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GITR blockade facilitates T-reg mediated allograft survival

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Abstract

BACKGROUND—Many models of transplant tolerance have been found to depend upon the induction of regulatory T cells (T-regs). Innate immune signals are known to down-regulate T-regs thereby augmenting immunity by abrogating regulatory T cell function. Such signals may also provide a barrier to transplantation tolerance mediated by T-regs. A number of cell surface molecules expressed by T-regs have been found to inhibit T-reg activity, the best characterized of which is the glucocorticoid-induced TNF receptor-related protein (GITR).

METHODS—Using an adoptive transfer model of allograft rejection we can study the effects of inflammation and antigen-specific T-regs on graft survival. Inflammation resulting from the transplant procedure counter-regulates the suppressor activity of T-regs. To assess whether T-reg activity could be enhanced by blocking GITR signaling we compared the capacity of T-regs to prolong the survival of grafts in the presence or absence of AITRL-Fc, a novel construct that binds GITR.

RESULTS—We report that interruption of GITR-GITRL binding by AITRL-Fc resulted in long-term T-reg-dependent acceptance of skin grafts in the setting of innate immune signals that otherwise interfere with T-reg activity.

CONCLUSIONS—Inflammation and other innate immune signals may activate antigen presenting cells (APC) to upregulate GITRL. GITR-GITRL interaction is one pathway by which APCs may

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enhance the adaptive response to foreign antigen by counter-regulating T-regs and by costimulating effector T cells. By blocking this interaction with AITRL-Fc, one can sustain the benefit conferred by graft-protective T-regs.

Keywords

RODENT; TRANSPLANTATION; TOLERANCE/SUPPRESSION/ANERGY

Introduction

The recent recognition of the ability of regulatory T cells to control specific immune responses offers new avenues for development of transplantation tolerance, an objective that has heretofore remained elusive. Therapies targeting T cell activation and/or co-stimulation have yielded impressive examples of transplant tolerance, and in many instances appear to rely on the induction of T cells with regulatory or suppressive capacity (1–4). The means by which such cells function to promote allograft survival have begun to be delineated, but less understood are the means by which regulatory activity is itself regulated. We have found that naturally occurring “counter-regulatory” mechanisms are crucial to the generation of allo-immunity and thus may also surface as formidable obstacles that thwart T-reg mediated tolerance induction strategies.

A variety of innate inflammatory mediators recently have been shown to attenuate the suppressive capacity of T-regs (5–8). Compared with heart, kidney, and pancreas/islet grafts, organs like skin, lung, and intestine that are naturally colonized with commensal organisms have often proven more refractory to traditional tolerization protocols likely in part due to enhanced innate activation by microbes that promote both effector cell activation and T-reg counter-regulation (9,10,11). For example, injury to the skin is associated with enhanced TLR4 reactivity (9), and intact MyD88-dependent signaling was recently shown to preclude tolerization of fully MHC-mismatched skin grafts by a regimen of anti-C154 (10,11), a T-reg inducing antibody (12). In addition, since surgical injury and ischemia/reperfusion are acute, profound inducers of innate pathways (9,13–15), this potentially poses a significant barrier to tolerance induction in clinically relevant models that are T-reg dependent.

