNγ and TNF in response to donor stimulation (8.01% +/- 0.869%). In contrast, CoB-treated recipients at this time point showed 60-fold lower frequencies of antigen-specific cytokine-producing CD8 T cells (0.133% +/- 0.067 %), at a level not significantly different from naïve responses (0.085 % +/- 0.037

%, p=0.618). Importantly, at POD 25, when CoB-treated recipients were losing their grafts, the frequency of donor-specific dual cytokine-producing CD8 T cells had increased significantly over naïve responses and became readily discernable on flow plots of CD8 T cells from the spleen of CoB-treated recipients, with 0.713% +/- 0.077 % of CD8 T cells producing both IFNγ and TNF in response to donor stimulation ($p<0.0001$, comparison with naïve responses). Though the observed frequency of dual cytokine-producing CD8 T cells from CoB-treated recipients at the peak of the response was less than one percent, the cytokine production from these cells was donor-specific and not due simply to the inflammatory milieu in the animal nor to assay background, as un-restimulated responders alone in our assays showed 0.009% +/- 0.001% of CD8 T cells positive for IFNγ and TNF.

Because we were interested in population expansion of donor-specific effector T cells, we used the frequency of donor-specific, dual cytokine-producing T cells in each recipient to calculate the total number of donor-responsive effector T cells in the spleen of each animal through data from TruCount bead analysis (BD Biosciences) of total numbers of CD4 and CD8 T cells within the corresponding individual spleen sample. In this way, we were able to track changes in the total number of cells over time in order to visualize population expansion of donor-specific effectors in the spleen after graft placement. The total number of donor-specific effector CD4 T cells in the spleen of isotype control-treated recipients peaked at POD 7 then rapidly contracted to levels seen in naïve mice, and CoB was able to prevent the expansion of the donor-reactive CD4 T cell population such that their absolute numbers throughout the first five weeks after graft placement did not rise significantly above that observed for naïve mice (data not shown). As seen in figure 1C, the absolute number of donor-specific effector CD8 T cells in isotype control-treated recipients had increased by POD 7 and peaked at POD 14 ($\log_{10}$[cell number] = 5.9 ± 0.075), coinciding temporally with the MST for grafts in this group.

CoB treatment prevented the expansion of the donor-specific effector CD8 T cell population at both of these early time points (log_{10}[cell number] = 3.5 ± 0.19 and 3.7 ± 0.22), as no significant difference in absolute number of these cells was found in CoB-treated recipients compared to ungrafted, naive controls (indicated by shaded horizontal bar, log_{10}[cell number] = 3.4 ± 0.10). However, a breakthrough population of donor-specific, dual cytokine-producing CD8 T cells did emerge in CoB-treated recipients, evident as a significant departure from naïve responses by POD 21 (p=0.017). The donor-specific effector CD8 T cell population continued to expand to a peak at POD 25 in CoB-treated recipients (log_{10}[cell number] = 4.7 ± 0.077), temporally correlating with the MST of grafts seen with CoB treatment. Thus, as CoB was able to prevent the increase in number of activated and effector CD4 T cells responding to BALB/c skin grafts, CoB was able to blunt activated CD8 T cell numbers and to delay and diminish but not completely prevent effector CD8 T cell responses to BALB/c grafts. The expansion of a donor-specific effector CD8 T cell population seen at the time of graft loss in CoB-treated recipients (POD 24-25) we define as the CD8 breakthrough response.

Disruption of IFNγ or IFNγR in recipient mice results in divergent effects on allograft survival after treatment with costimulation blockade

As IFNγ has long been known to promote cell-mediated immune responses and because of its potent effect on accumulation of CD8 effector T cells, we hypothesized that IFNγ contributes to the CD8 breakthrough response correlated with graft rejection under CoB (27, 41). Although previous studies had shown IFNγ-deficient recipients to be aggressive rejectors of grafts even in the presence of CoB (71-73), more recent evidence that grafts require IFNγ in order to protect against early graft necrosis suggested that conclusions about the role of IFNγ in the

rejection response under CoB may have been confounded by the lack of IFNγ available to act on the graft (75-78). When we placed BALB/c skin grafts on IFNγ-deficient recipients treated with CoB, we also found graft survival time consistently shortened compared with WT recipients under CoB. As seen in figure 2A, GKO recipients treated with CoB showed a median graft survival time (MST) of only 19d, a significant but not impressive prolongation of graft survival over the MST of 13d seen on isotype control-treated GKO recipients ($p<0.0001$). However, we hypothesized that graft survival might be greatly prolonged when the IFNγ signal deficit could be isolated to the recipient alone, so we substituted GRKO mice for GKO mice as recipients of BALB/c skin grafts under CoB. Indeed, CoB dramatically prolonged BALB/c skin graft survival on GRKO recipients with an MST of 88d as compared to isotype control-treated GRKO recipients, which potently rejected BALB/c grafts with an MST of 14d ($p<0.0001$, fig. 2B). Though WT, GKO or GRKO mice are capable of rejecting BALB/c skin grafts within the same time frame (13-14d), CoB affects graft survival time on each of these recipients differently. When IFNγ was available to the graft, graft survival time was greatly prolonged in recipients lacking IFNγ signals as well as CD28 and CD40 signals, and we hypothesized that this prolongation may have been due to a decreased CD8 T cell response in the GRKO recipient treated with CoB.

GRKO recipients show no CD8 breakthrough response under costimulation blockade

To address whether or not IFNγ affects the generation of a CD8 breakthrough response under CoB in vivo, we looked at T cells from spleens of CoB-treated WT or GRKO recipients of BALB/c skin grafts at various time points after graft placement. We analyzed these cells in the same manner as in the experiments described by figure 1 but with a focus on POD 14, 24 and 35. As observed in WT

recipients, CoB-treated GRKO recipients showed no increase in total number of activated ($CD44^{hi}$) CD8 T cells in the spleen at any time point (data not shown). As shown in figure 3A, WT recipients treated with CoB showed a CD8 breakthrough response of donor-specific dual-cytokine producing CD8 T cells at POD 24 (0.373% +/- 0.037 %). Importantly, this response was not seen in CoB-treated GRKO recipients (0.064 % +/- 0.026 % of CD8 T cells, p=0.0005 for difference between WT and GRKO). The lack of donor-specific dual cytokine-producing GRKO cells was not due to an inability of GRKO mice to produce IFNγ and TNF, as we detected dual cytokine production from these GRKO splenocytes after incubation with a non-specific stimulus of PMA and ionomycin (fig. 3A).

The frequencies of donor-specific dual cytokine-producing CD8 T cells were used to calculate the total population size of donor-specific effector CD8 T cells at three time points (fig. 3B). At POD 14, regardless of the expression of a functional IFNγR, CoB-treated recipients show naïve levels of total donor-specific effector CD8 T cells in the spleen (shaded horizontal bar, $\log_{10}$[cell number] = 3.5 ± 0.094). At POD 24, however, the population of donor-specific effector CD8 T cells had expanded in WT recipients treated with CoB ($\log_{10}$[cell number] = 4.5 ± 0.098) but remained at naïve levels in GRKO recipients treated with CoB ($\log_{10}$[cell number] = 3.7 ± 0.18). Untreated GRKO recipients were able to mount a donor-specific effector CD8 T cell response, as these mice did show increased numbers of dual-cytokine producing CD8 T cells at POD 14 and POD 24 (data not shown). The near-naïve levels of donor-specific effector CD8 T cells in CoB-treated GRKO recipients at time points when WT recipients showed peak numbers of donor-responsive cells suggested an impairment of antigen-specific effector CD8 T cell population expansion in recipients where critical costimulatory signals as well as IFNγ signals are lacking.

Paradoxical graft loss with no CD8 breakthrough response in IFNγ-deficient recipients treated with costimulation blockade

Previously, in experiments using GKO recipients or WT recipients treated with IFNγ-neutralizing antibody, IFNγ was found to be critical for the modest prolongation of graft survival seen with CoB (71-73). We also found that BALB/c skin grafts on GKO or IFNγ-neutralized WT recipients showed similarly shortened survival kinetics despite CoB (figs. 2A and 4A). As shown in figure 4A, BALB/c skin grafts on WT mice treated with CoB and with a neutralizing mAb to IFNγ showed a MST of 17.5d, significantly attenuated as compared to WT recipients treated with CoB alone (MST 24d, $p=0.0033$) but dramatically attenuated as compared to GRKO recipients treated with CoB (MST 88d, $p<0.0001$). Since the lack of the cytokine or the lack of the cytokine receptor should be expected to present with the same immunophenotype, we hypothesized that despite graft loss, IFNγ-deficient recipients must also harbor impaired anti-donor T cell responses under CoB.

Because our identification of donor-specific effector T cells depended on the expression of IFNγ for intracellular cytokine staining, for the next study we used WT recipients treated with neutralizing antibody to IFNγ throughout the experiment (WT+antiIFNγ), as GKO recipients would not be able to produce the necessary IFNγ in the assay system to enumerate donor-responsive T cells. To address CD8 T cell population expansion in vivo in IFNγ-deficient mice under CoB, splenocytes from graft recipients were analyzed as in the experiments above but with a finer focus on time points relevant to the peak cytokine responses of CD8 T cells in WT recipients, namely POD 14 and POD 24. As had been seen in WT recipients, CoB blocked in vivo T cell responses in recipients where IFNγ was neutralized, in terms of activated ($CD44^{hi}$) CD8 and CD4 T cell numbers as well as total numbers

of donor-specific effector CD4 T cells at both time points (data not shown). As shown in figure 4B, which summarizes results from all experiments, the percent of total CD8 T cells responding to donor stimulation was negligible at POD 14 in recipients treated with CoB, whether the recipient was WT (0.055% +/- 0.024%), lacked IFNγ (0.031% +/- 0.006%) or lacked the IFNγR (0.053% +/- 0.014%). At POD 24, when WT recipients under CoB showed a breakthrough anti-donor CD8 T cell response (0.471% +/- 0.077%), donor-specific CD8 T cell responses remained at naïve levels (0.074% +/- 0.020%) in WT recipients treated with neutralizing IFNγ mAb and CoB (0.047% +/- 0.024%) as well as in GRKO recipients treated with CoB (0.040% +/- 0.015%). Thus, an intact IFNγ axis in the recipient is required for the generation of a costimulation blockade-resistant anti-donor CD8 T cell response.

When these frequencies were used to calculate total numbers of donor-specific effector CD8 T cells in the spleen at the time of the CD8 breakthrough response in WT recipients (POD 24, fig. 4C), neither IFNγ-neutralized WT recipients ($\log_{10}$[cell number] = 3.2 ± 0.21) nor GRKO recipients ($\log_{10}$[cell number] = 3.4 ± 0.21) under CoB showed a population size above that seen for naïve mice ($\log_{10}$[cell number] = 3.5 ± 0.089), and the responses of these two groups of recipients were not significantly different from one another (p=0.50). The lack of IFNγ signaling in recipient cells, whether by the lack of the cytokine or the cytokine receptor, led to a significant reduction in the population size of donor-specific effector CD8 T cells at POD 24 under CoB, as WT recipients ($\log_{10}$[cell number] = 4.4 ± 0.15) showed a significant increase in this population as compared to IFNγ-neutralized WT recipients (p=0.00020) or to GRKO recipients (p=0.0010). Despite the failure of a donor-specific effector CD8 T cell population to expand in IFNγ-deficient animals treated with CoB, skin allografts had failed on these recipients. Therefore, we hypothesized that graft death on IFNγ-deficient recipients treated

with CoB may be due to lack of IFNγ available to act on the graft and that this type of death may have masked the otherwise beneficial effect of an impaired CD8 T cell response in IFNγ-deficient recipients treated with CoB.

IFNγ is required for prolonged skin graft survival on GRKO recipients treated with costimulation blockade

To test the hypothesis that IFNγ action on the graft is necessary for graft survival in our model of skin transplantation, we neutralized IFNγ in GRKO recipients treated with CoB (which otherwise would show long-term graft survival) such that only the cells with an intact receptor for IFNγ (*i.e.* the graft cells) would be affected by the absence of the cytokine. As shown in figure 5A, BALB/c skin graft survival was dramatically attenuated when IFNγ was neutralized in GRKO recipients treated with CoB, bringing graft survival from an MST of 107d in the GRKO+CoB+Rat IgG1 group down to an MST of 26d in the GRKO+CoB+antiIFNγ group ($p<0.001$). Neutralizing IFNγ reverted the acceptor phenotype of the CoB-treated GRKO recipient to a rejector phenotype with graft survival kinetics similar to that of the CoB-treated WT recipient (MST 23.5d).

The results from the cytokine neutralization experiments above did not rule out the possibility of an alternate, as yet undiscovered, receptor for IFNγ in the recipient that would both have tolerogenic properties as well as not use the known ligand-binding subunit, IFNγR1, mutated in GRKO mice. Therefore, to allow IFNγ to remain in the system while addressing the impact of IFNγ directly on the graft tissue, we sought to replace normal BALB/c skin in our model with fully-allogeneic donor tissue lacking IFNγR1. As GRKO mice on a BALB/c background were not commercially available, we bred BALB/c mice to B6-background GRKO mice and

typed PBMC from the F2 generation by flow cytometry for class I MHC and IFNγR status. F2 pups chosen as skin donors were K^d homozygous (fig. 5B) to ensure a strong direct alloresponse and either completely lacked CD119 (the IFNγR1 subunit of the IFNγR) or had full expression of CD119 (fig. 5C). We grafted skin from these F2 donors onto B6 background GRKO mice treated with CoB, and found that F2 grafts lacking IFNγR were swiftly lost with an MST of 19d (fig. 5D), identical to that of BALB/c grafts on GKO recipients treated with CoB (fig. 2A), and significantly different from that of grafts from F2 $CD119^{+/+}$ littermate controls on GRKO recipients (51.5d, p=0.0003). In sum, graft survival could not be prolonged on GRKO recipients treated with CoB when either IFNγ was absent from the system (*i.e.* IFNγ neutralization) or when the graft, too, could not respond to IFNγ, demonstrating for the first time in a model of costimulation blockade-based immunomodulation that IFNγ must act directly on the graft to prolong its survival.

