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Human Regulatory T Cells with Alloantigen Specificity Are More Potent Inhibitors of Alloimmune Skin Graft Damage than Polyclonal Regulatory T Cells

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Abstract

Graft rejection by the immune system is a major cause of transplant failure. Lifelong immunosuppression decreases the incidence of graft rejection; however, nonspecific immunosuppression results in increased susceptibility to infection and cancer. Regulatory T cells (T_{regs}), which suppress the activation of the immune system and induce tolerance, are currently under evaluation for use in clinical transplantation. Ex vivo expanded polyclonal T_{regs} that are introduced into transplant recipients alter the balance of T effector cells to T_{regs}; however, experimental data suggest that alloantigen-specific T_{regs} would be more effective at preventing graft rejection. We have developed a method to enrich alloantigen-specific human T_{regs} based on the coexpression of activation markers, CD69 and CD71. These T_{regs} could be readily expanded in vitro and demonstrated potent antigen-specific suppression. In a humanized mouse model of alloimmune-mediated injury of human skin grafts, alloantigen-specific T_{regs} resulted in a significant reduction in clinically relevant indicators of dermal tissue injury when compared with polyclonal T_{regs}, restoring a histology comparable to healthy skin. This method of human allospecific T_{reg} selection should be scalable to the clinic. The improved in vivo efficacy of alloantigen-specific T_{regs} over polyclonal T_{regs} shown here suggests that generating “customized” T_{regs} with defined anti-donor allospecificities may improve current practice in clinical immunotherapy.

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SUPPLEMENTARY MATERIAL

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Fig. S1. Cell proliferation and activation marker expression analysis of alloactivated T_{regs}.

Fig. S2. In vitro assessment of T_{reg}-mediated suppression of autologous T_{eff} proliferation.

Fig. S3. Use of dermal DCs to generate donor skin alloantigen-specific T_{regs}.

Fig. S4. Immune reconstitution characteristics in the humanized mouse.

Fig. S5. Improved allograft protection by antigen-specific T_{regs} is also observed in a BALB/cRag2^{-/-} model of human skin alloimmune injury.

Fig. S6. Antigen-specific T_{regs} traffic to skin allografts in vivo and interact with HLA-DR⁺ cells.

Table S1. Human lymphocyte engraftment characteristics in NSG mice.

INTRODUCTION

Patients who receive organ transplants undergo lifelong immunosuppressive therapy to maintain their allograft. Immunosuppression is required to control the vigorous immune response triggered by allogeneic major histocompatibility complex (MHC) antigens from donor tissue, which pose the main obstacle to achieving transplantation tolerance. Two main pathways of antigen presentation, direct and indirect, are responsible for donor alloantigen recognition: Each pathway makes a distinct contribution to the development of the alloimmune response. Although immunosuppression can effectively manage acute rejection, it is unable to prevent generation of anti-donor alloreactivity over time. As a consequence, many patients succumb to immune-mediated chronic allograft rejection (1, 2) or the associated toxicities and side effects of long-term immunosuppression. Treatment of chronic rejection often requires retransplantation, further compounding the shortage of organs available for transplantation. Development of regulatory T cells (T_{regs}) as novel therapeutics is therefore based on the need for an alternative to the current clinical management of transplant recipients.

Naturally occurring T_{regs} are a functionally and phenotypically distinct lymphocyte subset constituting 1 to 5% of total $CD4^+$ T cells. In humans, T_{regs} are defined by constitutive expression of the transcription factor FoxP3 (Forkhead box P3), the surface profile $CD25^{\text{hi}}CD127^{\text{lo/-}}$, and mechanistically broad immunosuppressive effects (3, 4). Although their important immunomodulatory role has been recognized for many years (5), T_{regs} have only recently made the transition from bench to bedside; they are currently being evaluated as cellular therapeutics in several clinical transplantation settings (6, 7).