Glucocorticoid-induced tumor necrosis factor family-related receptor 18 (GITR) is constitutively expressed at high levels on CD4+CD25+FoxP3+ naturally occurring T-regs and is upregulated on CD4+CD25-FoxP3- effector cells upon activation (16,17). Its ligand, GITRL, is constitutively expressed on resting peritoneal B1 B cells as well as several non-lymphoid tissues (18,19). GITRL transcripts and protein are transiently upregulated in antigen-presenting B cells, bone marrow-derived DCs and macrophages *in vitro* after LPS treatment (18,20), and on antigen presenting cells in the draining lymph node after herpes simplex virus exposure *in vivo* (21). Engagement of GITR on T cells by GITRL on APCs or by agonistic anti-GITR antibody DTA-1 appears to have differing impact on allo-destructive effectors versus allo-protective regulatory cells. GITR co-stimulates effector cells and may render them resistant to regulation (18,22,23), while simultaneously directly diminishing the suppressive capacity of T-regs and promoting their proliferation to T cell receptor (TCR) stimulation (20,24,25). Such an interaction indicates one pathway by which APCs, activated by innate stimuli to express GITRL, enhance the adaptive immune response. For example, activation of GITR *in vivo* has been demonstrated to exacerbate autoimmune disease and inflammation-mediated injury, and also to improve the tempo of immune response against tumors and pathogen (26–29). In the context of transplantation however GITR-GITRL ligation may lead to the loss of benefit otherwise conferred by graft-protective T-regs. Thus, we hypothesized that blocking the GITR-GITRL interaction might promote graft survival simultaneously through effector co-stimulatory blockade and by interceding in counter-regulatory pathways.

MATERIALS AND METHODS

Mice

TS1, HA104, and HA28 transgenic mice have been described in detail (30,31). Briefly, TS1 transgenic mice possess a high frequency of CD4⁺ T cells specific for the immunodominant (Site 1) epitope of the influenza hemagglutinin (HA) protein in the context of MHC Class II I-E^d (31). HA104 mice provide a source of HA-expressing grafts as they carry an HA transgene controlled by the SV40 early region promoter/enhancer which results in ubiquitous HA expression (32,33). (TS1xHA28)F1 mice were created and described by Jordan et al (30). TS1 Thy1.1 mice were created by crossing TS1 mice onto Thy1.1 mice (Jackson Laboratory, Bar Harbor, ME). TS1, TS1 Thy1.1, HA28, and HA104 transgenic lines are maintained as hemizygotes backcrossed with BALB/c mice (Jackson Laboratory). All animals were maintained in a pathogen-free environment in the University of Pennsylvania animal facility under IACUC approved protocols.

Transplantation Procedures

Skin grafts were transplanted to mice according to the technique of Billingham and Medawar (34). Rejection was recorded when more than 75% tissue destruction was evident.

Statistical Analysis

Survival data was compared with the Kaplan-Meier method and analyzed by the log-rank test. For normally distributed data, student's t-test was applied. P-values less than 0.05 were considered significant.

Expression and purification of soluble AITRL-Fc

To create the AITRL-Fc recombinant molecule, the DNA encoding the Fc portion of human IgG1 was fused to DNA encoding the C-terminal end of the extracellular domain of the human AITRL (amino acids 42 - 170), cloned into the pMT/Bip/V5 expression plasmid and then stably transfected into *Drosophila* Schneider S2 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). AITRL-Fc was purified from the culture supernatants on protein A – Sepharose bead (Pharmacia, Piscataway, NJ).

Flow Cytometry and Cell Sorting

Approximately one million cells were suspended in biotin-free RPMI containing 0.1% azide and 3% FCS and surface stained in 96-well plates with different mAbs. Antibodies used included: anti-CD4 APC (RM4-5), anti-CD4 PE (GK1.5), anti-CD25 APC/PE (PC61), anti-CD25 FITC (7D4), anti-CD45RB PE (16A), anti-GITR FITC (DTA-1), anti-GITRL (YGL-386), anti-human IgG PE, anti-B220 PerCP (RA3-6B2), anti-CD11c APC (N418); these antibodies were purchased from ebioscience. The clonotypic antibody 6.5 biotin recognizes the S1-specific TCR from TS1 transgenic mice (31). Biotin-conjugated mAbs were subsequently detected with streptavidin-RED670 (Life Technologies), streptavidin-PE, or streptavidin-PerCP (BD Biosciences); cells were washed no fewer than three times prior to the addition of the secondary reagent. All samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software.

