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Combined LFA-1 and costimulatory blockade prevents transplant rejection mediated by heterologous immune memory alloresponses

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Abstract

Background—Recent evidence suggests that alloreactive memory T cells are generated by the process of heterologous immunity, whereby memory T cells arising in response to pathogen infection cross-react with donor antigens. Due to their diminished requirements for costimulation during recall, these pathogen-elicited allo-crossreactive memory T cells are of particular clinical importance, especially given the emergence of costimulatory blockade as a transplant immunosuppression strategy.

Methods—We utilized an established model of heterologous immunity involving sequential infection of a naïve C57BL/6 recipient with lymphocytic choriomeningitis virus and vaccinia virus, followed by combined skin and bone marrow transplant from a BALB/c donor.

Results—We demonstrate that coupling the integrin antagonist anti-LFA-1 with costimulatory blockade could surmount the barrier posed by heterologous immunity in a fully allogeneic murine transplant system. The combined costimulatory and integrin blockade regimen suppressed proliferation of alloreactive memory T cells and attenuated their cytokine effector responses. This combined blockade regimen also promoted the retention of FoxP3⁺ Tregs in draining lymph nodes. Finally, we show that in an *in vitro* mixed lymphocyte reaction system using human T cells, the combination of belatacept and anti-LFA-1 was able to suppress cytokine production by alloreactive memory T cells that was resistant to belatacept alone.

Conclusions—As an antagonist against human LFA-1 exists and has been used clinically to treat psoriasis, these findings have significant translational potential for future clinical transplant trials.

Keywords

Costimulatory blockade; memory T cells; integrins; LFA-1; heterologous immunity

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W.K. and M.F. designed the experiments, analyzed the data and wrote the manuscript. W.K. performed all murine experiments with technical assistance provided by M.W. and M.S. D.H. performed all human allostimulation experiments.

Introduction

Belatacept, a second-generation CD28 antagonist, is the first biologic to receive clinical approval for use in long-term transplant immunosuppression. This new drug class prolongs graft survival through costimulatory blockade (CoB), a novel immunosuppression strategy that disrupts the vital costimulatory signals (such as CD28-B7 and CD40-CD154 interactions) required for full activation of alloreactive T cells (1–3). Conventional immunosuppression regimens typically rely upon calcineurin inhibitors, all of which suffer from serious metabolic side effects such as hypertriglyceridemia, hypertension and hyperglycemia (4). Calcineurin inhibitors are also nephrotoxic, contributing to chronic renal graft failure. Importantly, the phase III BENEFIT trial of belatacept demonstrated dramatically improved long-term renal function in belatacept-treated transplant recipients compared to conventional regimens (5, 6). However, the BENEFIT trial also paradoxically revealed that patients treated with belatacept suffered a higher incidence and severity of acute rejection.

Accumulating evidence suggests that alloreactive memory T cells may play a critical role in mediating this CoB-resistant transplant rejection. Memory T cells possess a lower costimulatory threshold than naïve T cells, and in experimental transplant systems, alloreactive memory T cells have proven resistant to costimulatory blockade (7–11). Besides their contribution to CoB-resistant rejection, these donor-specific memory T cells are of broader interest to the transplant community, as pre-transplant levels of donor-reactive memory T cells are associated with acute rejection and worsened long-term graft function, even in patients treated with calcineurin inhibitors (7, 12–14). Thus, understanding the origins of alloreactive memory T cells and the mechanisms by which they contribute to transplant rejection is essential for improving the clinical outcomes of organ transplants, especially considering the increasing prominence of CoB as an immunosuppression strategy.

Alloreactive memory T cells can arise from prior exposure to donor MHC, whether through a failed prior transplant, blood transfusion or pregnancy. More recently, several groups have described how alloreactive memory T cells can arise in transplant recipients without prior exposure to donor MHC through the process of heterologous immunity. Heterologous immunity is a by-product of infection, whereby a subset of pathogen-specific memory T cells can cross-react with donor antigens, enabling their recruitment into a rejection response (15). Recently published findings have highlighted the significant contribution of heterologous immunity to alloreactive memory responses in humans, finding that >40% of T cells raised against common viruses possess alloreactive potential (16).