Summary

In light of recent evidence that IFNγ is required by the graft to avoid early necrosis after transplantation, the decade-old observation that graft survival in GKO recipients cannot be prolonged by CoB may have limited our view of the impact of IFNγ on alloresponses in the absence of T cell costimulation (75). In this study, we sought to isolate the effects of IFNγ to either the recipient or the graft in order to better understand the impact of this cytokine on costimulation blockade-resistant rejection. We found that recipients lacking the receptor for IFNγ showed greatly prolonged graft survival under CoB, bringing into question old ideas about the role of IFNγ in costimulation blockade-resistant rejection. Some previous conclusions about why grafts aggressively fail on IFNγ-deficient recipients despite CoB were based on data suggestive of "runaway" proliferation or failure of contraction of T cells in the presence of the allostimulus and the absence of IFNγ, though these studies did not address the resulting population size of the donor-specific T cell pool present *in vivo* during the course of graft survival and death (73, 74). To investigate the impact of IFNγ on CD8 T cells under CoB, we initially tracked the population expansion of donor-specific CD8 effector T cells during the course of the immune response to skin allografts in wild-type recipients and found that the total number of these cells in the spleen increased after graft placement and peaked around the time of graft loss. Though CoB delayed the emergence of this population of donor-specific CD8 effector T cells, a small breakthrough population did expand just prior to graft loss in treated recipients. These results unify findings from previous studies showing that CD8 T cells are necessary for rejection in the face of CoB and that the frequency of IFNγ-secreting cells of unknown origin increases around the time of graft rejection under CoB (17-19, 67). Importantly, we found that the expansion of

this donor-specific CD8 effector T cell population under CoB depended on IFNγ, for when recipients did not express the IFNγR or when IFNγ was neutralized *in vivo*, this population did not expand. That grafts were rapidly lost on IFNγ-neutralized recipients, in the absence of a detectable population of donor-specific effector CD8 T cells, can be explained by our finding that the grafted skin itself required IFNγ in order to survive after allotransplantation under CoB. Previous reports have demonstrated the importance of graft-specific action of IFNγ on the survival of spontaneously accepted murine liver allografts as well as on the prevention of early necrosis of heart or kidney allografts in untreated recipients (75-77, 86), though ours is the first report showing the importance of graft-directed IFNγ for graft survival in a model where the recipient T cell response is subdued by blockade of major costimulatory pathways. Evidence for a positive, direct impact of IFNγ on graft survival in this setting dictated a reappraisal of results from studies from our group and others that showed GKO recipients to be resistant to the graft-prolonging effects of CoB (71-73). Our new data indicate that accelerated graft loss on these recipients is due not to uncontrolled expansion of CoB-resistant alloreactive T cells but rather to the lack of direct IFNγ signals to the graft to support tissue survival even at times when the recipient T cell response is undetectable.

Materials & Methods

Mice: Male mice [C57BL/6J (WT), B6.129S7-*Ifngr1*^{tm1Agt}/J (GRKO), and B6.129S7-*Ifng*^{tm1Ts}/J (GKO)] aged 6-12 weeks were purchased from The Jackson Laboratories and used as recipients. BALB/cJ (BALB/c) female mice aged 6-12 weeks, also purchased from The Jackson Laboratories, were used as donors. F2 BALBxGRKO mice were generated in-house as follows: female BALB/c mice were mated with male GRKO mice on a C57BL/6 background, then F1 pups were inter-bred. Blood samples from F2 pups were screened by flow cytometry using anti-K^d FITC, anti-K^b PE and anti-CD119 (IFNγR1) biotin followed by streptavidin APC (all BD Pharmingen). F2 mice used as graft donors were K^d homozygous and either CD119$^{-/-}$ or CD119$^{+/+}$. Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

Skin Grafting: Full-thickness ear and tail skin grafts were placed bilaterally on the dorsal thorax and secured with an adhesive bandage for six days. Grafts were scored by visual inspection for signs of necrosis, and rejection was declared when less than 10% of the original graft remained viable.

Costimulation Blockade: 500 μg CTLA-4Ig (abatacept, Bristol-Myers Squibb) and 500 μg anti-CD154 monoclonal antibody (mAb), clone MR1 (BioXCell, New Hampshire), were delivered intra-peritoneally (i.p.) on post-operative day (POD) 0, 2, 4, and 6. Graft recipients not treated with CTLA-4Ig and MR1 were given isotype control reagents, human Fc IgG1 and hamster IgG (both BioXCell, New Hampshire), at the same dose and schedule.

IFNγ Neutralization: mAb to murine IFNγ, clone R4-6A2 (BioXCell, New Hampshire), was delivered i.p. on POD -1 (2 mg) and weekly thereafter (1 mg) either until graft rejection (graft survival kinetics experiments) or until terminal harvest of tissues (T cell responses in vivo). In graft survival kinetics experiments, mice not receiving anti-IFNγ mAb were given isotype control mAb, rat IgG1 (BioXCell, New Hampshire), at the same dose and schedule.

Measuring in vivo-*generated T cell alloresponses:* At indicated time points after graft placement, recipients were euthanized, and splenocytes were isolated. CD45+, CD4+ or CD8+ T cells were enumerated by TruCount bead analysis according to manufacturer's instructions (BD Biosciences). To assess for donor-reactive T cells, 106 recipient splenocytes were incubated with 2x106 BALB/c splenocytes per well in flat-bottom 96-well plates in the presence of 1 mg/mL Brefeldin A for 5 hours at 37° C. Subsequently, cells were stained with anti-CD4 Pacific Blue, anti-CD8 Pacific Orange and anti-K^d FITC (to exclude stimulator cells), then fixed, permeabilized and stained with anti-IFNγ PE and anti-TNF APC (BD Pharmingen) according to kit instructions (eBioscience). All cells were acquired on an LSR-II flow cytometer (BD Coulter), and flow data were analyzed using FlowJo software (TreeStar).

Statistical Analyses: Skin graft survival data were plotted using Prism (Graph Pad), and significance was determined using the Mann-Whitney U test. T cell response data were plotted as the geometric mean ± SEM at each time point using Prism software (Graph Pad), and significance was determined using the unpaired, two-tailed t-test.

Figure 2.1

Donor-specific dual cytokine-producing effector CD8 T cells preferentially expand during costimulation blockade-resistant rejection

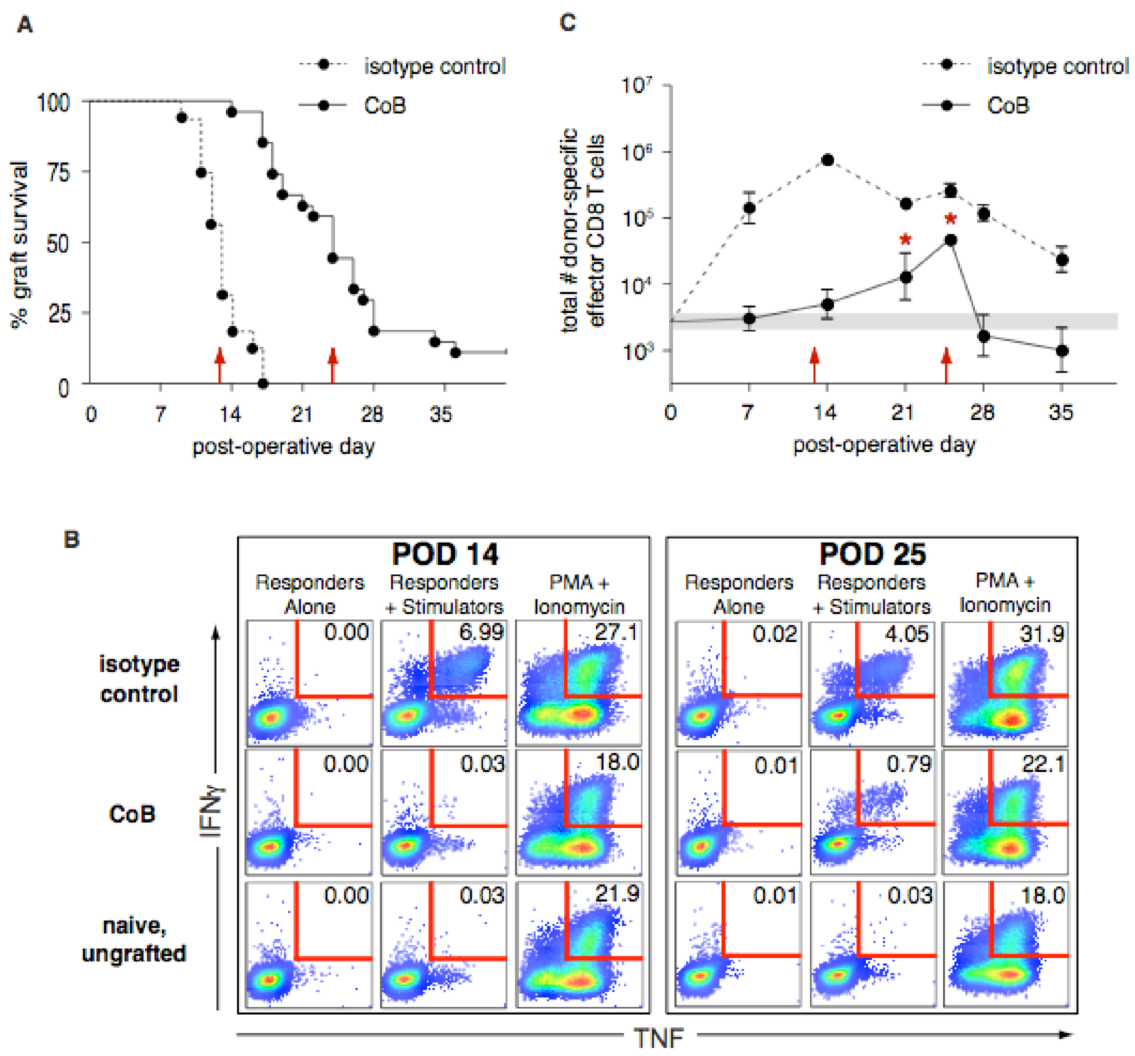

Figure 2.1

Donor-specific dual cytokine-producing effector CD8 T cells preferentially expand during costimulation blockade-resistant rejection

BALB/c skin grafts were placed on B6 recipients treated either with CoB or isotype control reagents. (A) CoB-treated recipients showed costimulation blockade-resistant rejection (n=27, MST=24d) with graft survival only modestly prolonged relative to isotype control-treated recipients (n=16, MST=13d, $p<0.0001$). Red arrows indicate MST for each group. (B&C) Splenocytes were harvested at indicated time points from recipients of BALB/c skin grafts, stimulated in vitro with BALB/c splenocytes, then analyzed by flow cytometry for recipient CD8 T cells expressing IFNγ and TNF. (B) Representative flow plots of recipient splenic CD8 T cells showing donor-specific dual-cytokine producers expressed as a percentage of total CD8 T cells. (C) Total number of donor-specific dual-cytokine producing CD8 T cells in the spleen. Data from naïve, ungrafted, mice (n=24) are represented as a shaded horizontal bar indicating the geometric mean ± SEM. Red arrows indicate MST with isotype control or CoB treatment.

* comparison of CoB-treated recipients with naive animals (POD 21, $p=0.017$; POD 25, $p=0.0001$). Data shown are from a single experiment with 3-4 recipients per group per time point. Similar results were found in four independent experiments with CoB-treated recipients.

Figure 2.2

Disruption of IFNγ or IFNγR in recipient mice results in divergent effects on allograft survival after treatment with costimulation blockade

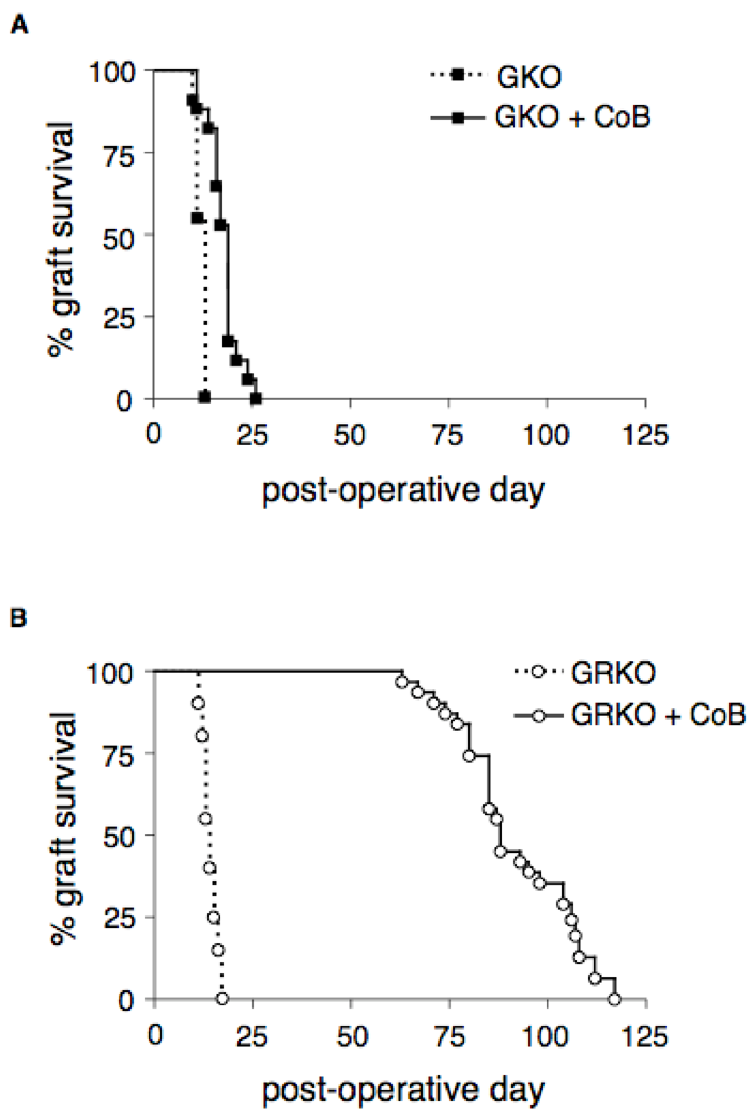

Figure 2.2

Disruption of IFNγ or IFNγR in recipient mice results in divergent effects on allograft survival after treatment with costimulation blockade

BALB/c skin grafts were placed on B6-background recipients treated with either CoB or isotype control reagents and monitored for graft survival. (A) GKO recipients showed normal graft rejection kinetics with isotype control treatment (n=11, MST=13d) and slightly prolonged graft survival when treated with CoB (n=17, MST=19d, $p<0.0001$). Data are cumulative from three independent experiments. (B) GRKO recipients showed normal graft rejection kinetics with isotype control treatment (n=20, MST=14d) and dramatically prolonged graft survival when treated with CoB (n=31, MST=88d, $p<0.0001$). Data are cumulative from seven independent experiments.