Cells were sorted on a BD FACSVantage SE (Becton Dickinson, San Jose, CA) high-speed cell sorter. The dual laser Vantage is equipped with 5W argon (Coherent Innova 305, Santa Clara, CA) and mixed gas argon-krypton (Coherent Spectrum) lasers. Antibodies used for cell sorting included anti-CD4 FITC, anti-CD25 APC, and anti-CD45RB PE. Sorted populations were gated on CD4 positive, CD25 positive, and CD45RB intermediate to obtain regulatory

T cells. Forward scatter pulse width (FSC-W) was used as an additional gated parameter to exclude cell aggregates. Purity checks on the sorted populations ranged from 94–98%.

CFSE Labeling

Lymphocytes were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) as previously described. Briefly, splenocytes or lymph node cells were resuspended at a concentration of 1×10^7 cells/ml in serum-free IMDM (Gibco/BRL, Gaithersburg, MD) at 37 °C. An equal volume of a dilution of the CFSE stock (5 mM in DMSO; 1:350 for in vitro experiments, 1:150–1:300 for adoptive transfer experiments) in 37 °C serum-free IMDM was then added to the cell preparation, which was subsequently vortexed briefly and incubated for 5 minutes at 37 °C. CFSE labeling was quenched by adding an equal volume of heat-inactivated FCS (HI-FCS). Cells were washed twice and resuspended in IMDM containing 10% HI-FCS for cell culture or washed twice and resuspended in PBS for adoptive transfer.

Determination of AITRL-Fc binding

293T cells grown in DMEM/10% FCS were transfected with an expression vector containing murine GITR cDNA by calcium phosphate precipitation. Cells were incubated with 2 µg/ml or 5 µg/ml of AITRL-Fc and binding was assessed by flow cytometry using a PE-labeled anti-human IgG antibody (Caltag). Control experiments were performed using FITC-labeled anti-GITR (DTA-1, eBioscience) and human IgG (Sigma) with labeled secondary antibody.

To demonstrate AITRL-Fc binds activated murine lymphocytes, peripheral lymph node cells were isolated from TS1 mice and were cultured with hemagglutinin S1 peptide for 96 hours at 37 degrees C in RPMI supplemented with 10% FCS, penicillin/streptomycin and 2-mercaptoethanol. Cells were then re-suspended and labeled with anti-CD4 APC, 6.5 biotin/streptavidin-RED670, and either AITRL-Fc (5 µg/ml) or human IgG followed by PE-labeled anti-human IgG secondary antibody. Binding was subsequently analyzed by flow cytometry.

Suppression assay

CFSE labeled TS1, Thy1.1 lymphocytes (7.5×10^5) were combined in RPMI medium (see above) with FACS purified (TS1xHA28)F1 lymphocytes (3.75×10^5) and cultured for five days in the presence or absence of S1 peptide, AITRL-Fc (5 µg/ml or 20 µg/ml), or DTA-1 (5 µg/ml or 20 µg/ml) and CFSE dilution by Thy1.1+ cells was assessed by flow cytometry. Unlabeled anti-GITR monoclonal antibody DTA-1 was purchased from Bio X Cell (New Lebanon, NH).

RESULTS

Skin transplantation acutely upregulates GITRL expression

Using an adoptive transfer model, we have previously shown a dramatic difference in regulatory T cell function in the established graft setting (skin graft 30 days prior to adoptive transfer) versus the acute graft setting (skin graft within 24 hours of adoptive transfer). BALB/c hosts receiving lymph node cells (LNC) from TS1 (HA-specific effectors) reject established, HA-bearing skin grafts, but co-transfer of HA-specific T-regs with HA-specific effectors at a 1:1 ratio results in prolonged survival of the grafts (33). These same T-regs, however, are unable to prevent rejection of acute skin grafts. Since GITR signaling is known to interfere with T-reg function, we hypothesized that if GITRL were upregulated in the draining lymph node (DLN), it would make an attractive and specific therapeutic target. To determine the effect of transplantation on GITRL expression, HA104 skin was transplanted to naïve BALB/c mice, and the cell surface expression of GITRL on ipsilateral versus contralateral LNC was compared