To improve the clinical efficacy of costimulation blockade against an alloreactive memory response, several groups have attempted to couple CoB with adjunct immunosuppressive agents that target the memory T cells. Integrins such as leukocyte functional antigen-1 (LFA-1) and very late antigen-4 (VLA-4) are attractive targets for these adjunct therapies, as integrin expression is markedly upregulated on the surface of memory alloreactive T cells (17). Integrins are heterodimeric cell surface receptors that play a vital role in T cell adhesion, trafficking and activation (18–23). Importantly, integrin antagonists are clinically-relevant adjunct immunosuppressants, as anti-VLA-4 (natalizumab) and anti-LFA-1 (efalizumab) are clinically approved for treating autoimmune diseases such as multiple sclerosis, psoriasis and Crohn's disease (24, 25).

In experimental transplant systems utilizing immunologically naïve recipients, integrin antagonists can dramatically prolong graft survival, either as monotherapy (26–35) or when coupled with costimulatory blockade (36–41). In addition to suppressing *naïve* alloresponses, we have previously demonstrated that combined costimulatory and integrin

blockade can prolong graft survival against *memory* alloresponses (17). However, the transplant system employed in this earlier work did not address the ability of LFA-1 antagonism to synergize with costimulation blockade in inhibiting polyclonal allo-crossreactive heterologous T cell responses, potentially limiting its relevance to the clinically-important phenomenon of heterologous immunity.

In this current report, we address these critical concerns about the clinical relevance of combined costimulatory and integrin blockade, demonstrating that a regimen of CoB + anti-LFA-1 can inhibit transplant rejection by alloreactive memory T cells in a fully-allogeneic transplant system that models heterologous immunity. This regimen effectively suppressed the ability of alloreactive memory T cells to proliferate, attenuated memory T cell effector functions as measured by cytokine release, and promoted a selective retention of allospecific FoxP3⁺ Tregs in the draining lymph nodes. Given that an LFA-1 antagonist has already been clinically developed, these findings may offer a clinically translatable strategy to improve the efficacy of biologics such as belatacept in prolonging transplant survival.

Results

Combined LFA-1 and costimulatory blockade prolongs skin graft survival against a heterologous immune alloresponse

To study the impact of combined LFA-1 and costimulatory blockade on transplant rejection mediated by an alloreactive memory response, we utilized a well-defined experimental model of heterologous immunity (15). In this system, naïve C57BL/6 mice are infected with lymphocytic choriomeningitic virus (LCMV), followed by an infection with vaccinia virus six weeks later. These sequential infections generate pathogen-specific memory T cells that are cross-reactive with BALB/c alloantigens (~10⁴ allo-crossreactive memory CD4⁺ and CD8⁺ T cells per 10⁸ splenocytes) (15). Six weeks after the final infection, the mice receive a simultaneous skin graft and bone marrow transplant from a fully allogeneic BALB/c donor (Fig. 1A). While uninfected transplant recipients treated with CoB alone demonstrated indefinite graft survival, sequentially-infected recipients treated with CoB alone promptly rejected their skin grafts with the same kinetics as untreated controls (Fig. 1B). Treatment with anti-LFA-1 alone also led to prompt rejection, but treatment with a combined regimen of CoB and anti-LFA-1 enabled prolonged skin graft survival, with a median survival time >100 days (Fig. 1B). A donor bone marrow transplant was important for prolonged graft survival in this stringent transplant system, as even uninfected recipients achieved only a 22 day median skin graft survival time when treated with CoB alone in the absence of donor bone marrow (Fig. 1B). Similarly, maintenance anti-LFA-1 was required for the duration of transplant, as administration of anti-LFA-1 only during the first 6 days after transplant failed to prolong graft survival (SDC, Fig. 1).