One of the main approaches to using T_{regs} in clinical transplantation (8) is to enrich T_{regs} from a prospective transplant recipient, expand them ex vivo, and then reintroduce them into the patient after transplantation. This strategy aims to alter the in vivo balance of T effector cells (T_{effs}) to T_{regs} by exploiting their natural suppressive functions. However, this approach would not negate all the caveats associated with standard immunosuppression, because polyclonal T_{regs} would also deliver pan-immunosuppressive effects. Experimental rodent models of transplantation (9-11) and immune monitoring of transplant patients (12, 13) indicate that these side effects could be avoided by specific suppression of the expansion of donor-alloreactive T_{effs} after transplantation, which can be achieved using donor allospecific T_{regs} . Collectively, these studies demonstrate that T_{regs} with defined specificities for donor alloantigens are more effective at preventing allograft rejection and improving clinical outcome than polyclonal T_{regs} . The advantage conferred by donor-specific T_{regs} may be attributed to their immunomodulatory functions being concentrated at the site of alloantigen source and immune activation, whereas polyclonal T_{regs} would be more systemically distributed (14, 15). Moreover, fewer donor-specific T_{regs} than polyclonal T_{regs} would be required to suppress the expansion of donor-reactive T_{effs} in vivo. However, challenges remain in the ability to test the relative efficacy of human polyclonal and antigen-specific T_{regs} , including the ability to generate large numbers of highly pure human alloantigen-specific T_{regs} and also the need of an appropriate in vivo model to test human T_{reg} function.

Current methods for ex vivo expansion of human donor-specific T_{regs} require repetitive stimulation of polyclonal T_{regs} with donor antigen-presenting cells (APCs) to increase the proportion of T_{regs} with (direct pathway) allospecificity (16). This approach is strictly limited by the logistical challenge of expanding such a small population, and persistence of T_{regs} with other specificities may hinder the expansion of alloantigen-specific T_{regs} , ultimately masking their true functional potency. To test human T_{reg} therapy, humanized mouse models have emerged as an alternative to nonhuman primates to study human

immunity, particularly in the context of allotransplantation (17-19). Although these models are crucial in validating the in vivo function of T_{regs} , much work remains to optimize their translation to clinical application, allowing us to evaluate the contribution of antigen specificity in determining T_{reg} function.

Here, we demonstrate a method that enriches human T_{regs} with direct pathway allospecificity, allowing them to be expanded to clinically useful numbers in vitro without the need of further restimulation. This protocol has permitted the in vivo demonstration of the superior efficacy of antigen-specific T_{regs} , compared to polyclonal T_{regs} , in protecting against alloimmune-mediated injury of human skin grafts using a humanized mouse model of xenotransplantation.

RESULTS

Activation marker screening can identify alloantigen-activated human T_{regs}

Cell surface markers indicating T_{eff} cellular activation have been studied comprehensively; however, few studies have reported on expression of activation markers by T_{regs} . To select alloantigen-specific T_{regs} , we hypothesized that by exposing a polyclonal pool of T_{regs} to an allogeneic stimulator, T_{regs} with specificity for the nominal alloantigen could be detected by display of activation markers. Freshly isolated human T_{regs} and autologous T_{effs} (Fig. 1A) were screened by flow cytometry for the basal expression of potential markers of cellular activation, in addition to markers associated with T_{reg} phenotype (Fig. 1, B and C) (20-22). As a positive control for cellular activation, T cell subsets were stimulated polyclonally using CD3CD28 beads for 1 to 5 days (Fig. 1D). T_{regs} responded to polyclonal stimulation with similar alterations in activation marker expression to T_{effs} , namely, steady up-regulation of all markers studied over 5 days, with the exception of CD62L, which was transiently down-regulated by a proportion of the T_{eff} cell subset. The most consistent and useful markers of activation were CD69 and CD71, because these had low basal expression in unstimulated cultures and were up-regulated by most of the T cell population after polyclonal stimulation.