by flow cytometry 8 days after surgery. GITRL expression on ipsilateral dendritic (CD11c+) cells was elevated compared to levels on contralateral dendritic cells (mean fluorescence 29.73 versus 15.5, respectively), but fell to similar levels by day 10 (Figure 1). No appreciable difference was observed in GITRL expression comparing ipsilateral and contralateral in lymph node cells of mice bearing established grafts (>30 days), consistent with the time course seen after acute grafting (data not shown). These experiments show that the acute phase of transplantation is accompanied by enhanced GITRL expression in antigen presenting cells concentrated in the draining lymph node, the primary site of counter-regulation. We hypothesize that this upregulation may inactivate Treg function.

Purification and binding of AITRL-Fc

The interaction of GITR with GITRL has been demonstrated to render T-eff resistant to regulation as well as to reduce the suppressive capacity of regulatory T cells, and the skin grafting procedure results in the upregulation of GITRL in local dendritic cells. To promote survival of acute grafts, we took advantage of a recently designed a fusion protein, denoted AITRL-Fc, to disrupt GITR-GITRL binding. This fusion construct combines 129 amino acids of the extracellular domain of activation-inducible TNF receptor (AITRL), the human ortholog of GITRL, with the human IgG1 constant region (Figure 2A) which facilitates potential clinical application in the future. AITRL-Fc was generated from stably transfected *Drosophila* Schneider S2 cells then purified from the culture supernatants on protein A – Sepharose bead (Figure 2B).

To verify that AITRL-Fc can bind GITR, we utilized HEK293 cells that were transiently transfected with GITR cDNA. The transfected cells were incubated with AITRL-Fc (2 µg/ml or 5 µg/ml) and then analyzed by flow cytometry using a labeled anti-human IgG secondary antibody. AITRL-Fc demonstrated enhanced, dose-dependent binding to GITR-transfected cells (Figure 3A, left). Induction of surface GITR expression was confirmed using a commercially available anti-GITR antibody, DTA-1 (Figure 3A, right).

To demonstrate that AITRL-Fc binds to endogenous GITR, we activated TS1 splenocytes with cognate S1 peptide and assessed AITRL-Fc binding. GITR is upregulated by effector T cells (24,35). Peptide-specific and non-specific T cells can be distinguished by the 6.5 clonotypic antibody which recognizes the TS1 TCR. As shown in Figure 3B, S1-specific CD4+6.5+ T cells demonstrated the highest degree of AITRL-Fc binding (left), whereas non S1-specific CD4+6.5- and CD4-6.5- cells had similar degrees of low level binding (Figure 3B, middle and right). These data show that AITRL-Fc binds antigen activated S1-specific T cells which upregulate GITR expression.

AITRL-Fc prolongs acute graft survival

Bushell and Wood demonstrate that administration of DTA-1 anti-GITR antibody reverses tolerance to heart transplant resulting in acute allograft rejection (36). Similarly, DTA-1 treatment exacerbates graft versus host disease in some bone marrow transplant models (37). We examined whether by interfering with GITR-GITRL interaction the reagent could prolong acute graft survival. HA-bearing skin was transplanted onto naïve BALB/c recipients and then within twenty hours of skin grafting these mice were given 10^6 (TS1xHA28)F1 cells in the presence of AITRL-Fc or control human IgG. As described previously, the lymphoid organs of the F1 mouse contain a roughly equal proportion of HA-specific T-regs and CD4+CD25-CD45RB^{high} HA-specific T effectors (30,38). AITRL-Fc or control human IgG antibody (100 µg or 500 µg) was administered intraperitoneally on post-operative days 0, 4, and 10 and graft survival was compared. Mice that received AITRL-Fc at either dose exhibited significantly prolonged graft survival compared to those receiving control antibody (Figure 4A: median

survival time 120 days vs. 19 days, $p = 0.018$ for 100 ug dose; panel B: MST 120 days vs. 27 days for 500 ug dose, $p = 0.004$).