Whereas grafts explanted from untreated recipients showed a prominent cellular infiltrate, explanted grafts taken either early (day 11) or late (>100 days) post-transplant from recipients treated with combined costimulatory/LFA-1 blockade had no infiltration, closely resembling isografts or grafts explanted from uninfected recipients treated with CoB alone (Fig. 2A–D). Further immunohistochemistry with anti-CD3 revealed a lack of T cells in the grafts treated with the combined immunosuppression regimen (Fig. 2E–H)

Combined blockade surmounts barrier posed by heterologous immunity to allogeneic bone marrow engraftment

We also examined BALB/c bone marrow engraftment eight weeks following transplant by assessing for hematopoietic chimerism in the peripheral blood of graft recipients. Using flow cytometry to determine the expression of donor MHC (H-2K^d), we found that sequentially-

infected recipients treated with either anti-LFA-1 or CoB alone failed to develop either lymphoid (CD3⁺) or myeloid (CD11b⁺) chimerism (Figs. 3A,B). In contrast, recipients treated with combined costimulatory and LFA-1 blockade demonstrated durable low-level (1–6%) lymphoid and myeloid chimerism.

Intriguingly, while our earlier published work found coupling CoB to either anti-LFA-1 or anti-VLA-4 could markedly prolong graft survival, treatment with CoB + anti-VLA-4 was ineffective in this current model of heterologous immunity (SDC, Fig. 2A). Treatment with CoB and anti-VLA-4 also failed to permit bone marrow engraftment and chimerism (SDC, Fig. 2B), consistent with previous evidence that VLA-4 is critical for homing of lymphocytes to the bone marrow (42–44).

Combined blockade inhibits alloreactive T cell proliferation and effector responses

Further *ex vivo* studies were performed to assess the mechanism by which combined costimulatory and LFA-1 blockade prolongs graft survival. First, we utilized an *in vivo* mixed lymphocyte reaction (MLR) to assess the ability of these different regimens to suppress the proliferation of alloreactive T cells after induction of heterologous immunity. Anti-LFA-1 alone failed to suppress the proliferation of alloreactive splenocytes, while CoB alone had a modest effect (Figs. 4A,B). In contrast, combined CoB and anti-LFA-1 demonstrated the most pronounced inhibition of alloreactive recall proliferation, demonstrating the synergy of these regimens (Figs. 4A,B). Next, we evaluated how these different regimens impacted alloreactive T cell effector mechanisms. Consistent with our previously published work (17), intracellular cytokine staining for IFN- γ and TNF revealed that following induction of heterologous immunity, transplant recipients treated with either CoB or anti-LFA-1 alone had a significant reduction in the percentage of splenocytes that were highly-activated double-producers of IFN- γ and TNF compared to untreated recipients (Figs. 4C,D). Combined CoB and anti-LFA-1 demonstrated an even more prominent inhibitory effect (Figs. 4C,D).

Combined blockade promotes retention of Tregs in draining LNs

Finally, we evaluated whether dominant tolerance mechanisms involving FoxP3⁺ Tregs could potentially contribute to the observed prolongation in graft survival in the combined integrin and costimulatory blockade recipients. Examining the draining lymph nodes of BALB/c graft recipients in which heterologous immunity had been induced, we found that the percentage of CD4⁺FoxP3⁺ Tregs was significantly higher in recipients treated with combined CoB and anti-LFA-1 at both early and late time-points compared to untreated recipients or recipients treated with CoB alone (Figs. 4E,F). Importantly, while this accumulation of Tregs in the draining lymph nodes may contribute to graft survival, it is not sufficient by itself, as a similar accumulation was observed in the recipients treated with anti-LFA-1 alone, despite their early graft rejection (Figs. 4E,F).