The kinetics of activation marker expression in response to direct pathway alloantigen presentation was next examined in mixed lymphocyte reaction (MLR) using HLA-DR 1-mismatched, blood-derived myeloid CD1c⁺ dendritic cells (DCs) as stimulators (Fig. 2A). Alloantigen-activated T_{regs} demonstrated similar modulation of CD39, CD62L, CD38, CD24, CD69, and CD71 expression as observed on polyclonal stimulation, although the proportion of activation marker expressing cells was considerably higher than expected for markers such as CD39 and CD38, suggesting that they were nonspecific markers of activation. Alloantigen-activated T_{regs} up-regulated expression of CD69 and CD71 with similar kinetics and in a similar proportion, with expression peaking at day 3 and sustained until day 5. When examined together, CD69 and CD71 were found to be discretely coexpressed by activated T_{regs} after either MLR or polyclonal stimulation (Fig. 2B). The use of CD69 and CD71 as dual parameters enabled more accurate identification of recently activated T cells, excluding any possible preactivated (CD69⁺CD71⁺) or bystander activated T cells (CD69⁺CD71⁻) (23), and was therefore used for analysis of MLR cultures after 4 days. Addition of HLA-DR 1-blocking antibody to cultures inhibited expression of CD69 and CD71 in MLR, but not polyclonally stimulated cultures, confirming that activation marker up-regulation by T_{regs} was specifically in response to the recognition of intact allogeneic HLA molecules (Fig. 2C). More detailed phenotypic analysis of enriched CD69⁺CD71⁺ activated subpopulations showed that alloantigen responder T_{regs} were CD27^{hi}, CTLA-4⁺, GITR⁺, TGF LAP⁺, HLA-DR⁺, CD73⁻, CD38^{+/-}, CD39⁺ and predominantly of a naïve CD62L⁺CD45RA⁺ phenotype (Fig. 2D).

Using sorted $CD4^+CD25^+CD127^{lo/-}$ T_{regs} , we determined the mean percentage of direct pathway alloreactive T_{regs} identified by $CD69^+CD71^+$ coexpression as $2.59 \pm 1.19\%$ (Table 1). Combining activation marker expression with cell proliferation analysis showed that after 4 days of MLR, most alloactivated $CD69^+CD71^+$ T_{regs} had undergone several divisions, whereas nonactivated $CD69^-CD71^-$ cells had remained largely undivided, although some CFSE^{dim} cells were detectable (fig. S1). This latter observation suggests that CFSE (carboxyfluorescein diacetate succinimidyl ester) dilution alone would not result in the specific enrichment of T cell receptor (TCR)–stimulated T_{regs} .

CD69 and CD71 can be used to enrich and expand T_{regs} with direct pathway alloantigen specificity

To validate whether the activation profile of CD69 and CD71 coexpression could be used to enrich T_{regs} with antigen specificity for the primary stimulator, we set up MLRs between T_{regs} and allogeneic myeloid DCs, after which $CD69^+CD71^+$ T_{regs} were sorted by flow cytometry. As expected, only a small number of cells were selected using this method (mean, $\sim 2.8 \times 10^4$ cells; range, 1×10^4 to 4.5×10^4 , $n = 5$). In parallel, T_{regs} from the same donor were stimulated polyclonally and $CD69^+CD71^+$ activated T_{regs} were sorted. Cells were rested for 48 hours before their suppressive function and antigen specificity was tested in vitro by CFSE dilution assays (Fig. 3A and fig. S2). Autologous naïve $CD4^+CD25^-$ T_{effs} were used as responders using allogeneic DCs from the primary T_{reg} MLR, and an additional allogeneic DC HLA-disparate to both the responder and the primary allostimulator, as Donor and 3rdParty stimulators, respectively. $CD69^+CD71^+$ T_{regs} enriched after activation with Donor DCs were able to mediate potent and specific suppression of autologous T_{eff} proliferation in response to Donor DC stimulation at T_{reg}/T_{eff} ratios as low as 1:100, which was not observed after stimulation with 3rdParty DCs (Fig. 3A). In contrast, polyclonally activated T_{regs} (Pc- T_{regs}) were able to suppress T_{eff} proliferation in response to both Donor and 3rdParty stimulation to similar levels. The $CD69^+CD71^+$ T_{reg} activation profile could be reproducibly used as a protocol to identify and enrich alloantigen-specific T_{regs} (AgS- T_{regs}) with potent antigen-specific suppressive properties (Fig. 3B).