AITRL-Fc (at 500 ug dose regimen) did not significantly prolong survival in BALB/c mice bearing acute HA104 skin grafts that received TS1 cells alone (Fig. 4B; $p > .25$ for TS1 + AITRL-Fc vs. TS1 alone) - excluding the possibility that AITRL-Fc was generally immunosuppressive in the absence of supra-physiological proportion of Tregs. In addition, we examined splenic B to T cell ratios five days after the completion of an AITRL-Fc course (100 ug day 0, 4, 10) and found no substantial change compared to uninjected naïve mice and mice receiving isotype control antibody, suggesting that AITRL-Fc does not deplete GITR-bearing T cells (data not shown), nor does it alter the percentage of regulatory T cells *in vivo* or *in vitro* (data not shown).

We have previously demonstrated that in the absence of inflammation (an established graft), antigen-specific regulatory T cells are able to prolong graft survival (38). However, this T-reg-mediated prolongation is disrupted in an inflammatory setting (an acute graft; Figures 4A & B). Administration of AITRL-Fc to mice receiving TS1 cells alone did not prolong graft survival which demonstrates that the reagent's function is dependent on the presence of Tregs. These data suggest that by interfering with GITR-GITRL interaction AITRL-Fc restored T-reg-mediated allograft prolongation that had been disrupted by the acute skin grafting procedure.

AITRL-Fc unlike DTA-1 preserves T-reg suppression in vitro

To further assess our hypothesis that AITRL-Fc restored T-reg function, we studied its effect on T-reg suppression of effector proliferation *in vitro*. Using FACS purified T-regs (CD4+CD25+CD45RB^{int}) derived from (TS1xHA28)F1 mice and CFSE-labeled spleen cells from TS1, Thy1.1+ mice (T-effectors), we performed suppression assays to compare T-reg function with and without the addition of AITRL-Fc. First, 7.5×10^5 CFSE-labeled TS1, Thy1.1+ effectors and 3.75×10^5 T-regs were combined in culture for five days in the presence or absence of S1 peptide (Figure 5A). In the absence of peptide, no proliferation was evident; however in the presence of peptide, T-effectors in the absence of T-regs underwent multiple rounds of division, as demonstrated by a serial two-fold reduction in CFSE intensity. Proliferation was completely prevented in the presence of T-regs, demonstrating that the T-reg population utilized was functionally suppressive.

To confirm that GITR signaling in this system would result in failure of T-reg suppression, we added DTA-1, an anti-GITR antibody that has been shown both to suppress the inhibitory function of GITR⁺ T-regs (23,24,35) and to block the ability of activated GITR⁺ T-eff to be suppressed by T-regs through GITR (18). In the presence of either low-dose or high-dose DTA-1, T-regs were unable to prevent T-effector proliferation to peptide as multiple rounds of division of the CFSE-labeled cells were observed (Figure 5B). The degree of proliferation with DTA-1 was similar to that observed in T-effectors (alone) combined with peptide. When T-regs and T-effectors were administered DTA-1 in the absence of peptide, no CFSE dilution was observed, excluding the possibility that DTA-1 was acting as a mitogen (data not shown). AITRL-Fc could not reverse the counter-regulation due to DTA-1; when T-eff, T-reg, and peptide were co-cultured with both DTA-1 and AITRL-Fc, proliferation proceeded similar to cultures solely with DTA-1 (data not shown).

To determine whether AITRL-Fc acts as an antagonist or agonist, we performed the same assay with AITRL-Fc, instead of DTA-1, at the same concentrations (Figure 5C). T-regs appeared to restrict T effector proliferation to peptide with potency similar to control conditions. In addition, culture of T-eff with peptide in the absence of T-regs does not enhance the proliferation of the T-eff cells which demonstrates that AITRL-Fc is not acting as a co-

stimulatory reagent (Figure 5D). Collectively, these results show that despite binding GITR, AITRL-Fc does not behave like a classic GITR agonist *in vitro*.