Combined blockade suppresses cytokine responses of human memory CD8⁺ T cells

We next extended our findings to human alloreactive memory T cells. Peripheral blood mononuclear cells (PBMCs) were obtained from human responder-stimulator pairs, none of which had prior history of transfusion, pregnancy or solid organ transplant. These responder and stimulator PBMCs were co-cultured along with different immunosuppressant reagents, after which IFN- γ and TNF cytokine production by CD8⁺CD45RA⁺ memory T cells was determined through intracellular cytokine staining. Given the wide variation in alloreactive T cell precursor frequency between different responder-stimulator pairings (45), the data was normalized against the peak cytokine response obtained with no treatment. Whereas treatment with belatacept alone failed to attenuate the percentage of cytokine producers amongst the alloreactive memory T cell population compared to untreated controls,

combined therapy with belatacept and anti-LFA-1 mAb led to a statistically-significant reduction in IFN- γ production by the alloreactive memory T cells, as well as a trend towards lower TNF production (Figs. 5A,B). Thus, combined integrin and costimulatory blockade also appears to have efficacy against human heterologous alloreactive memory T cell effector responses *in vitro*.

Discussion

Compared to immunosuppression with calcineurin inhibitors, costimulatory blockade offers improved long-term graft function. However, relatively higher rates of blockade-resistant transplant rejection pose a potential impediment to the widespread clinical adoption of immunosuppressants based on CoB, such as belatacept (5). Compelling evidence now suggests that alloreactive memory T cells may be prime mediators of this blockade-resistant rejection, as these memory T cells are known to possess diminished requirements for costimulation (7–11). Recipients possessing higher precursor frequencies of donor-reactive memory T cells may therefore be uniquely vulnerable to CoB-resistant rejection.

Importantly, the obstacle posed by alloreactive memory T cells may impact a large proportion of transplant patients, not just those previously sensitized to donor MHC via a failed prior transplant, blood transfusion or pregnancy. Instead, recent evidence highlights heterologous immunity as a prominent source of alloreactive memory responses (15, 16). Thus, the clinical success of belatacept in this subset of patients may ultimately require adjunct immunosuppression to surmount the barrier posed by alloreactive memory T cells.

In the search for adjunct immunosuppressants that enhance the efficacy of CoB, we have focused on integrin antagonists. Earlier work by our group demonstrated that in a murine transplant system, donor-specific memory T cell effector responses are dependent on LFA-1 engagement (17). Our previous work utilized an experimental transplant system in which ovalbumin-specific transgenic T cells were primed by infection with ovalbumin-expressing *Listeria*. After memory induction, these mice were challenged with a skin graft from a transgenic mouse that ubiquitously expresses membrane-bound ovalbumin. Several limitations with this system restricted the clinical relevance of our earlier findings. First, this system utilized a fully MHC-matched transplant pairing, with rejection targeted against only a nominal antigen (ovalbumin). Second, this earlier transplant system did not model cross-reactive heterologous immunity, as the epitope used to prime the memory T cells was *identical* to the antigen recognized on the donor graft. In true heterologous immunity, pathogen-specific memory T cells likely recognize a cross-reactive, non-identical donor antigen, for which the T cells may possess altered affinity.

To address these limitations, we turned to a system previously employed by our group to model heterologous immunity in a fully allogeneic transplant pairing (15). Consistent with our previous results, we find that while CoB alone could not prolong graft survival against a heterologous immunity memory response, an immunosuppression regimen using combined costimulatory and LFA-1 blockade did enable durable graft survival.

In contrast to our earlier results, however, the regimen of CoB + anti-VLA-4 failed to prolong graft survival. This difference may be explained by the impact of VLA-4 blockade on bone marrow engraftment. Several groups have demonstrated that VLA-4 is required for T cell and hematopoietic stem cell homing to the bone marrow (42–44, 46), and unlike recipients treated with CoB + anti-LFA-1, those treated with CoB + anti-VLA-4 failed to demonstrate successful engraftment of the BALB/c bone marrow transplant. Establishment of durable mixed chimerism may be required for long-term allogeneic skin graft survival in this very stringent transplant system, explaining why CoB + anti-VLA-4 failed to prolong

graft survival. Alternatively, this difference may reflect different integrin utilization between low-affinity memory T cells (e.g. cross-reactive T cells generated by heterologous immunity) and high-affinity memory T cells (e.g. ovalbumin-specific transgenic T cells utilized in our previous transplant system) (47).