Figure 2.3

GRKO recipients show no CD8 breakthrough response under costimulation blockade

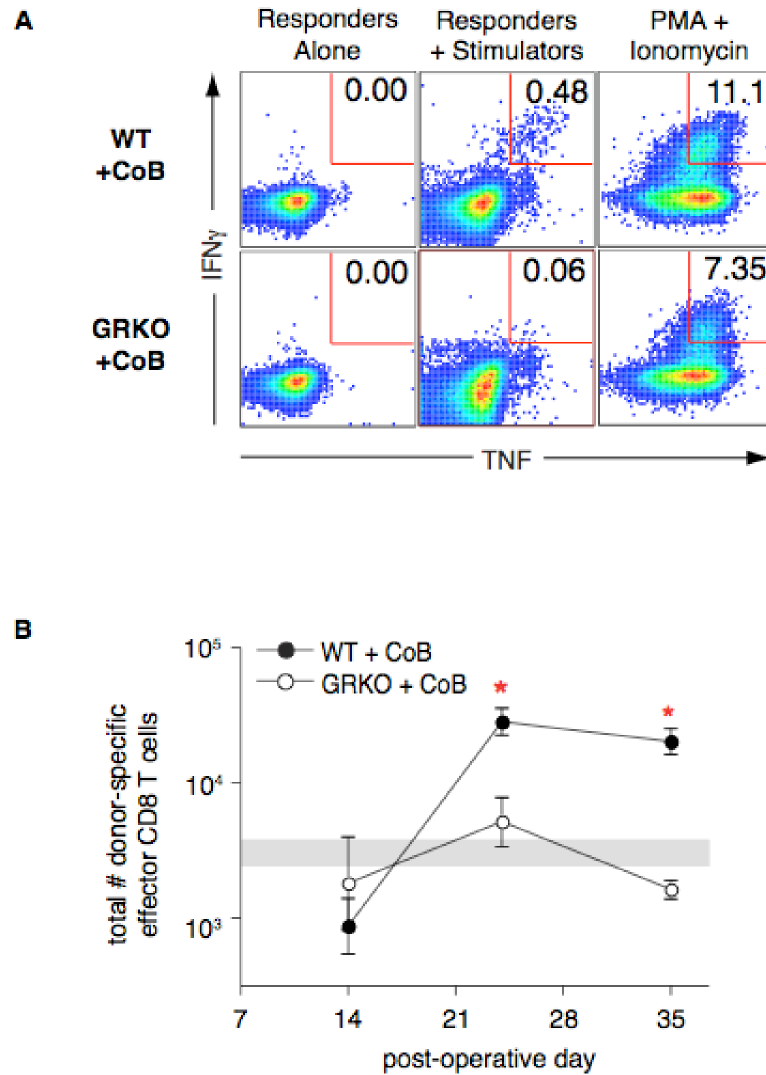

Figure 2.3

GRKO recipients show no CD8 breakthrough response under costimulation blockade

Splenocytes were harvested from CoB-treated WT or GRKO recipients of BALB/c skin grafts, stimulated in vitro with BALB/c splenocytes, then analyzed by flow cytometry for CD8 T cells expressing IFNg and TNF. (A) Representative flow plots of CD8 T cells from the spleen of recipients at POD 24 showing donor-specific dual-cytokine producers expressed as a percentage of total recipient CD8 T cells. (B) Total number of donor-specific dual-cytokine producing CD8 T cells in the spleen. Data from naïve, ungrafted, mice (n=16) are represented by a shaded horizontal bar indicating the geometric mean ± SEM.

* comparison of CoB-treated WT recipients with CoB-treated GRKO recipients (POD 24, $p=0.012$; POD 35, $p<0.001$). Data are from a single experiment with three animals per group per time point. Similar results were found in three independent experiments with CoB-treated GRKO recipients.

Figure 2.4

Paradoxical graft loss with no CD8 breakthrough response in IFNγ-deficient recipients treated with costimulation blockade

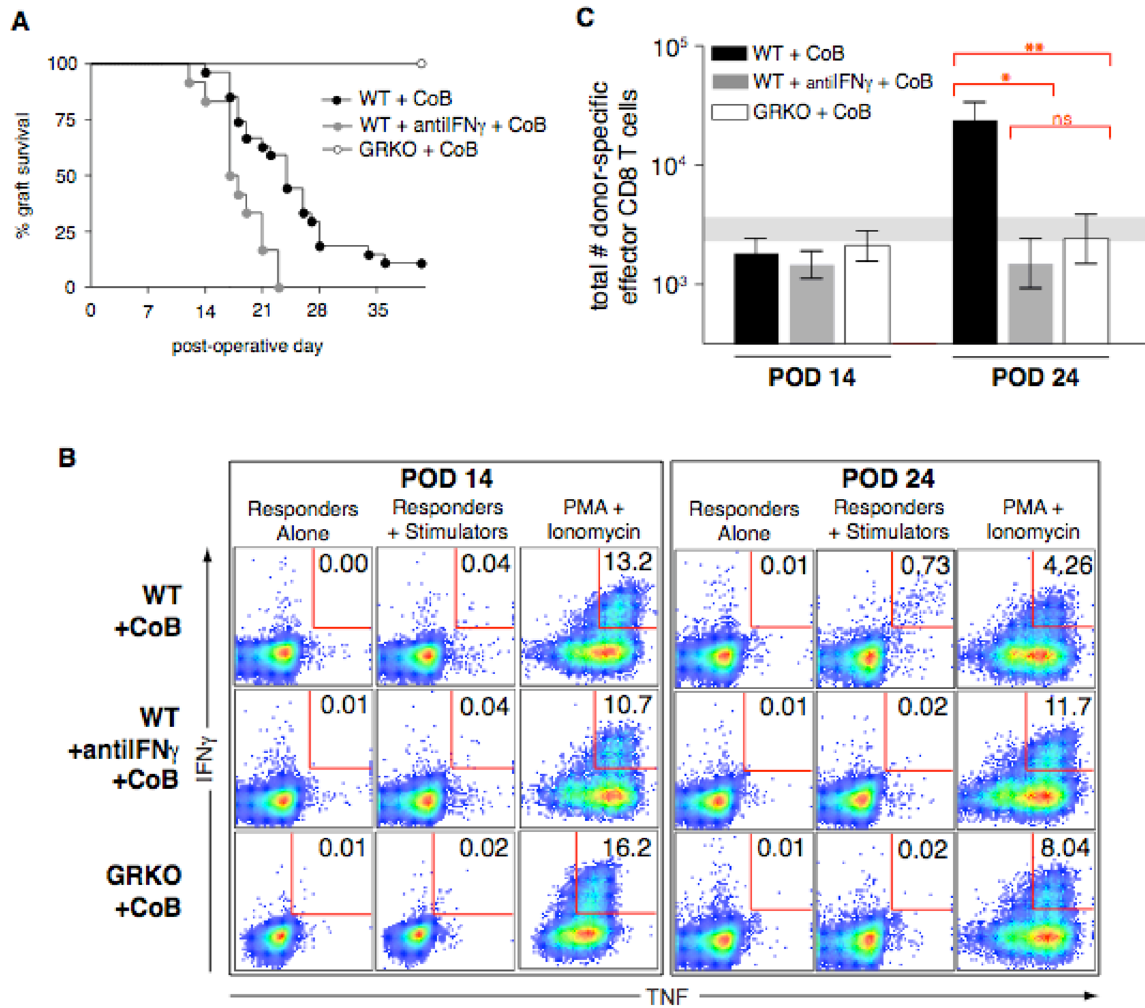

Figure 2.4

Paradoxical graft loss with no CD8 breakthrough response in IFNγ-deficient recipients treated with costimulation blockade

BALB/c skin grafts were placed on B6-background recipients treated with either CoB or isotype control reagents. (A) Graft survival kinetics. Neutralization of IFNγ in WT recipients treated with CoB (WT+antiIFNγ+CoB) shorted graft survival (MST=17.5d, n=12) as compared with WT+CoB recipients (MST=24d, n=27, p=0.0033) and as compared to GRKO+CoB recipients (MST=88d, n=31, p<0.0001). Data are from three independent experiments involving each of the groups presented. (B) Representative flow plots of recipient splenic CD8 T cells showing donor-specific dual cytokine producers expressed as a percentage of total recipient CD8 T cells. (C) Total number of donor-specific dual cytokine producing CD8 T cells in the spleen at POD 14 (WT+CoB, n=13; WT+antiIFNγ+CoB, n=7, GRKO+CoB, n=8) and POD 24 (WT+CoB, n=10; WT+antiIFNγ+CoB, n=7, GRKO+CoB, n=10).

* p=0.0002; ** p=0.0010. Data from naïve, ungrafted, mice (n=28) are represented by a shaded horizontal bar indicating the geometric mean ± SEM. Data are combined from independent experiments with a minimum total of seven mice per group per time point.

Figure 2.5

IFNγ is required for prolonged skin graft survival on GRKO recipients treated with costimulation blockade

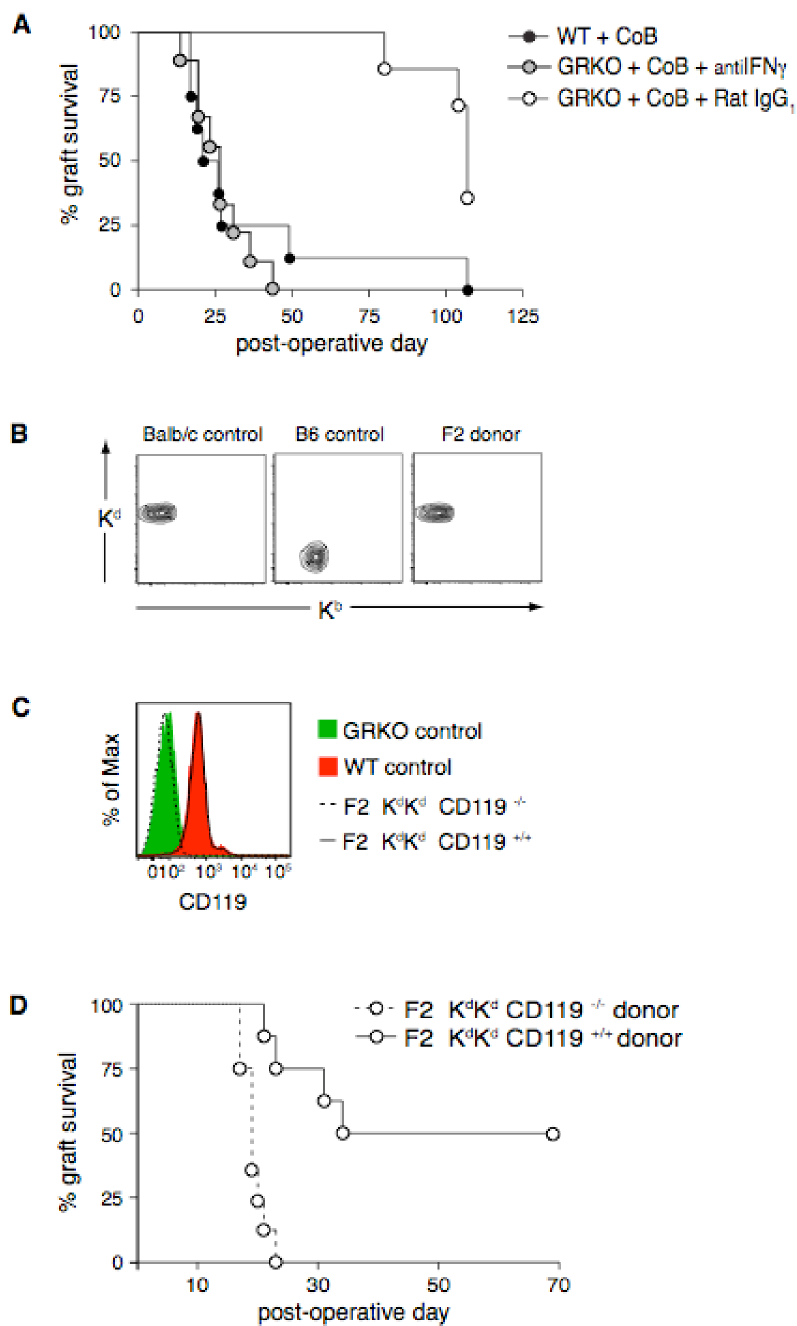

Figure 2.5

IFNγ is required for prolonged skin graft survival on GRKO recipients treated with costimulation blockade

(A) BALB/c skin grafts were placed on CoB-treated WT or GRKO recipients, which were treated with either anti-IFNγ mAb (n=9) or isotype control Rat IgG1 mAb (n=7). Neutralization of IFNγ in GRKO+CoB recipients precipitated graft loss (MST=26d) with kinetics significantly different from GRKO recipients treated with CoB and Rat IgG1 (MST=107d, p=0.00017). (B and C) F2 pups from the breeding of BALB/c mice with GRKO mice on a B6 background were typed by flow cytometry of PBMC. (B) MHC status of F2 pups used as graft donors (K^d and K^b molecules). (C) CD119 (IFNγR1 subunit) expression in F2 pups used as graft donors. (D) Skin grafts from F2 donors were placed on GRKO recipients on a B6 background treated with CoB. F2 K^dK^d $CD119^{-/-}$ grafts showed an MST of 19d, significantly different from the MST of F2 K^dK^d $CD119^{+/+}$ grafts (51.5d, p=0.0003). Data are from two independent experiments with a total of eight mice per group.