Despite evidence for activation of NFkB and MAPK pathways via its binding of GITR (our unpublished results), AITRL-Fc does not break suppression either *in vitro* or *in vivo* when used alone. The inability of AITRL-Fc to break suppression is consistent with the data that DTA-1 enhances anti-CD3 induced proliferation while AITRL-Fc does not (25). AITRL-Fc binding to GITR may provide a signal that is qualitatively different from that provided by both GITRL and DTA-1, perhaps acting as a partial agonist-antagonist. It may prevent effective cross-linking of GITR resulting in a signal that, though detectable under *in vitro* conditions using supraphysiological AITRL-Fc concentrations, is not of functional significance *in vivo*. Corroborating this is our observation that GITR-Fc has similar capacity to permit T-regs to prolong acutely transplanted skin grafts [unpublished data] indicating that interruption of the GITR-GITRL ligand interaction rather than signaling through GITR is required to prevent counter-regulation.

DISCUSSION

In the reported experiments, we took advantage of an experimental T cell receptor transgenic model in which skin graft survival is Treg-dependent in the absence of counter-regulation to examine the effect of blocking GITR signaling on Treg-mediated graft survival. Taken collectively, our results suggest that the GITR-GITRL interaction is a crucial mediator of counter-regulatory processes invoked by innate inflammatory signaling that accompanies the acute phase of transplantation, and its disruption with AITRL-Fc represents an attractive means by which it can be overcome to engender T-reg dependent graft survival.

Efforts at achieving donor-specific hypo-responsiveness that target T cell activation or co-stimulation appear to often be critically dependent on the generation of regulatory T cells (39). The possibility of *ex vivo* expansion of donor-permissive T-regs for transfer to organ recipients has been widely proposed and is under intense investigation (40,41). Successful translation will require understanding how innate immune activation, as that encountered with organ procurement, transplantation surgery, and reperfusion affects regulatory cell function. APCs are activated via receptors for various classes of innate stimuli including cytokines, complement components, circulating immunoglobulin, bacterial cell-wall mannose, and pathogen-associated molecular patterns. As natural regulatory cells are hypo-proliferative relative to non-regulatory T cells on encounter with cognate antigen and co-stimulatory molecules, activated DCs may permit effector cells to out proliferate T-regs overwhelming their regulatory capacity and enhancing antigen clearance. However, as T-regs are now recognized to bear receptors like GITR or TLRs that can directly regulate their function, counter-regulation does not appear simply a manifestation of relative proliferative potency.

The relevance of innate immune activation, and more specifically TLRs to models of transplantation tolerance has recently been clearly demonstrated. Mice with targeted disruption of the TLR signal adaptor MyD88 were unable to reject both major (10,42) and minor (43) MHC incompatible skin grafts. Infection by virus known to require MyD88 for antiviral response prevented the establishment of co-stimulatory blockade induced tolerance (11,44) and injection of TLR2, -3, -4, and -9 agonists shortened the co-stimulatory blockade-mediated prolongation of skin allografts (11,45).

Our data emphasize the role of the GITR-GITRL interaction in counter-regulation by acute inflammation. The relative contribution of direct GITR-mediated attenuation of T-reg suppression compared with GITR-mediated costimulation of T effectors conveying resistance to suppression by T-regs is uncertain. Utilization of lymphocytes from a GITR^{-/-} mouse by

two different groups derived contradictory conclusions about the specific importance of GITR expression on T-eff in the counter-regulatory response to DTA-1 or GITRL (18,25). In *in vitro* suppressor assays co-culturing GITR^{+/+}CD4⁺CD25⁺ T cells with GITR^{-/-}CD4⁺CD25⁻ T cells in the presence of DTA-1 antibody, Stephens et al. suggest that it is stimulation of GITR on CD4⁺CD25⁻ effector T cells rather than on CD4⁺CD25⁺ regulatory T cells that renders them resistant to the suppression imposed by T-regs. GITR^{-/-}CD4⁺CD25⁻ effector cells cannot receive stimulation through GITR, and thus are suppressed by T-regs even in the presence of the agonist anti-GITR antibody, DTA-1. However, Ronchetti *et al.* find that in the presence of DTA-1, GITR^{-/-}CD4⁺CD25⁻ effector cells are not suppressed by GITR^{+/+} T-regs which suggests that DTA-1 is in fact acting on the T-regs.