We explored several mechanisms by which combined costimulatory and LFA-1 blockade could prolong graft survival. Generally, the combined costimulatory/integrin blockade appeared to potently suppress recall proliferation of alloreactive T cells following induction of heterologous immunity. Anti-LFA-1 also attenuated allospecific T cell effector responses such as cytokine production when coupled with CoB. Finally, the combined blockade strategy promoted the potential for dominant immunosuppression through FoxP3⁺ Tregs, as the relative ratio of Tregs to CD4⁺FoxP3⁻ effector cells in the dLNs was markedly increased, potentially enabling better immunoregulation and suppression of alloresponses. This effect seems mediated predominantly by anti-LFA-1, as we have previously reported (48).

The clinical potential of immunosuppression based on combined costimulatory and LFA-1 blockade is perhaps best reflected in its ability to inhibit human alloreactive memory T cells, as assessed by cytokine production. In this *in vitro* experiment, belatacept alone was relatively ineffective in suppressing these effector mechanisms. While it is obviously impossible to identify the source of alloreactive memory in our human responder-stimulator pairs, these alloreactive memory T cells may have arisen through heterologous immunity, as none of our subjects had a previous history of solid organ transplant, blood transfusion, or pregnancy. Importantly, the frequency of alloreactive memory CD8⁺ T cells in our subjects was very low (as is often the case, (45)), and it remains possible that this combined blockade regimen may not be as effective at attenuating effector responses in individuals possessing a higher precursor frequency of alloreactive memory T cells (49).

Besides our findings in the *in vitro* human allostimulation assay, the clinical potential of LFA-1 antagonists in transplantation is also enhanced by the development of an FDA-approved antagonist, efalizumab. However, there are several important limitations that might impact the clinical translation of combined costimulatory and integrin blockade. Belatacept therapy confers an increased risk of post-transplant lymphoproliferative disease (5), and it will be vital to define whether the addition of efalizumab would further increase these risks. An additional limitation of this regimen is that the costimulatory blockade we utilized included anti-CD154, the clinical development of which has been complicated by its known pro-thrombotic effects (2). Earlier work has demonstrated that even in uninfected C57BL/6 recipients of BALB/c skin grafts, treatment with combined CTLA-4Ig and anti-LFA-1 (in the absence of anti-CD154) achieved a median survival time of only 45.5 days (39). While anti-CD154 is critical for prolonged skin graft survival, it may not be required for the less stringent immune barrier posed by kidney, liver or heart transplantation. Furthermore, the ongoing clinical development of both domain-specific antibodies against CD154 that lack thrombogenic side-effects and CD40-specific monoclonals may also improve the translational potential of this regimen (50, 51).

Perhaps the most important constraint on the clinical development of combined costimulatory and LFA-1 blockade is the known risks of LFA-1 antagonists themselves, as the report of several cases of progressive multifocal leukoencephalopathy (PML) in patients receiving efalizumab led to its voluntary recall and withdrawal from the market in June 2009 (52–55). Importantly, accumulating evidence suggests that the risk of PML following integrin antagonism is directly related to the duration of therapy (55). Indeed, all patients who developed PML while on efalizumab had received the drug for longer than 3 years (52). While an extremely short induction regimen of efalizumab would likely not be effective

(SDC, Fig. 1), if efalizumab was employed solely as an induction agent to protect the graft during its most vulnerable period (*i.e.* the initial months post-transplant, when the rates of CoB-resistant rejection are highest (5)), the risk of PML in transplant patients might be reduced. Furthermore, the risk-benefit calculus of using efalizumab may be notably different in the setting of transplantation compared to psoriasis. If a combined regimen of belatacept and efalizumab could avert graft loss from CoB-resistant heterologous immune responses, it might justify a nominal absolute risk of PML (56). Future non-human primate trials with anti-LFA-1 and belatacept immunosuppression may better evaluate the clinical potential of this promising regimen of combined costimulatory and LFA-1 blockade, and to define its potential risks.