Chapter Three

Source and Activity of Interferon-Gamma in Prolonging Graft Survival in Recipients under Costimulation Blockade

Figure 3.1 *Prolonged graft survival on GRKO recipients treated with CoB is not dependent on the presence of CD8+ cells or NK1.1+ cells or on the production of IFNγ by the graft.* 87

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Introduction

In Chapter One, we showed that fully-allogeneic skin graft survival is dramatically prolonged on GRKO mice treated with CoB. In Chapter Two, we provided evidence that allograft survival is dependent on the presence of IFNγ from the start of the response, even when recipient cells are unable to respond to it. We went on to show that the action of IFNγ necessary for prolonged survival is directly upon the graft IFNγ receptors, as skin from IFNγR1-deficient BALB/c background donors did not show the same prolonged survival times. Given that IFNγ is such a necessary force contributing to tissue survival during an immune response, we sought to determine the source of the necessary IFNγ in our system and to investigate the mechanisms by which IFNγ action on the graft tissue allows for its prolonged survival.

More than a decade ago, Koniezsky *et al* observed that allografts placed on recipients deficient in IFNγ are swiftly lost, and this loss could not be prevented with costimulation blockade (45). Furthermore, IFNγ neutralization in mice attenuates the graft survival induced by combined blockade of CD28 and CD154 (45). These results led to the belief that IFNγ may have tolerogenic properties in some situations, and subsequent studies in various models of autoimmunity and transplantation have investigated the possible mechanisms by which IFNγ generates or maintains peripheral tolerance.

Interferon gamma produced by Treg, NK cells or CD8+ T cells can be important for tolerance

Sawitzki *et al* found that IFNγ production by donor-responsive CD4+CD25+ Treg was necessary for graft acceptance in a model of skin graft rejection where grafts were prolonged using donor blood transfusion during CD4 blockade (87). While natural killer (NK) cells were not found to be necessary for solid organ rejection or for CoBRR of skin grafts (17, 88), NKT cells have been shown to be required for tolerance to heart grafts after costimulation blockade (54). Furthermore, NKT cells produce IFNγ in addition to other suppressive cytokines such as IL-10 and TGF-β (89), their expression of IFNγ has been shown to be crucial in preventing diabetes in NOD mice (52). In a GvHD model, NK1.1 positive cells were shown to secrete IFNγ and to prevent GvHD while NK1.1 negative cells did not (55). Surprisingly, in a model of rat heart transplantation where rejection was prevented via infection of the donor heart with an adenovirus expressing a fusion protein, CD40:Ig, to prevent costimulatory signals, CD8+ T cells producing IFNγ were shown to be necessary for donor-specific tolerance seen in these recipients (56). The authors go on to show that these IFNγ-secreting CD8+ T cells from tolerant recipients could be adoptively transferred into naïve recipients, conferring donor-specific tolerance to these rats upon heart transplantation. Infectious tolerance via these CD8+ T cells producing IFNγ could be seen in up to four passages through naïve recipients.

Importance of graft responsiveness to interferon gamma for early graft survival

In line with the proposed protective role for IFNγ in tissue transplantation is the observation that allografts undergo early necrosis in the absence of IFNγ. Halloran's group found when WT hearts and kidneys were transplanted into untreated IFNγ$^{-/-}$ hosts, extensive necrosis is seen by day 7 (46). Partial rescue of graft necrosis was

possible when recombinant IFNγ was given to IFNγ$^{-/-}$ recipients. On the other hand, no early necrosis was seen in allografts transplanted into WT hosts. The authors postulated that large amounts of IFNγ produced within the allograft acts on graft tissue to prevent necrosis during the initial stages of immune rejection.

Investigating the importance of IFNγ action on liver grafts in a model of spontaneous graft acceptance, Mele *et al* transplanted IFNγR$^{-/-}$ livers into WT hosts as well as WT livers into IFNγ$^{-/-}$ hosts (47). In both situations, they found early necrosis (by day 10), though differences were seen in that IFNγR$^{-/-}$ liver grafts in WT recipients displayed decreased inflammatory cell infiltrates as compared to WT liver in IFNγ$^{-/-}$ recipients (47). The authors conclude that the spontaneous acceptance of liver allografts is dependent on the action of IFNγ on the graft (47).

When IFNγR$^{-/-}$ B6 kidney grafts were transplanted into untreated WT CBA recipients, significant graft necrosis was seen by day 10 (48). Furthermore, grafts deficient in IFNγR expression showed less cellular infiltrates and less iNOS mRNA by RT-PCR than did WT grafts (48). Grafts expressing IFNγR showed no necrosis even up to day 21, though rejection of grafts eventually occurred. These experiments highlight the importance of expression of the IFNγR on graft tissue specifically at early time points after transplantation.

Early expression of interferon gamma predicts tolerance

In an oral tolerance model where colonic proteins are fed to prevent disease after colitis-induction treatment, early production of IFNγ as detected by RT-PCR in lymphocytes from spleen was seen in tolerized, non-diseased mice while late IFNγ production, starting six days after colon damage, was seen in non-tolerized, diseased

mice (49). Also, in two transplant models as described below, early expression of IFNγ correlated with graft acceptance.

In a model of rat liver and kidney transplantation, where liver is spontaneously accepted and kidney is rejected, a higher ratio of CD25+ cells to total T cells was found in liver grafts as compared to kidney grafts (51). Furthermore, beginning at day 1 post-transplant, IFNγ mRNA expression was 10-fold higher in liver grafts than in kidney grafts. IFNγ mRNA expression continued to increase in liver grafts until day 5 while IFNγ mRNA expression in kidney grafts remained virtually unchanged from day 0 to day 5 (51).

In a model of rat cardiac transplantation with donor-specific blood transfusion to promote tolerance, early expression of IFNγ mRNA within the graft was associated with graft survival (50). IFNγ mRNA expression beginning later at day 5, however, was associated with graft rejection. A significant mononuclear infiltrate was seen in both tolerized and rejecting grafts, hinting that the IFNγ produced could be from recipient cells invading the graft. The authors hypothesized that the early Th1 response was necessary for tolerance induction, and warned that use of certain immunosuppressants like calcineurin inhibitors which block Th1 cytokine production may actually interfere with tolerance induction (50).

Interferon gamma, tolerogenic DC, IDO and PD-L1

In a collagen-induced arthritis model, Kelchtermans *et al* found that expression of the IFNγR on APC was necessary for functional Treg generation (90). *In vitro*, IFNγ acting on immature DC in the absence of CD40 ligation leads to tolerogenic DC, whereas IFNγ acting on a mature DC in the presence of CD40 ligation renders immunostimulatory DC (59). IFNγ potently induces IDO expression (60), and the

action of IFNγ leading to IDO expression in DC is postulated to be either autocrine or paracrine (91). IFNγ-activated DC expressing IDO were shown to prevent priming to T-cell antigens (57). In the transplant setting, functional IDO is necessary for spontaneous acceptance of liver allografts (92), and for prolonged survival for allografts after treatment with CTLA-4Ig (93, 94). In a tumor model, plasmacytoid DC expressing IDO were shown to induce CD8+ T cell anergy in tumor-draining lymph nodes (59). In several models, therefore, IDO has a clear role in suppression of immune responses and tolerance induction. Programmed Death Ligand 1 (PD-L1) is a cell surface molecule induced by IFNγ and is one of two known ligands for Programmed Death Receptor 1 (PD-1) expressed on T cells during a state of activation as well as on partially-functional or exhausted T cells in chronic infections (95). Unlike the other known ligand for PD-1, PD-L2, which is expressed only on cells of hematopoietic origin, PD-L1 can be expressed on cells of both hematopoietic and non-hematopoietic lineage (81). Furthermore, the expression of PD-L1 on pancreatic islet cells was shown to be important for their survival after transplantation (82).

In this chapter, we address questions arising from the results of studies in Chapter Two. In general, we hoped to understand the mechanism by which IFNγ action on the graft tissue led to its prolonged survival. The first, and perhaps easiest, question to address was where the necessary IFNγ comes from that is so necessary for graft survival. Knowing the source of this graft-supporting IFNγ would be important when designing new therapies to prevent graft rejection, as it might be wise to consider strategies that preserve the IFNγ-secreting function of these cells.

Secondly, we wanted to understand what IFNγ is doing to the graft that might explain the prolonged survival seen under CoB. Based on the previous work of others, we hypothesized that IFNγ may have a role in early graft responses as well as a likely different role in later stages for maintenance of graft survival, perhaps involved in a type of active peripheral tolerance.

Results

Prolonged graft survival on GRKO recipients treated with CoB is not dependent on the presence of CD8+ cells or NK1.1+ cells nor on the production of IFNγ by the graft

Several types of cells can make IFNγ, and the likely suspects identified through transplant studies to induce tolerance via IFNγ in some manner include CD8+ T cells, NK cells and Treg (52-56). Furthermore, antigen-presenting cells within the skin can also produce IFNγ, so the graft itself may be the source of IFNγ for its own survival. Therefore, we eliminated each of these possibilities via either mAb depletion or gene deletion in order to determine the necessity of each of these components in the prolonged graft survival observed in our system. Skin from BALB/c background mice was grafted onto B6 background recipients treated with CTLA-4Ig and MR1. A set of GRKO recipients received anti-CD8b mAb to deplete CD8b+ cells (T cell-specific), another set received anti-NK1.1 mAb to deplete NK1.1+ cells (NK or NKT cells), and a third set received CoB alone. As shown in figure 3.1, prolonged graft survival was seen on GRKO recipients compared with WT recipients (n=6, MST 26d) regardless of whether the recipients were treated with anti-CD8 mAb (n=8, MST >60d, p=0.02), anti-NK1.1 mAb (n=5, MST 85d, p=0.004) or no additional antibody (n=6, MST 85d, p=0.004). These results suggested that neither CD8b+ cells nor NK1.1+ cells are the source of the necessary IFNγ for graft survival, though we cannot exclude the explanation that depletion of these cells obviated the need for the protective effects of IFNγ. Interestingly, grafts from IFNγ-deficient (GKO) BALB/c donors also showed prolonged survival on GRKO recipients (n=6, MST>60d, p=0.02), indicating that the graft itself is not the necessary source for IFNγ in prolonged graft survival on GRKO recipients treated with CoB.

Skin grafted in the absence of IFNγ shows vessel congestion, hemorrhage and cellular infiltrates regardless of the presence of costimulation blockade.

Previous studies in vascularized allograft models had shown that IFNγ acts on the graft to reduce early necrosis of graft tissue with evidence of microvascular thrombosis and increased neutrophil invasion after transplantation (75-78). This effect was postulated to happen via IFNγ action on the IFNγR expressed by the donor-derived vascular endothelium of the graft, and the authors of at least one paper speculated that this early effect of IFNγ (or the lack thereof) might not be seen in non-vascularized grafts such as skin (86). In order to investigate in our system, the mechanism by which IFNγ acts on the grafted skin to prolong its survival, we initially looked at histological samples of POD 7 skin grafts for evidence of vessel congestion, hemorrhage and cellular infiltrates that had been reported in untreated vascularized allografts at similar time points. As usual, we transplanted full-thickness dorsal ear skin from BALB/c mice onto B6 background mice in the presence or absence of CoB. Half of each treatment group was also given neutralizing antibody to IFNγ. POD 7 grafts were harvested, formalin-fixed and paraffin-embedded for hematoxylin / eosin staining. As seen in figure 3.2, grafts from untreated recipients show evidence of dermal cellular infiltrates as well as focal hemorrhage, and when anti-IFNγ mAb was given to otherwise untreated recipients, this cellular infiltrate becomes massive, blurring the dermal-epidermal boundary. Extensive hemorrhage was seen by histology in grafts from these IFNγ-neutralized recipients even when gross clinical scores of grafts were minimally decreased. Though CoB treatment of recipients led to decreased cellular infiltrates in the graft at POD 7, when IFNγ was neutralized in CoB-treated recipients, grafts still showed more evidence of hemorrhage, vessel congestion and cellular infiltrates than un-

neutralized CoB-treated recipients. These results suggested that the presence of IFNγ within the first week after transplantation can dampen leukocyte invasion and hemorrhage within the skin graft, and though treating the recipient with CoB can ease the vessel congestion, hemorrhage and cellular infiltrates seen in the graft tissue at POD 7, the positive impact of IFNγ on maintaining tissue integrity is still evident in the setting of CoB treatment.