Moreover it was recently demonstrated that DTA-1 did not co-stimulate CD4⁺CD25⁻ or CD8⁺CD25⁻ cells responding to fully MHC mismatched alloantigen *in vitro* (in contrast to previous studies using peptide or CD3 stimulation) (35). GITR ligation also did not potentiate rejection when CD4⁺CD25⁻ cells were transferred to cardiac allograft bearing Rag^{-/-} hosts suggesting that GITR cross-linking primarily serves to attenuate the function of regulatory T cells. The inability of AITRL-Fc to delay rejection of acute grafts in mice receiving effectors alone favors the notion that prevention of the GITR-GITRL interaction specifically on T-regs is the predominant mode of action. Nevertheless, prevention of GITR ligation on both T-regs and T effectors could offer significant synergy to inhibit responder T cell activation and promote graft survival.

The counter-regulation of T-regs indirectly via APC-derived factors (i.e. GITRL, IL-6 (8), OX40L (46)) may be influenced by inflammatory molecules acting directly on T-regs. Both CD4⁺CD25⁻ and CD4⁺CD25⁺ subsets have been shown to express TLRs (47), and T-regs are constitutively TNFR^{high} (6). Though TNF- α clearly appears to inhibit suppression, the consequences of TLR ligation on regulatory T cells appears complex. TLR4 and TLR5 appear to enhance suppressive capacity (48,49), while TLR2 and TLR8 appear to abrogate suppression (50,51). Our emphasis on the GITR-GITRL interaction does not exclude a direct role of innate mediators on T cell costimulation or counter-regulation. Since we have shown that AITRL-Fc preserves suppression despite acute inflammation *in vivo*, we postulate that GITR blockade inhibits a critical early signal that allows regulatory cells (or effectors) to respond to local innate mediators. By blocking the responsiveness of T cells to innate signals at an “upstream” step, AITRL-Fc could maintain suppression in the face of numerous classes of directly acting inflammatory mediators with pleiotropic effects on both regulators and effectors. Anti-inflammatory effects via blockade of GITR-GITRL interaction have previously been described using soluble GITR-Fc (26,52,53). Our data are consistent with the effects of GITR-Fc; however our AITRL-Fc reagent blocks by binding GITR instead of by binding GITRL. Using a similar GITR-Fc reagent to the ones described, we see significant prolongation of BALB/c skin graft survival onto C57BL/6 recipients when used in combination with anti-CD40L (54). This demonstrates that blocking the interaction of GITR-GITRL has application in a fully MHC-mismatched model.

The pathways by which inflammation induces and sustains GITRL expression by APCs continue to be defined. Our results contrast with *in vitro* observations showing TLR ligation caused only transient enhancement in GITRL expression by DCs (< 36 hours) and down-regulation on B cells (18,20). We found enhanced GITRL expression in CD11c⁺ cells of the graft ipsilateral lymph node between eight days after transplantation, and it is likely that the convergence of numerous other TLR and non-TLR innate pathways upon the APC permits prolonged and more physiologically relevant GITRL expression *in vivo*. Sustained counter-regulatory signals in the context of “danger” would permit optimal antigen eradication by responder T cells, while allowing return of regulation and preservation of self-tolerance once “danger” signals have subsided.

We have demonstrated that surgically-induced inflammation impairs T-reg ability to prolong graft survival and that the GITR-GITRL interaction appears to be of crucial significance in mediating this counter-regulatory phenomenon. Our work highlights the potent and under-recognized barrier of innate immunity on efforts to achieve transplantation tolerance and offers a novel means by which it might be overcome. As AITRL-Fc is a human construct, potential for translation to clinical trials of tolerance induction and autoimmunity deserves further consideration.