Materials and Methods

Mice

Male 6–8-week-old C57BL/6 and BALB/c mice (NCI-Frederick) were obtained. Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines. Viral infections were conducted by intraperitoneal injection of 2×10^5 pfu LCMV Armstrong (gifted by R. Ahmed) and 10^6 pfu Vaccinia virus (gifted by J.R. Bennick).

Skin and bone marrow transplantation

Bone marrow recipients were pre-treated with 600 μ g of busulfan (GlaxoSmithKline) *i.p.* The following day, 2×10^7 BALB/C bone marrow cells (harvested by femur flushing) were adoptively transferred via tail vein injection into the recipients. Full thickness tail skin grafts ($\sim 1 \text{ cm}^2$) were transplanted onto the recipient dorsal thorax. Where indicated, transplant recipients were treated with costimulatory blockade [500 μ g each of hamster anti-mouse-CD154 mAb (MR-1, BioXcell, West Lebanon, NH) and human CTLA-4 Ig (Bristol-Meyers Squibb, New York, NY)], 250 μ g of rat anti-mouse-VLA-4 mAb (PS/2, BioXcell), and/or 250 μ g of rat anti-mouse-LFA-1 mAb (M17/4, BioXcell). All monoclonal antibodies were administered *i.p.* on post-transplant day 0, 2, 4 and 6. Integrin antagonists were continued once weekly for the duration of transplant survival.

Flow cytometric analyses

Splenocytes, blood, and/or cells obtained from axillary draining lymph nodes (dLNs) were stained with H-2K^d-FITC, CD8a-APC and CD4-V500 (Pharmingen) for analysis on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo Software (Tree Star, San Carlos, CA).

Intracellular cytokine staining

Splenocyte suspensions from transplant recipients were co-cultured with BALB/c splenocyte stimulators at a 1:2 responder-to-stimulator ratio in the presence of 10 μ g/ml Brefeldin A (Pharmingen). Replicates with responders alone were also performed. After 5 hours, cells were stained intracellularly with anti-TNF-PE and anti-IFN- γ -AlexaFluor700 (Pharmingen) according to manufacturer's instructions. For FoxP3 staining, FoxP3-AlexaFluor700 (eBioscience) was used per manufacturer protocol.

In vivo MLR

Splenocytes were harvested on POD#60 from previously sequentially-infected graft recipients treated with different immunosuppression regimens. These splenocytes were labeled for 5 minutes with 10 μ M CFSE, and $2\text{--}3 \times 10^7$ of these labeled responders were adoptively transferred *i.v.* into irradiated BALB/c mice (700 rads). Splenocytes were

harvested after 72 hours and analyzed by flow cytometry to assess the CFSE dilution and thus proliferation of H-2K^d-negative (responder) T cells.

Human allostimulation assay

After receiving informed consent, peripheral blood mononuclear cells (PBMCs) were obtained from six human donors to form three responder—stimulator pairings. A 1:1 mixture of responders and irradiated stimulators (3500 cGy) was prepared in triplicate (~10⁶ total cells/well). Cells were either left untreated or were treated with belatacept (100 µg/ml, provided by Bristol-Myers Squibb) and/or anti-human-LFA-1 (250 µg/ml, clone TS-1 [BioXcell]). After 6 hours, intracellular cytokine staining was performed as described.

Immunohistochemistry

Explanted skin grafts were fixed in OTC and frozen. Hematoxylin and eosin staining was employed to visualize rejection. Sections were stained with anti-CD3e mAb and developed with horseradish peroxidase. Representative images (of at least 4 transplants per group) are magnified 20X.