Exposing donor skin to recombinant IFNγ prior to transplantation is not sufficient to prolong its survival when transplanted onto WT recipients under costimulation blockade

Because of the positive effects of IFNγ on tissue integrity inferred by histological specimens when IFNγ was neutralized, we hypothesized that graft survival might be boosted if this tissue were given an IFNγ stimulus prior to transplantation. Therefore, we prepared BALB/c skin tissue as if for transplantation, but instead cultured the tissue overnight in media containing recombinant IFNγ (rIFNγ). As shown in figure 3.3, exposing donor skin to 10 ng/mL rIFNγ prior to transplantation is not sufficient to prolong its survival when transplanted onto B6 recipients under CoB. IFNγ-stimulated grafts showed an MST of 33 days, and the MST of media-alone cultured grafts was 24 days. However, neither of these groups showed a significant difference in MST from freshly harvested BALB/c grafts placed on WT recipients treated with CoB (MST 31d). This lack of an impact of pre-transplant IFNγ stimulation on graft survival was disheartening given the impact of the IFNγ-deficiency seen by histology, but WT recipients treated with CoB still hold the ability to mount an effective anti-graft CD8 T cell response at later time points (as seen in chapter two). Therefore, the gains in early tissue integrity may not be sufficient to counterbalance the eventual CD8 response in this system and lead to

overall graft survival. Also, IFNγ may be necessary to act on the graft tissue beyond the time window in which the effects of *in vitro* stimulation could be seen. Importantly, the results of this experiment led us to question whether or not IFNγ activity is necessary in the subsequent phases graft maintenance in the absence of detectable anti-graft CD8 T cell responses.

IFNγ is required even at late time points for sustained graft survival on IFNγR-deficient recipients treated with costimulation blockade.

To determine whether or not the presence of IFNγ is necessary for the maintenance of graft survival, we neutralized IFNγ in GRKO recipients that had been treated with CoB during the first week after transplantation and still showed perfect-scoring grafts at POD 45, roughly halfway through their predicted survival time of 88d. As shown in figure 3.4, neutralization of IFNγ at POD 45 in GRKO+CoB recipients precipitated graft loss (MST 73d, p=0.03). This result indicated that on-going action of IFNγ on graft-derived cells was necessary for graft survival and could partly explain why pre-treatment with IFNγ alone could not prolong graft survival in the previous experiments. Furthermore, this result was interesting because it pointed toward a continued requirement for *graft*-derived cells to receive IFNγ signals, as the GRKO recipients in which IFNγ was neutralized would not have been able to respond to the cytokine in the first place. Therefore, we hypothesized that IFNγ acts on the skin to induce changes early after graft placement which may set up a state of peripheral tolerance responsible for the prolonged graft survival seen in GRKO recipients treated with CoB.

Tolerance-associated transcripts are upregulated in skin after treatment in vitro *with rIFNγ as well as in POD 7 skin grafts on IFNγR-deficient recipients under costimulation blockade.* To begin to address the question of how IFNγ action on the graft prolongs its survival, we investigated whether or not IFNγ can upregulate transcription of tolerance-associated genes in skin tissue. We prepared full-thickness dorsal ear skin from WT, GRKO or BALB/c mice as if for transplantation but instead cultured the skin tissue overnight in media with or without 10 ng/mL rIFNγ. The samples were then processed for RT-PCR to look for changes in mRNA transcripts for PD-L1 & IDO, as well as for immune-related molecules known to be upregulated by IFNγ (Class I MHC and CD86). As shown in figure 3.5A, WT skin cultured with rIFNγ showed significantly increased expression of PD-L1 (5.5+/-0.53, *p*=0.01), IDO (6.9+/-0.49, *p*=0.007), and H-2K^b (2.0+/-0.09, *p*=0.02) relative to media-alone cultured skin (1.1+/-0.07, 1.1+/-/0.11, and 1.1+/-0.09, respectively). Importantly, regardless of the addition of IFNγ to the culture media, GRKO skin showed no difference in expression of PD-L1 or IDO compared to media-alone cultured WT skin. These results indicated that IFNγ stimulation *in vitro* can upregulate expression of PD-L1 and IDO transcripts in skin and that this upregulation is due specifically to IFNγ action and not due simply to the culture conditions, as tissue lacking the receptor for IFNγ did not show upregulation of these transcripts despite the presence of IFNγ in the culture media.

To get closer to our *in vivo* system involving BALB/c skin grafts, we also stimulated BALB/c skin *in vitro* with IFNγ to see if the same tolerance-associated genes could be upregulated. While overnight culture in media alone led to a meager but significant increased the quantity of PD-L1 transcripts relative to fresh ear skin (2.0+/-0.27 versus 0.94+/-0.16, *p*=0.02), the addition of IFNγ, either 2 ng/mL (8.0+/-

0.82, *p*=0.002) or 10 ng/mL (11+/-1.1, *p*=0.002), significantly increased the expression of PD-L1 over that seen in media-alone cultured skin. The culture conditions alone did not significantly increase the quantity of transcripts for IDO relative to fresh skin, but addition of IFNγ to the media, either 2 ng/mL (3.7+/-0.10, *p*=0.001) or 10 ng/mL (8.0+/-0.51, *p*=0.0003), significantly increased the expression of IDO transcripts compared to media-alone treatment (1.4+/-0.26). H-2K^{d} mRNA expression increased in BALB/c skin after overnight culture alone, (media-alone skin 2.0+/-0.043; fresh skin 1.1+/-0.12, *p*=0.002), but the addition of IFNγ (2 ng/mL, 2.8+/-0.11, *p*=0.002; 10 ng/mL, 3.2+/-0.13, *p*=0.0009) further boosted the quantity of Class I MHC transcripts significantly over media-alone culture. These results indicated that IFNγ could significantly increase in a dose-dependent manner the expression level of transcripts for two tolerance-associated molecules, PD-L1 and IDO, in the skin from mice we use as donors in our model of transplantation. However, it remained to be determined whether or not the presence of IFNγ *in vivo* would be enough to increase the expression of these molecules after transplantation as a way to explain the prolonged survival of grafts seen on GRKO recipients under CoB.

Therefore, to investigate the impact of IFNγ *in vivo* on the expression level of transcripts for tolerance-associated molecules within the skin allograft, we harvested POD 7 grafts from GRKO mice treated with CoB. Because IFNγ-deficient recipients treated with CoB show an inhibited T cell response equivalent to that in GRKO recipients under CoB, we compared the transcript levels found in grafts from GRKO+CoB recipients to those found in grafts from WT recipients treated with neutralizing antibody to IFNγ, as the graft experience could be assumed to be comparable in these situations but for the presence or absence of IFNγ.

On POD 7, BALB/c skin grafts were harvested and processed for RT-PCR to assay the quantity of transcripts (relative to fresh BALB/c skin) for tolerance-

associated molecules (PD-L1 and IDO) as well as CXCL-11, a chemokine produced by keratinocytes, the most abundant cell type within skin tissue, in response to IFNγ. As shown in figure 3.5B, cells within POD 7 skin grafts from GRKO+CoB recipients showed significant increases in expression of PD-L1 (7.8+/-0.81), IDO (640+/-250) and CXCL-11 (360+/-47) relative to fresh BALB/c skin, as well as when compared to BALB/c skin grafts harvested from recipients in which IFNγ had been neutralized (p=0.05 for all analyses by one-tailed Mann Whitney test). These results indicate early differences within the graft when it is exposed to IFNγ or not on recipients with equally impaired T cell responses. The upregulation of transcripts for tolerance-associated molecules by POD 7 in grafts which would go on to long-term survival and the significant decrease in quantity of expression of these molecules could partly account for the dramatic difference in graft survival on IFNγ-deficient versus IFNγR-deficient recipients under CoB. Furthermore, these results led us to question again whether or not the prolonged survival of grafts on GRKO+CoB recipients could be under the control of regulatory T cells, as IDO has been shown to be important in the generation of Treg, and IFNγ has been implicated in the generation and maintenance of Treg populations (57, 96).

CD4+ cells are required for maintenance of prolonged graft survival on IFNγR-deficient recipients treated with costimulation blockade.

To begin to address the question of whether or not the presence of Treg are important in maintaining the prolonged graft survival seen on GRKO+CoB recipients, we hypothesized that depletion of CD4+ cells at late time points would precipitate graft loss. To test this hypothesis, as before, BALB/c skin was grafted onto GRKO recipients treated with CTLA-4Ig and MR1. On POD 45, as set of

these recipients were initiated on a CD4+ cell-depleting regimen via i.p. delivery of an known CD4-depleting mAb, GK 1.5. Mice receiving anti-CD4 mAb from POD 45 (n=5) swiftly rejected their grafts with an MST of 60d, significantly different from that of un-neutralized recipients (MST 88d, $p<0.001$). CD4 depletion in POD 45 GRKO+CoB recipients was repeated two more times with similar results, indicating that CD4+ cells are necessary for maintenance of graft survival even at late time points.

Neutralization of IFNγ in IFNγR-deficient recipients of long-term surviving grafts alters transcript profiles within graft-draining lymph nodes.

As another approach to addressing the question of whether or not active control of the anti-graft immune response occurs at late time points in GRKO recipients initially treated with CoB, we investigated the impact of IFNγ withdrawal on the transcriptional activity with the graft-draining lymph nodes of these recipients. We speculated that a form of peripheral tolerance was instilled by the action of IFNγ on the graft-derived cells, and that this action had a secondary effect on dampening the recipient immune response to the graft. We hypothesized that lifting the action of IFNγ on the graft would also lift the damper on the recipient immune response to the graft, and that this would lead to increased transcription for immune response genes in the lymph nodes draining the long-term surviving skin grafts.

To start, we sought to establish baseline levels of transcripts present in lymph nodes draining long-term surviving grafts on GRKO+CoB and to determine if there were any differences between these nodes and those at POD 7 in terms of transcripts for T cells and tolerance-associated molecules. GRKO and WT mice were grafted with BALB/c skin and treated with CoB during the first week after transplantation.

On POD 7 or POD45, graft-draining lymph nodes (axillary & inguinal) were harvested and processed for RT-PCR, and the resulting transcript levels were quantified relative to that found in naïve lymph nodes. As shown in figure 3.7A, no significant differences were found between POD 7 WT and GRKO lymph nodes in terms of transcripts for CD4, CD8b, FoxP3, PD-L1 and IDO ($p>0.05$ by two-tailed Mann-Whitney test). These results suggest that, unlike the transcriptional activity in the grafts themselves, transcriptional levels of tolerance-associated molecules in lymph nodes draining the graft cannot predict long-term graft survival. When lymph nodes from GRKO+CoB recipients at POD 7 were compared with those at POD 45, no significant increases in quantity of transcripts for T cell or tolerance-associated molecules were found ($p>0.05$ by two-tailed Mann-Whitney test). Despite the lack of differences in transcripts for CD4, CD8, or FoxP3 in nodes harvested at POD 7 and POD 45, lymph nodes from POD 45 on gross inspection appeared as if they might harbor an active immune response, as they were larger than those harvested at POD 7 which appeared more like those of naïve mice (data not shown). This discrepancy in size of the node versus relative quantity of transcripts could be explained by simple increase in cellularity without an increase in transcripts per cell, as might occur when cells are more metabolically active as in an active immune response.

To probe the effect of IFNγ withdrawal on the transcriptional activity within lymph nodes draining long-term surviving grafts on GRKO recipients, on POD 45, recipients were neutralized of IFNγ by anti-IFNγ mAb. Seven days later, graft-draining lymph nodes were harvested and processed for RT-PCR, and the resulting transcript levels were quantified relative to that found in naïve lymph nodes. As shown in figure 3.7B, increased relative quantity of mRNA for genes associated with cell-mediated immunity was found in graft-draining lymph nodes after IFNγ neutralization in GRKO mice harboring long-term surviving grafts. Transcript levels

of CD8, T-bet and Granzyme B were significantly increased in graft-draining lymph nodes from GRKO recipients seven days after initiation of IFNγ neutralization (harvested on POD 52, open circles) compared with levels just prior to IFNγ neutralization (harvested on POD 45, filled circles, $p=0.05$ by one-tailed Mann-Whitney test). Because the graft-derived cells were the only cells capable of receiving an IFNγ signal, therefore would be the only cells changed by its neutralization, these results indicate that IFNγ action on graft cells allows them to mediate a suppressive effect on the recipient immune system, keeping dormant a cell-mediated anti-graft response for months after transplantation.

Summary

In this chapter, we provide evidence that the necessary source of IFNγ for prolonged graft survival seen on GRKO recipients treated with CoB is not a CD8+ T cell nor an NK or NKT cell, cell types shown to be important for graft survival in other transplant systems as described earlier in this chapter. We also found that the graft itself does not provide the necessary IFNγ for its own survival. Another cell-type capable of producing IFNγ early during immune responses and shown in some models to be responsible for allograft survival is the CD4+ Treg (53), and depletion of CD4+ cells in the recipient prior to grafting does shorten graft survival time on WT animals treated with CoB (17). When we depleted CD4+ cells prior to grafting in GRKO mice treated with CoB, we also saw attenuated graft survival (data not shown). Though it is tempting to conclude from these experiments that CD4+ cells supply the necessary IFNγ for prolonged graft survival seen on GRKO recipients under CoB, we cannot exclude the possibility that CD4+ cells are an otherwise necessary component of the system, that when these cells are removed, the primary immune response changes altogether.