Relevant for cell therapy, sorted alloantigen-activated T_{regs} could be readily expanded in vitro in the presence of high doses of exogenous interleukin-2 (IL-2) (250 U/ml), without further restimulation; these cells expanded several log fold (mean, ~ 1000 -fold expansion; range, 340- to 5130-fold, $n = 4$) over 4 to 6 weeks to numbers sufficient for in vivo use in patients (mean numbers expanded, $\sim 2.8 \times 10^7$; range, 1.5×10^7 to 5.1×10^7) (Fig. 3C). Both antigen-specific and polyclonal T_{reg} lines maintained expression of T_{reg} phenotypic markers after 4 to 6 weeks of in vitro culture and did not demonstrate a significant outgrowth of interferon- γ (IFN- γ)– or IL-17–producing populations, suggesting that the purity of T_{regs} was maintained throughout culture (Fig. 3D). In vitro expanded T_{regs} were rested in media supplemented with low IL-2 (10 U/ml) before being tested for maintenance of their antigen specificity (Fig. 3E) and subsequent in vivo use.

Alloantigen-specific T_{regs} are more efficient than polyclonal T_{regs} at protecting human skin allografts from alloimmune injury

We developed our previously described model of human skin xenotransplantation (24) into a model of allogeneic CD4-mediated allograft injury to compare the relative protective function of allospecific and polyclonal T_{regs} (Fig. 4A). Skin donor alloantigen-specific T_{regs} were enriched using the activation profile protocol described above with dermal $CD1c^+$ DCs extracted from donor skin as allostimulators (fig. S3) (25), which in all experiments were equally effective as blood-derived DCs.

Mice transplanted with human skin grafts received 5×10^6 human allogeneic T_{effs} (HLA-DR 1 mismatched to skin), which resulted in engraftment of human $CD3^+CD4^+$ populations (table S1 and fig. S4). Skin allografts were harvested after 4 to 6 weeks and analyzed by histological immunostaining (Fig. 4B). Histopathological analysis of dermal tissue provided a more clinically relevant assessment of tissue damage, compared to topical assessment of allografts, which was not considered. Compared to phosphate-buffered saline (PBS) control animals (Nil), animals receiving allogeneic T_{effs} showed intense human $CD45^+$ mononuclear cellular infiltrates within allografted tissue. The presence of band-like human $CD45^+$ infiltrates localized to the dermo-epidermal junction was associated with allograft pathology, clinically resembling the immunohistological appearance of human cutaneous graft-versus-host disease (GVHD). Allogeneic T_{effs} also induced an increase in proliferating keratinocytes across epidermal stratum basale, loss of involucrin expression in upper stratum spinosum and granulosum, and loss of the stratum corneum (Fig. 4B). Combined, these parameters were indicative of active skin inflammation and loss of viable dermo-epidermal integrity and concur with previous studies examining T cell-mediated rejection of skin allografts in xenograft models (26, 27).