Statistical analyses

Skin graft experiments are presented on Kaplan-Meier survival curves and compared with log-rank test. All other assays were compared with Mann-Whitney nonparametric tests. Statistical analyses utilized GraphPad Prism (La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CFSE	carboxyfluorescein succinimidyl ester
CoB	costimulatory blockade
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
dLNs	draining lymph nodes
ELISA	enzyme-linked immunosorbent assay
IFN-γ	interferon-gamma
LCMV	lymphocytic choriomeningitis virus
LFA-1	leukocyte functional antigen-1
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
OVA	ovalbumin

PBMC	peripheral blood mononuclear cells
PML	progressive multifocal leukoencephalopathy
SEM	standard error of mean
TCR	T cell receptor
TNF	tumor necrosis factor
VLA-4	very late antigen-4

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Figure 1.

Combined costimulatory and LFA-1 blockade prolongs graft survival against a heterologous immune response. (A) Experimental model of transplant rejection mediated by a heterologous immune response. (B) Survival curves of BALB/c skin grafts transplanted onto C57BL/6 recipients that had been previously infected with LCMV and Vaccinia to generate an anti-donor heterologous immune response. These sequentially-infected recipients were either untreated ($n=6$, MST=11.5 days) or treated with CoB alone ($n=14$, MST=12 days), anti-LFA-1 alone ($n=3$, MST=12 days) or CoB + anti-LFA-1 ($n=10$, MST>100 days, $p<0.0001$). Uninfected C57BL/6 mice treated with CoB in the absence ($n=3$, MST=3) or presence of BALB/c bone marrow transplant ($n=5$, MST>100 days) were also grafted.

Figure 2.

Combined costimulatory and LFA-1 blockade prevents graft infiltration. Representative H&E stains (panels A–D) and anti-CD3 immunohistochemistry stains (panels E–H) of explanted skin grafts are shown at 20x magnification. Grafts were explanted from naïve recipients (panels A and E) or sequentially-infected recipients (panels D and H) treated with CoB and BALB/c BM transplant. Other grafts were explanted from sequentially-infected recipients treated with CoB + anti-LFA-1 + BALB/c BM transplant (panels B, C, F and G). Grafts were harvested on POD#11 or POD#100, as indicated.

Figure 4.

Mechanisms of graft survival after combined costimulatory and LFA-1 blockade in recipients with a robust anti-donor heterogeneous immune response. (A) Representative histograms of CFSE dilution for CD4⁺ and CD8⁺ T cells after *in vivo* mixed lymphocyte reaction in presence of no treatment, CoB alone, anti-LFA-1 alone, or combined blockade (n= 3–8 mice/group). (B) Summary of CD8⁺ T cell proliferation during *in vivo* MLR as measured by percentage of total CD8⁺ T cells that had undergone >4 divisions after 72 hours. (C) Representative flow plots of intracellular cytokine staining for IFN γ and TNF in CD8⁺CD44⁺ splenocytes harvested on POD#60 from skin graft recipients treated with the specified immunosuppression regimen (n=3 mice/group). (D) Summary of the impact of different immunosuppression regimens on the percentage of CD8⁺CD44⁺ splenocytes that were highly activated dual producers of IFN γ and TNF. (E) Representative plots of the frequency of FoxP3⁺ Tregs in the spleen and draining lymph nodes of skin graft recipients treated with the specified immunosuppression regimen (n=3 mice/group). (F) Summary of how different immunosuppression regimens impact the accumulation of FoxP3⁺ Tregs in the draining lymph nodes on POD#11 or POD#60 after a BALB/c skin graft. All error bars represent the mean $\pm$ SEM.

Figure 5. LFA-1 blockade (but not costimulation blockade) inhibits human CD8⁺ memory T cell effector responses. Allostimulation assays performed with human PBMCs that were either untreated or incubated in the presence of belatacept alone, anti-LFA-1 alone, or combined belatacept and anti-LFA-1. Intracellular cytokine staining assessed IFN γ and TNF production by CD45RA alloreactive memory CD8⁺ T cells. Results show (A) representative flow plots of IFN γ production by responder cells or (B) summary figure of data normalized against untreated controls (four replicate experiments). All error bars represent the mean $\pm$ SEM.