We also found that the presence of IFNγ dampened leukocyte invasion and decreased incidence of hemorrhage within grafts, and the positive effects of IFNγ presence on tissue integrity were seen even when recipients were treated with CoB. We tested whether or not treatment of graft tissue with IFNγ prior to transplantation would overcome CoBRR, prolonging graft survival. However, we found that the gains in early tissue integrity alone were not sufficient to counterbalance the eventual anti-graft CD8 T cell response in the WT recipient, and IFNγ-stimulated grafts failed with kinetics similar to unstimulated graft tissue. However, this result led us to question whether or not IFNγ action on graft tissue is

necessary beyond the time window in which the effects of *in vitro* stimulation could be seen.

When IFNγ was neutralized in IFNγR-deficient recipients harboring long-term surviving grafts, we found that grafts were lost, indicating that IFNγ was necessary even during the maintenance phase of graft survival and partly explaining why pre-treatment with IFNγ alone could not prolong graft survival in the previous experiments. From these results, we questioned whether or not IFNγ action on the skin can induce changes early after graft placement which may set up a state of peripheral tolerance responsible for the prolonged graft survival seen in GRKO recipients treated with CoB.

We found that IFNγ action on skin specifically upregulates mRNA transcripts for the tolerance-associated genes PD-L1 and IDO *in vitro* and *in vivo*, and that these changes *in vivo* are evident early after transplantation within grafts that would go on to long-term survival. Furthermore, CD4+ cell depletion in recipients harboring long-term surviving grafts precipitated graft loss, indicating that CD4+ cells are necessary for maintenance of graft survival even at late time points and suggesting that some form of active tolerance is at play.

Since IFNγ-neutralization in IFNγR-deficient recipients of long-term surviving grafts also precipitated graft loss, and because in this system the only cells able to respond to IFNγ (and therefore the only ones to note its absence), we hypothesized that lifting the action of IFNγ on the graft might also lift the damper on the recipient immune response to the graft. Indeed, we found that IFNγ neutralization at late time points led to increased transcript levels for cellular-immune response genes in the lymph nodes draining long-term surviving skin grafts.

These results suggest that IFNγ action on graft cells allows them to mediate a suppressive effect on the recipient immune system, keeping dormant a cell-mediated anti-graft response for months after transplantation.

Materials & Methods

Mice: Male mice {C57BL/6J (WT), B6.129S7-*Ifngr1*^{tm1Agt}/J (GRKO)} aged 6-12 weeks were purchased from The Jackson Laboratories and used as recipients. BALB/cJ (BALB/c) female mice aged 6-12 weeks, also purchased from The Jackson Laboratories, were used as donors. Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

Skin Grafting: Full-thickness ear and tail skin grafts were placed bilaterally on the dorsal thorax and secured with an adhesive bandage for six days. Grafts were scored by visual inspection for signs of necrosis, and rejection was declared when less than 10% of the original graft remained viable.

Costimulation Blockade: 500 μg CTLA-4Ig (abatacept, Bristol-Myers Squibb) and 500μg anti-CD154 monoclonal antibody (mAb), clone MR1 (BioXCell, New Hampshire), were delivered intra-peritoneally (i.p.) on post-operative day (POD) 0, 2, 4, and 6. Graft recipients not treated with CTLA-4Ig and MR1 were given isotype control reagents, human Fc IgG1 and hamster IgG (BioXCell, New Hampshire), at the same dose and schedule.

IFNγ Neutralization: mAb to murine IFNγ, clone R4-6A2 (BioXCell, New Hampshire), was delivered i.p. on the first day of treatment (2 mg on either POD -1 or POD 45) then weekly doses of 1 mg were given if indicated by the experiment. Control animals were given isotype control mAb, rat IgG1 (BioXCell, New Hampshire) in graft kinetics experiments.

Measuring in vivo-*generated T cell alloresponses:* At indicated time points after graft placement, recipients were euthanized, and splenocytes were isolated. CD45+, CD4+ or CD8+ T cells were enumerated by TruCount bead analysis according to manufacturer's instructions (BD Biosciences). To assess for donor-reactive T cells, 10^6 recipient splenocytes were incubated with $2x10^6$ BALB/c splenocytes per well in flat-bottom 96-well plates in the presence of 1 mg/mL Brefeldin A for 5 hours at 37° C. Subsequently, cells were stained with anti-CD4 Pacific Blue, anti-CD8 Pacific Orange and anti-K^d FITC (to exclude stimulator cells), then fixed, permeabilized and stained with anti-IFNγ PE and anti-TNF APC (BD Pharmingen) according to kit instructions (eBioscience). All cells were acquired on an LSR-II flow cytometer (BD Coulter), and flow data were analyzed using FlowJo software (TreeStar).

Cell Depletion: NK and NKT cells were depleted by i.p. injection of mAb to NK1.1 (clone PK136) given on POD –3, -2, -1 then every three days at 100 μg per dose. CD8+ cells were depleted by i.p. injection of mAb to CD8b (clone YTS169, BioXCell, New Hampshire) given on POD –3, -2, -1 then weekly at 100 μg per dose. CD4+ cells were depleted by mAb to CD4 (clone GK1.5, BioXCell, New Hampshire) given as an induction dose of 100μg i.p. for three consecutive days then followed by weekly doses of 100 μg.

IFNγ stimulation of skin in vitro*:* Skin tissue was prepared as if for transplantation but instead was cultured at 37•C and 5% CO_2 for 18 hours by floating on the surface of 5mL RPMI+10%FCS with or without 10 ng/mL recombinant murine IFNγ (eBioscience). Afterward, tissue was rinsed in sterile PBS and either transplanted or prepared for RT-PCR.

RT-PCR: Tissues were harvested and immediately placed into 1.5 mL tubes of RNA-Later (Ambion), left for 24 hrs at RT, then placed at –80° C until RNA isolation. RNA was isolated by modified Trizol method, and cDNA were prepared with First Strand cDNA kit (Roche) using 2.5 μg RNA per sample according to kit instructions. Real time PCR was performed using inventoried TaqMan assays (ABI) along with 100 μg "RNA" from cDNA samples. Assays were run on a 7900 thermalcycler (Applied Biosystems), and data were analyzed using RQ Manager software (SDS).

Statistical Analyses: Skin graft survival data were plotted using Prism (Graph Pad), and significance was determined using the Mann-Whitney U test. Transcript data from RT-PCR assays for *in vitro* samples was analyzed for significant significant differences in the means by two-tailed t test. Transcript data from RT-PCR assays for *in vivo* samples was analyzed for significant increase in the mean by one-tailed Mann Whitney test, and for significant differences in the means by two-tailed Mann Whitney test.

Figure 3.1

Prolonged graft survival on GRKO recipients treated with CoB is not dependent on the presence of CD8+ cells or NK1.1+ cells nor on the production of IFNγ by the graft.

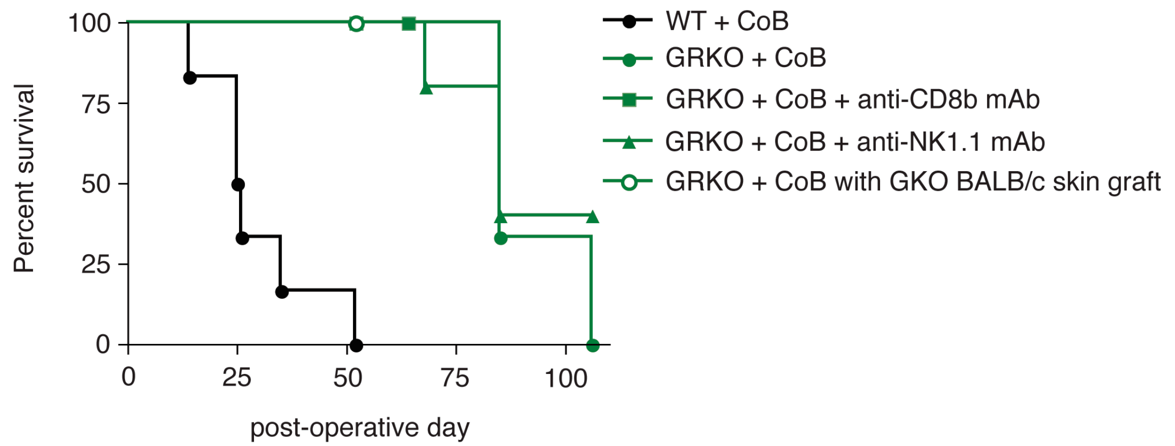


Figure 3.1

Prolonged graft survival on GRKO recipients treated with CoB is not dependent on the presence of CD8+ cells or NK1.1+ cells or on the production of IFNγ by the graft.

Skin from BALB/c background mice was grafted onto B6 background recipients treated with CTLA-4Ig and MR1. Prolonged graft survival was seen on GRKO recipients compared with WT recipients (n=6, MST 26d) regardless of whether the recipients were treated with anti-CD8b mAb (n=8, MST >60d, p=0.02), anti-NK1.1 mAb (n=5, MST 85d, p=0.004) or no additional antibody (n=6, MST 85d, p=0.004). Grafts from GKO BALB/c donors also showed prolonged survival on GRKO recipients (n=6, MST>60d, p=0.02).

Figure 3.2

Skin grafted in the absence of IFNγ shows vessel congestion, hemorrhage and cellular infiltrates regardless of the presence of costimulation blockade.

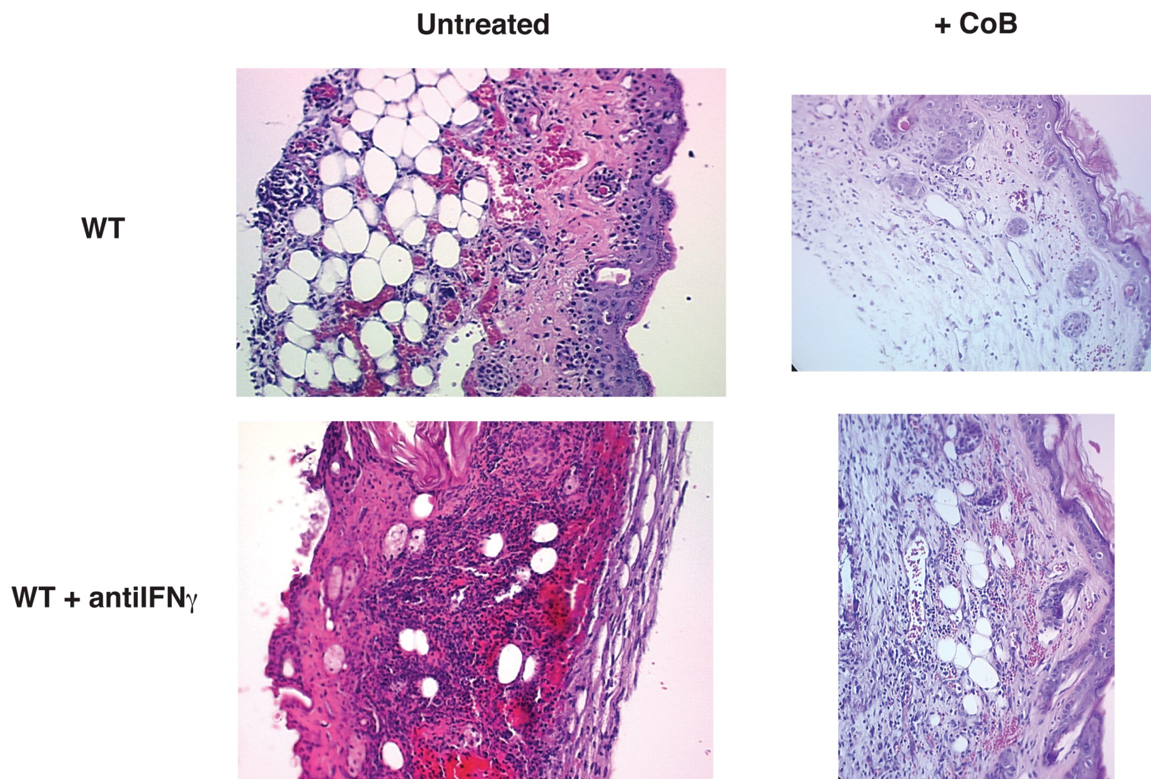


Figure 3.2

Skin grafted in the absence of IFNγ shows vessel congestion, hemorrhage and cellular infiltrates regardless of the presence of costimulation blockade.

Full-thickness dorsal ear skin from BALB/c mice was grafted onto B6 background mice in the presence or absence of CoB and with or without neutralizing antibody to IFNγ. POD 7 grafts were harvested, formalin-fixed and paraffin-embedded for hematoxylin / eosin staining. Images (20X) are oriented such that the epidermal surface is on the right side of the image and are representative of grafts harvested from three mice per treatment group.

Figure 3.3

Exposing donor skin to recombinant IFNγ prior to transplantation is not sufficient to prolong its survival when transplanted onto WT recipients under costimulation blockade.