Using this model of alloimmune-mediated injury of human skin allografts, we compared the protective function of donor alloantigen-specific T_{regs} (AgS- T_{regs}) and polyclonal T_{regs} (Pc- T_{regs}) by transfer of T_{effs} alone or in combination with each T_{reg} (ratio of 5:1) (Fig. 5A). This ratio was selected because in vitro both AgS- T_{regs} and Pc- T_{regs} mediated a comparable suppression of T_{eff} proliferation in response to donor allostimulation at T_{reg}/T_{eff} ratios between 1:1 and 1:10. High numbers of human $CD45^+$ cell infiltrates were detected in allografts, which was equivalent between all treatment groups and reflective of human $CD45^+$ splenic engraftment (fig. S4). However, fewer $CD45^+$ cells were localized to the dermo-epidermal junction in both T_{reg} -treated groups compared to the group treated with T_{effs} alone. Donor skin from both T_{reg} -treated groups also demonstrated reduced inflammatory-associated keratinocyte proliferation, which was largely undetectable in AgS- T_{reg} -treated animals. In addition, although both T_{reg} -treated groups displayed preservation of the stratum corneum and involucrin expression, skin histology of AgS- T_{reg} -treated animals was more comparable to that of animals receiving no allogeneic T_{effs} (Fig. 5A).

More comprehensive quantitative analysis of clinically relevant parameters of dermal pathology revealed that AgS- T_{regs} provided significantly improved protection against allogeneic T_{eff} -mediated damage compared to Pc- T_{regs} (Fig. 5, B to I). Keratinocyte proliferation in allografted tissue showed an epidermal hyperproliferative compartment in T_{eff} -treated animals with strong suprabasal keratinocyte expression of Ki67, whereas Ki67 expression in grafts from untreated animals was more discrete and restricted to fewer basal epidermal keratinocytes (T_{effs} versus Nil, $P < 0.0001$) (Fig. 5B). Histological quantification showed that T_{eff} -induced keratinocyte hyperproliferation was completely inhibited by cotransfer of AgS- T_{regs} , delivering a histology similar to that of untreated animals (T_{effs} versus AgS, $P < 0.0001$; Nil versus AgS, $P = 1.0$), whereas Pc- T_{reg} -treated animals displayed moderately reduced levels of keratinocyte proliferation (T_{effs} versus Pc, $P = 0.289$; Nil versus Pc, $P = 0.006$; Pc versus AgS, $P = 0.021$) (Fig. 5F). Similarly, cotransfer of AgS- T_{regs} completely inhibited inflammatory-associated tissue damage (Fig. 5C), because fewer apoptotic cells were detected in allografted dermal tissue from this group, which was comparable to that of untreated animals (Nil versus AgS, $P = 1.0$; T_{effs} versus AgS, $P < 0.0001$) (Fig. 5G). In contrast, similar numbers of apoptotic cells were detected in T_{effs} alone and Pc- T_{reg} -treated animals (T_{effs} versus Pc, $P = 0.084$), suggesting that Pc- T_{regs} were unable to regulate allogeneic inflammatory processes as efficiently as AgS- T_{regs} (Pc versus AgS, $P = 0.012$). Consistent with these observations, analysis of human $CD31^+$ endothelial vessels in the superficial dermis of allografts (Fig. 5D) showed that microvessel injury induced by allogeneic T_{effs} (Nil versus T_{effs} , $P < 0.0001$) could be significantly reduced by

cotransfer of AgS- T_{regs} compared to Pc- T_{regs} (T_{effs} versus AgS, $P < 0.0001$; T_{effs} versus Pc, $P = 0.58$) (Fig. 5H). Combined, these data show that allografted tissue is more fully protected from immune-mediated injury by donor-allospecific T_{regs} , preserving skin pathology similar to that of untreated animals, despite the presence of an allogeneic cellular infiltrate. These data suggest that active regulation of allogeneic effectors had occurred, rather than deletion or inhibition of homeostatic proliferation. These findings were also reproducible using the BALB/c *Rag2*^{-/-} immunodeficient mouse strain (fig. S5). A significantly higher proportion of CD3⁺FoxP3⁺ cells detectable within allografts of AgS- T_{reg} -treated animals (T_{effs} versus AgS, $P = 0.001$; Pc versus AgS, $P = 0.006$) correlated well with reduced indicators of inflammation and allograft damage detected within this treatment group (Fig. 5, E and I). Finally, investigation of early trafficking of adoptively transferred T_{regs} showed that although similar numbers of AgS- T_{regs} and Pc- T_{regs} were detected in skin allografts, a higher proportion of AgS- T_{regs} interacting with skin-resident HLA-DR⁺ cells was observed compared to Pc- T_{regs} ($P = 0.036$) (fig. S6). These data support the speculation that AgS- T_{reg} -mediated suppression occurs primarily at the site of alloantigen source, potentially through modulation of APCs, resulting in the active regulation or earlier, more rapid control of infiltrating donor-reactive T effector responses in vivo.