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Abbreviations

T-regs	regulatory T cells
GITR	glucocorticoid-induced TNF receptor-related
GITRL	glucocorticoid-induced TNF receptor-related ligand
HA	hemagglutinin
CFSE	5-(and-6)-carboxyfluorescein diacetate succinimidyl ester
AITRL	activation-inducible TNF receptor

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Figure 1. Expression of GITRL on CD11c+ antigen presenting cells is enhanced acutely post-transplantation

BALB/c recipients of HA104 skin grafts were sacrificed at 2, 4, 8, and 10 days after surgery, and GITRL expression was compared by flow cytometry in the ipsilateral graft draining nodes and contralateral lymph nodes. In CD11c+ cells, enhanced expression was only observed in ipsilateral lymph nodes; expression peaked at day 8 and returned to baseline by day 10. This data is representative of at least three separate experiments.

Figure 2.

(A) AITRL-Fc construct design. The AITRL (human GITRL) ecto domain (amino acids 42-170) was cloned into a pMT/Bip/V5 vector containing human IgG1 Fc fragment and transfected into *Drosophila* Schneider S2 cells.

(B) Purification of AITRL-Fc. Lane 1: culture supernatant after induction; lane 2: unbound fraction; lane 3: washing fraction; lane 4: elution fraction; lane 5: human IgG. Arrow: AITRL-Fc. Arrowhead: heavy chain of IgG is from the tissue culture serum.

Figure 3. AITRL-Fc binds GITR transfected HEK293 cells

(A) GITR cDNA was transiently transfected into HEK293 cells which were then combined with labeled anti-GITR antibody or AITRL-Fc with labeled anti-human IgG1 antibody. AITRL-Fc demonstrated similar binding to GITR as compared to anti-GITR antibody.

(B) **AITRL-Fc binds to activated T cells.** TS1 splenocytes were activated with S1 peptide and combined with AITRL-Fc. Binding was assessed by flow cytometry using a labeled anti-human IgG1 secondary antibody. AITRL-Fc binding to endogenous GITR was best observed in CD4+6.5+ (S1-specific) T cells (left); lower degree binding was seen in non-S1 specific CD4+ (middle) and CD4- (right) populations.

Figure 4. AITRL-Fc reverses counter-regulation by the acute graft setting to engender long-term graft survival

Donor skin from HA104 mice was placed onto naïve BALB/c recipients that received 1×10^6 (TS1xHA28)F1 or 5×10^5 TS1 cells one day after grafting. Recipients were administered via i.p. injection AITRL-Fc 100 ug (A) or 500 ug (B) or human IgG isotype control antibody on post-operative days 0, 4, and 10. Graft survival was indefinitely prolonged in mice receiving F1 cells and AITRL-Fc, whereas most grafts were promptly rejected in mice receiving control antibody or TS1 cells with AITRL-Fc. AITRL-Fc requires the presence of antigen-specific T-regs to prolong graft survival.

Figure 5. T-reg suppression of T-effectors is reversed by DTA-1 and not by AITRL-Fc
(A) CFSE-labeled TS1, Thy1.1+ splenocytes (“T-effectors”, 7.5×10^5) were cultured for five days in the presence or absence of S1 peptide and HA-specific T-regs (3.75×10^5) sorted from (TS1xHA28)F1 mice. In the presence of peptide, T-effectors underwent multiple divisions cycles which was completely suppressed in the presence of T-regs. **(B)** The addition of the known GITR-agonist DTA-1 to the combination of T-effectors and T-regs released effectors from T-reg mediated proliferative arrest. **(C)** The addition of AITRL-Fc failed to reverse suppression, and effector cell proliferation to peptide was again. **(D)** The addition of AITRL-Fc does not enhance the proliferation of TS1 cells in the presence of S1 peptide. Number indicates percentage of CD4+Thy1.1+ T cells are proliferating.