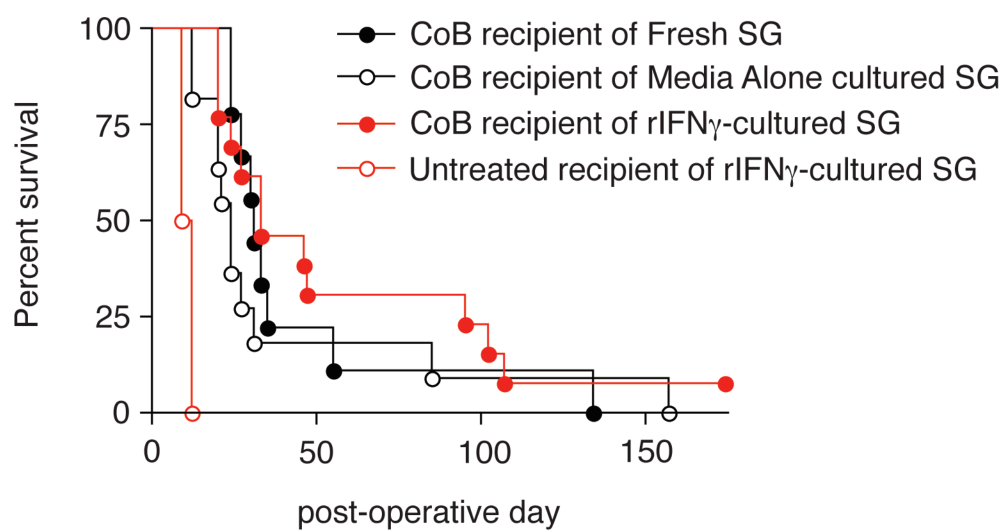


Figure 3.3

Exposing donor skin to recombinant IFNγ prior to transplantation is not sufficient to prolong its survival when transplanted onto WT recipients under costimulation blockade.

Skin prepared from BALB/c donors was cultured overnight in media with or without recombinant IFNγ before grafting onto WT recipients treated with or without CTLA-4Ig and MR1. n=8 fresh skin onto CoB-treated recipients (MST 31d); n=11 media alone-cultured skin on CoB-treated recipients (MST 24d); n=13 rIFNγ-cultured skin on CoB-treated recipients (MST 33d); n=6 rIFNγ-cultured skin on untreated recipients (MST 11d). No significant differences in graft survival were found between any of the CoB-treated groups.

Figure 3.4

IFNγ is required even at late time points for sustained graft survival on IFNγR-deficient recipients treated with costimulation blockade.

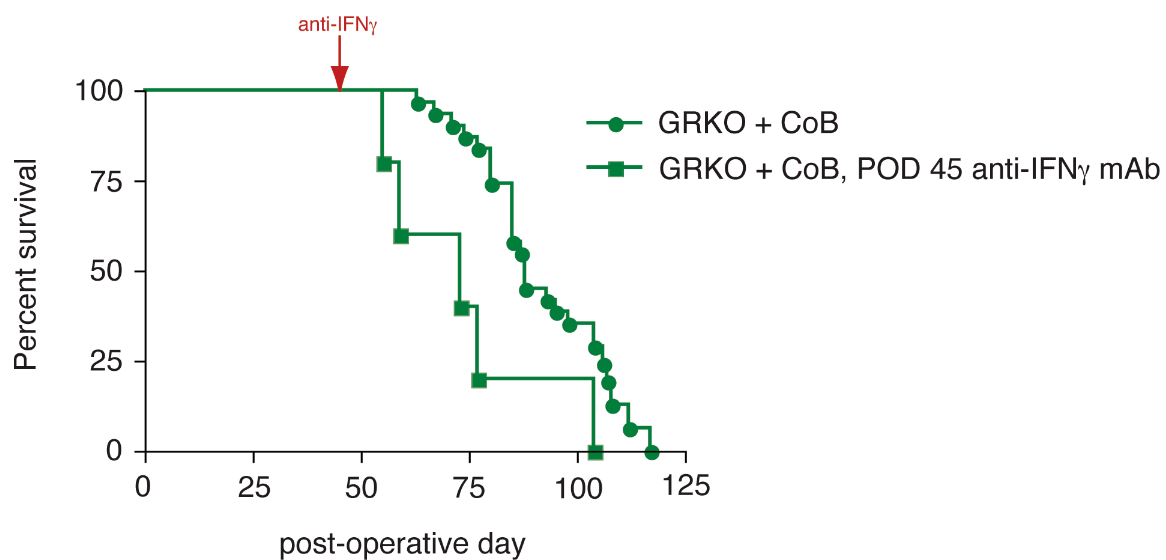


Figure 3.4

IFNγ is required even at late time points for sustained graft survival on IFNγR-deficient recipients treated with costimulation blockade.

BALB/c skin was grafted onto GRKO recipients treated with CTLA-4Ig and MR1. On POD 45, recipients were either started on an IFNγ-neutralizing regimen via mAb to IFNγ or given isotype control mAb, Rat IgG1. Mice receiving anti-IFNγ mAb beginning on POD 45 (n=5) rejected their grafts with an MST of 73d, significantly different from that of un-neutralized recipients (MST 88d, p=0.03).

Figure 3.5

Tolerance-associated transcripts are upregulated in skin after treatment in vitro *with rIFNγ as well as in POD 7 skin grafts on IFNγR-deficient recipients under costimulation blockade.*

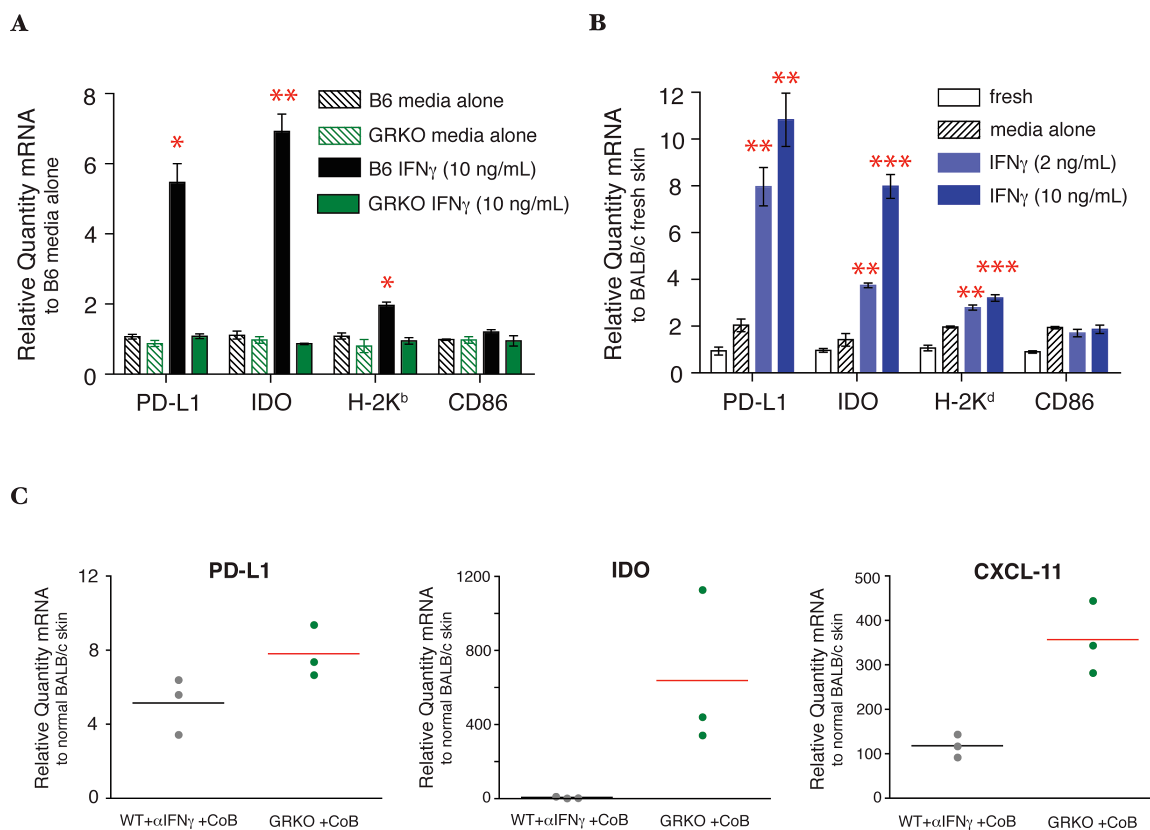


Figure 3.5

Tolerance-associated transcripts are upregulated in skin after treatment in vitro *with rIFNγ as well as in POD 7 skin grafts on IFNγR-deficient recipients under costimulation blockade.* A&B) Full-thickness dorsal ear skin from WT B6, GRKO B6 or BALB/c mice was prepared as if for transplantation, but instead was cultured overnight in media +/- 10ng/mL rIFNγ then processed for RT-PCR to look for changes in mRNA transcripts for tolerance-associated molecules (PD-L1 & IDO), as well as immune-related molecules known to be upregulated by IFNγ (Class I MHC and CD86). A) WT B6 and GRKO B6 skin. IFNγ-treated WT skin showed significantly increased expression of PD-L1 (5.5+/-0.53, p=0.01), IDO (6.9+/-0.49, p=0.007), and H-2K^b (2.0+/-0.09, p=0.02) relative to media-alone treated WT B6 skin (1.1+/-0.07, 1.1+/-/0.11, and 1.1+/-0.09, respectively), while GRKO skin showed no difference from WT media alone skin regardless of the addition of IFNγ. B) BALB/c skin, expression normalized to fresh skin samples. Data shown are of 3 specimens per group and are representative of two independent experiments. While overnight culture in media alone did meagerly but significantly increase PD-L1 transcripts compared to fresh ear skin (2.0+/-0.27 versus 0.94+/-0.16, p=0.02), the addition of rIFNγ, either 2 ng/mL (8.0+/-0.82, p=0.002) or 10 ng/mL (11+/-1.1, p=0.002), significantly increased the expression of PD-L1 over that seen in media-alone cultured skin. IDO was not significantly increased upon media-alone culture relative to fresh skin, but addition of rIFNγ to the media, either 2 ng/mL (3.7+/-0.10, p=0.001) or 10 ng/mL (8.0+/-0.51, p=0.0003), significantly increased the expression of IDO transcripts compared to media-alone treatment (1.4+/-0.26). Culture alone did lead to an increase in expression of H-2K^d transcripts in BALB/c skin (2.0+/-0.043 over 1.1+/-0.12, p=0.002), but the addition of rIFNγ (2 ng/mL, 2.8+/-0.11, p=0.002; 10 ng/mL, 3.2+/-0.13, p=0.0009) further boosted the expression of Class I MHC significantly over media

alone culture. C) mRNA expression levels in POD 7 BALB/c skin grafts. WT B6 mice in which IFNγ had been neutralized or GRKO mice were grafted with BALB/c skin and treated with CTLA-4Ig and MR1. On POD 7, grafts were harvested and processed for RT-PCR to look for upregulation of tolerance-associated molecules (PD-L1 and IDO) as well as CXCL-11, a chemokine produced by keratinocytes and known to be upregulated by IFNγ. Cells within the POD 7 skin graft showed significant increases in expression of PD-L1 (7.8+/-0.81), IDO (640+/-250) and CXCL-11 (360+/-47) relative to fresh BALB/c skin, or relative to BALB/c skin harvested from recipients in which IFNγ had been neutralized (p=0.05 for all analyses by one-tailed Mann Whitney test).

Figure 3.6

CD4+ cells are required for maintenance of prolonged graft survival on IFNγR-deficient recipients treated with costimulation blockade.

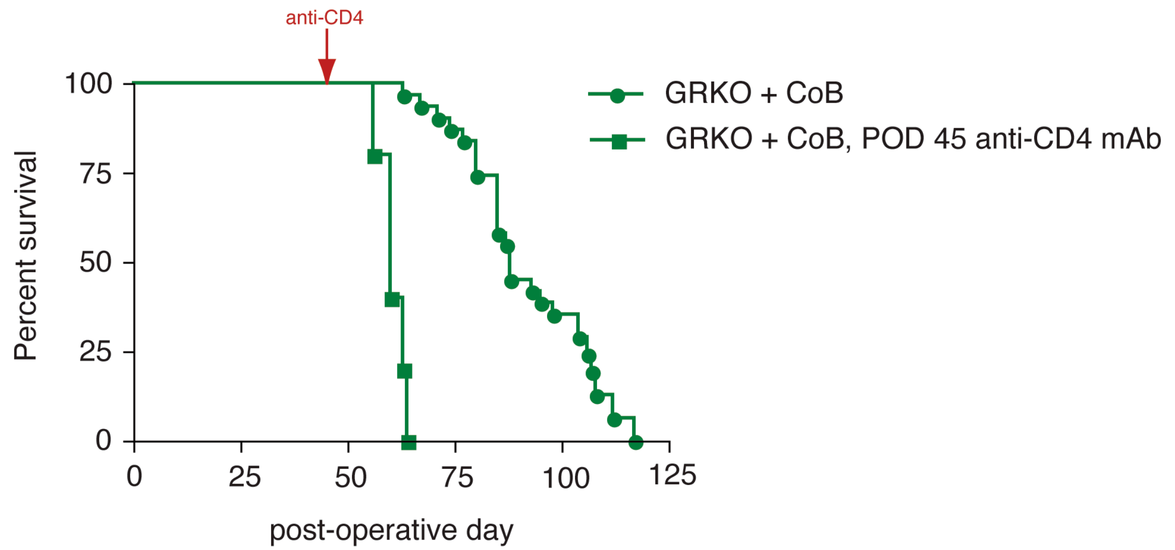


Figure 3.6

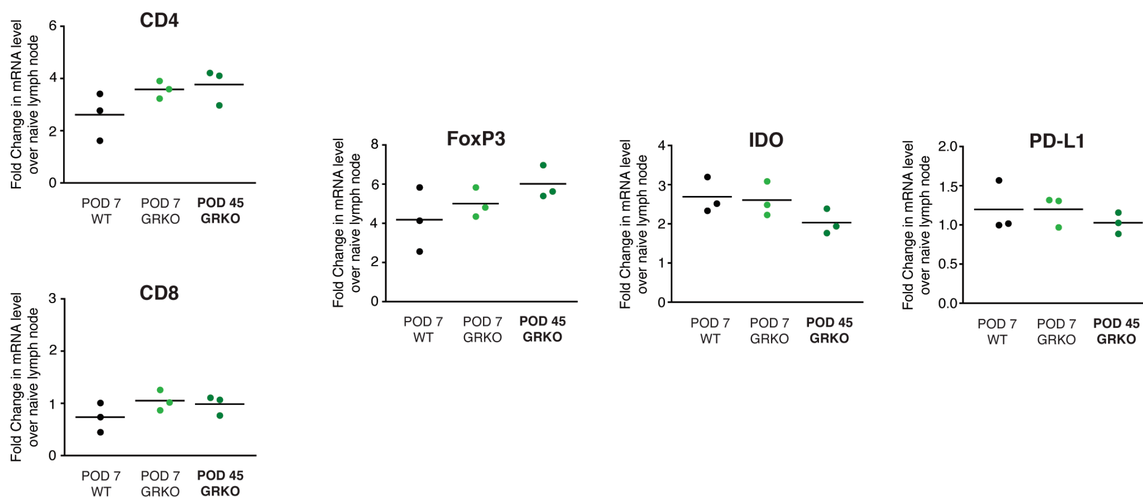
CD4+ cells are required for maintenance of prolonged graft survival on IFNγR-deficient recipients treated with costimulation blockade.

BALB/c skin was grafted onto GRKO recipients treated with CTLA-4Ig and MR1. On POD 45, some recipients were started on a CD4+ cell-depleting regimen via anti-CD4 mAb, GK 1.5. Mice receiving anti-CD4 mAb beginning on POD 45 (n=5) swiftly rejected their grafts with an MST of 60d, significantly different from that of un-neutralized recipients (MST 88d, $p<0.001$).

Figure 3.7

Neutralization of IFNγ in IFNγR-deficient recipients of long-term surviving grafts alters transcript profiles within graft-draining lymph nodes.

A



B

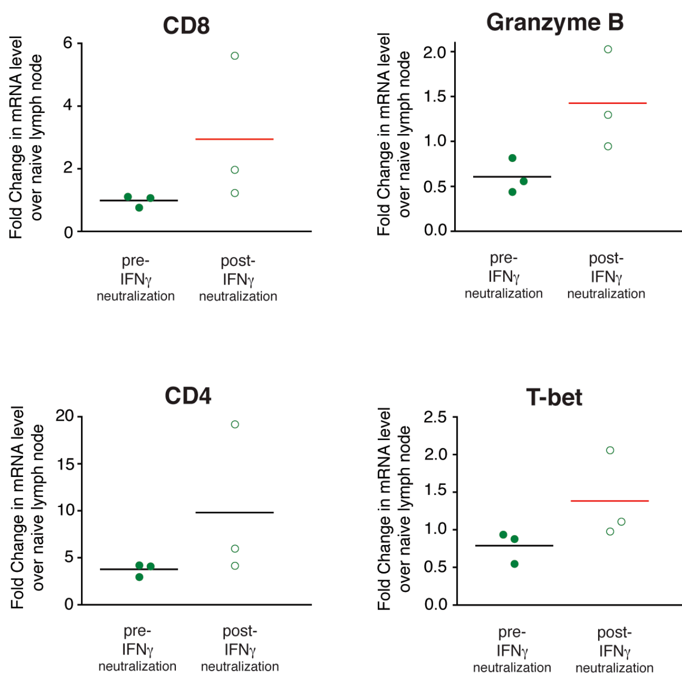


Figure 3.7

Neutralization of IFNγ in IFNγR-deficient recipients of long-term surviving grafts alters transcript profiles within graft-draining lymph nodes.

A&B) Relative quantity mRNA in graft-draining lymph nodes by RT-PCR. WT or GRKO mice were grafted with BALB/c skin and treated with CTLA-4Ig and MR1. Quantity of mRNA in all samples is relative to that detected in naïve WT lymph nodes. A) mRNA for T cell markers and tolerance-associated molecules are similar in graft-draining lymph nodes of WT and GRKO recipients treated with CTLA-4Ig and MR1. No significant differences in quantity of CD4, CD8, FoxP3, IDO or PD-L1 transcripts were found in graft-draining lymph nodes from WT and GRKO recipients at POD 7 ($p>0.05$ by two-tailed Mann-Whitney test). No significant increases in FoxP3, IDO or PD-L1 were found in graft-draining lymph nodes from GRKO recipients at POD 45 compared with POD 7 ($p>0.05$ by one-tailed Mann Whitney test). B) Increased mRNA transcripts of genes associated with cell-mediated immunity in graft-draining lymph nodes after IFNγ neutralization in GRKO mice harboring long-term surviving grafts. Transcript levels of CD8, T-bet and Granzyme B were significantly increased in graft-draining lymph nodes from GRKO recipients seven days after initiation of IFNγ neutralization (harvested on POD 52, open circles) compared with levels just prior to IFNγ neutralization (harvested on POD 45, filled circles). Red line indicates significant increase ($p=0.05$) by one-tailed Mann-Whitney test.

Discussion

Our data help to reconcile a seeming contradiction in the literature that IFNγ-deficient recipients of tissue transplants are resistant to T cell costimulation blockade-based tolerance regimens even though this cytokine is known to boost responses of CD8 T cells, which are critical for rejection under costimulation blockade. Using a novel system where either the recipient or the graft is unable to respond to IFNγ, but leaving intact the presence of the cytokine, we dissected the requirements for IFNγ signals in each party independently. In this study, we demonstrate that IFNγ is an important factor impacting not only responses of antigen-specific CD8 T cells in the absence of costimulation but also direct survival of grafted tissue in a model of allogeneic transplantation. Specifically, we found that IFNγ signals were required for recipients to expand a population of donor-specific CD8 T cells in the absence of CD28 and CD40 signals. However, when the graft also was deprived of IFNγ signals, grafts failed even in the absence of detectable T cell responses. These data demonstrate a critical role for IFNγ in the generation of costimulation blockade-resistant anti-donor T cell responses as well as in directly determining the fate of allografts. Thus, these data demand a reappraisal of results from previous studies performed in IFNγ-deficient recipients treated with costimulation blockade, in that graft death in this case may have been due to lack of IFNγ action on the graft and that this type of death may have masked the otherwise beneficial effect of an impaired CD8 T cell response.

How could IFNγ promote the population expansion of anti-donor CD8 T cells in the setting of costimulation blockade? IFNγ may partially supplant the role of CD40 stimulation in the initial activation of APCs, as IFNγ is known to upregulate

MHC class I and class II as well as to effectively upregulate CD86 on murine APC (27, 64). Alternatively, or additionally, IFNγ might directly support CD8 effector T cell survival in order to amass a large enough population to reject the graft. Whitmire *et al* found that the IFNγR was necessary on CD8 T cells for accumulation of CD8 effector T cells during acute LCMV infection, though division capacity and differentiation were not different from normal CD8 T cells, implying that IFNγ aids effector CD8 T cell population expansion predominantly via a pro-survival effect (41). Also, IFNγ might foster homeostasis of cross-reactive memory T cells or aid effector T cell trafficking to the graft, as direct IFNγ signals have been shown to aid trafficking of CD8 T cells to the lung during influenza A infection in mice and to be important in maintenance of CD8 memory T cell populations after LCMV infection (69, 70). Recent studies have shown that IFNγ signals in T cells can activate protein kinase C theta (PKCθ), a signaling kinase also activated downstream of TCR-mediated signals (39, 97). PKCθ has been shown to synergize with calcineurin, to activate NF-kB, and importantly, to provide an alternate pathway to activate AP-1, a transcription factor induced downstream of CD28 stimulation (39). As activation of PKCθ in CD8 T cells was shown to have a greater impact on cell survival than on proliferation (25), PKCθ activation via IFNγ signaling may be one way for donor-responding CD8 T cells to survive in the absence of critical costimulatory signals in order to amass a large enough population to mediate graft destruction. Though T cell proliferation and graft survival in the absence of IFNγ have been monitored in various transplant models (73, 74), this is the first study demonstrating the necessity of IFNγ signals to recipient cells for the population expansion of donor-specific CD8 effector T cells in the absence of major costimulatory signals.

In addition, other recipient cell types downstream of the CD8 T cell response may contribute to graft destruction and may also be impaired by the lack of IFNγ signals. Several models have been proposed to explain the role of IFNγ in CD8 T cell-mediated tissue destruction. Schuler and Blankenstein found in a tumor model that CD8 T cells must secrete IFNγ and that the host must express the IFNγR in order for tumors to be eliminated, leading them to propose a model where CTL recognize tumor cells directly then secrete IFNγ which acts on a third cell-type in the host which is responsible for tumor cell killing (98). Diamond and Gill also found that IFNγ secretion by CD8 T cells was necessary for efficient rejection of pancreatic islet allografts, but whether the IFNγ produced by the CD8 effectors killed islets directly or promoted expansion of the CD8 effector population was not determined (99). Valujskikh *et al* found that CTL kill allografts in an IFNγ-dependent manner by indirect recognition of alloantigens expressed on endothelial cells of graft-infiltrating vessels and suggested that IFNγ was required for endothelial cells to process and present donor antigens (100). Further experiments are needed to define precisely where in the process of the immune response to allografts IFNγ tips the balance to promote CD8 population expansion and graft rejection in the absence of CD28 and CD40 signals.

Despite a limited population size of donor-specific CD8 effector T cells in recipients treated with CoB and lacking IFNγ, graft survival time was dramatically reduced compared to recipients treated with CoB and lacking the receptor for IFNγ. The discordant outcomes in graft survival between recipients with concordant CD8 T cell responses under CoB presented a quandary initially. As transplantation by definition brings together cells derived from two separate entities, we propose a model wherein the cytokine, as a diffusible element, acts both on the recipient receptors to exert a

pro-rejection effect as well as on the graft receptors to exert a pro-survival effect. Our graft survival data from IFNγ-neutralized recipients agrees with previously published studies where IFNγ-deprivation within the entire transplant system accelerates graft loss (73, 75-77). Our current data showing prolonged graft survival on recipients unable to respond to IFNγ, as well as accelerated graft loss on these recipients after IFNγ-neutralization or when the graft lacks the IFNγR, point toward a necessary action of IFNγ on the graft to activate survival mechanisms crucial for weathering the alloresponse environment.

In line with the idea of a protective effect of IFNγ in the milieu of an immune response, the presence of IFNγ has been shown to ameliorate disease in several autoimmune models such as collagen-induced arthritis, experimental autoimmune conjunctivitis, and most notably in experimental autoimmune encephalomyelitis (EAE) (58, 101, 102). Importantly, a recent study in EAE showed that IFNγ delivered specifically to the central nervous system promotes survival of oligodendrocytes after disease induction, and this survival effect was mediated by pancreatic endoplasmic reticulum kinase (PERK), a stress-response kinase activated downstream of IFNγR signals (103). The timing and location of IFNγ action may certainly be important in determining disease outcome, as intravenous delivery of IFNγ to patients with multiple sclerosis exacerbated existing disease, with an increase in activated mononuclear cells in the peripheral blood of those who relapsed under IFNγ treatment (104). However, Wang *et al* found that IFNγ-treated CD4 T cells could suppress EAE upon adoptive transfer (96). Sawitzki *et al* found a similar requirement for IFNγ in generating functional regulatory T cells in a transplant model where adoptively transferred CD4+CD25+ cells generated via a tolerance-inducing regimen

of donor splenocyte transfusion in WT mice were able to suppress allograft rejection, but CD4+CD25+ cells generated in GKO mice did not have the same suppressive capacity (53).

Lastly, it is possible that the pleiotropic effects of IFNγ on the graft and on host immune cells involve cell type-specific signaling pathways downstream of the IFNγ receptor (97, 105). Of particular interest is the finding that immune cells have IFNγ signaling pathways that are distinct from those used by other cells of the body (106). Exploiting the cell type-specific signaling pathways for IFNγ may be one way to target blockade of IFNγ action to just the immune cells while sparing the protective action of IFNγ on the graft. Alternately, it may be possible to expose grafts or donors to IFNγ prior to transplantation to initiate pro-survival pathways. Recipients could then be treated with anti-IFNγ antibodies to blunt T cell alloresponses. Similarly, by delaying administration of anti-IFNγ, it may be possible to allow the pro-survival effects on the graft but keep the potential mounting CD8 response in check. In addition, one could envision exploiting the species specificity of IFNγ in xenotransplantation. For example, recipients of porcine pancreatic islet grafts could be treated with porcine IFNγ to enhance specifically the survival of grafted islet cells while avoiding the pro-rejection effects of IFNγ on recipient immune cells. Though the pro-survival impact of IFNγ on the graft may not be sufficient to defend the graft from a competent T cell alloresponse, the action of IFNγ on the graft is a necessary survival force that must be considered when designing strategies to promote allograft tolerance.

In total, the ideas and experimental results presented in this dissertation underscore the role of IFNγ in supporting opposing sides of the struggle toward donor-specific tolerance and clarify the need for more precise manipulation of IFNγ responses when considering any new tolerance-inducing regimen. The modifying effects of IFNγ shown here have the potential to impact a broad range of immunological studies, from autoimmunity studies where the cytokine may well serve a protective role for parenchymal cells, to studies of viral infection and vaccine development where stimulation of IFNγ activity would be necessary to achieve a substantial population of effector CD8 T cells to clear virus or to generate effective memory T cell responses. In a broad sense, these results suggest what could be an elegantly efficient role for IFNγ in the normal setting of a strong immune stimulus when an important balance must be reached between mounting a CD8 T cell response of sufficient size to clear the pathogen and limiting the collateral damage to surrounding tissues in order to preserve the functionality of the organism. Often, approaches to modulate immune behavior focus on removing a problem factor completely. In considering modulating IFNγ for therapeutic benefit, more refined approaches will be necessary to achieve the desired outcome without perturbing the entire system.

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