DISCUSSION

Here, we addressed several key questions relating to the use and translation of antigen-specific T_{regs} as cellular therapeutics in clinical transplantation. We have identified a clinically transferable approach to enrich human T_{regs} with direct pathway alloantigen specificity based on the detection of an activation profile of CD69⁺CD71⁺ coexpression. Alloreactive T_{regs} demonstrated potent alloantigen-specific suppression at $T_{\text{reg}}/T_{\text{eff}}$ ratios as low as 1:100 and could be readily expanded ex vivo (up to ~5000-fold). Enriched CD69⁺CD71⁺ T_{regs} maintained their antigen-specific suppressive function after in vitro expansion and, when tested in an in vivo model of immune-mediated allograft injury, provided significantly improved protection of human skin allografts compared to polyclonal T_{regs} . Previous experimental studies using MHC-mismatched combinations in murine skin and heart allotransplantation (10, 28, 29) have shown that alloantigen-specific T_{regs} are more effective at inducing indefinite allograft survival than polyclonal T_{regs} . In the study reported here, we have translated these observations into a human in vivo model system.

Modulating a transplant recipient's immune response toward allograft acceptance using polyclonal T_{reg} preparations is now technically achievable using Good Manufacturing Practice (GMP) facilities. Compared to the relative ease of expanding polyclonal T_{regs} , allospecific T_{regs} are difficult to isolate and expand. Clinical-grade cell sorting and associated reagents have recently been established, and therefore, enrichment of CD69⁺CD71⁺ allospecific T_{regs} represents a feasible approach for clinical-grade cellular preparation. CD69 is one of the most widely used markers of cellular activation in T cell biology (30, 31), although few studies have examined its expression by T_{regs} . CD71, also a well-defined marker of T_{eff} activation (32), has only recently been reported as an activation marker of human T_{regs} , where elevated numbers of CD71⁺ T_{regs} could be detected in peripheral blood of patients with juvenile idiopathic arthritis, compared to healthy individuals (33). In addition, a recent study has applied the use of CD71 to clinical hematopoietic stem cell transplantation (HSCT), using it to deplete alloreactive T_{effs} from donor lymphocyte infusions prepared for patients, as a strategy to prevent GVHD (34). Previous approaches to generate allospecific T_{regs} have used allogeneic PBMCs or B cells to expand T_{regs} , which in vitro were shown to mediate antigen-specific suppression (16, 22, 35). Here, we selected irradiated mature myeloid DCs as allostimulators, first, to provide a potent allostimulatory signal and, second, for its compatibility with clinical-grade T_{reg} preparation, eliminating the potential persistence of allostimulators within cultures.

Although these cells are effective, a parallel comparison of other cells of donor origin would be useful in determining which allostimulator delivers optimal T_{reg} activation and expansion.

The main limitation of current protocols for allospecific T_{reg} expansion is that they rely on repetitive allostimulation to maintain the efficient in vitro expansion and function of T_{reg} lines. Selection of $CD69^{+}CD71^{+}$ alloactivated T_{regs} eliminates the need for restimulation, because selected T_{regs} readily expand in the presence of exogenous IL-2 to numbers permitting in vivo use. This proliferative capacity may be associated with their initial enrichment from nonactivated T_{regs} , which could otherwise compete for IL-2 consumption and capacity within cultures. Detailed phenotypic analysis also shows that $CD69^{+}CD71^{+}$ T_{regs} fall within subpopulations defined by markers associated with potent suppressive function and stability, namely, $CD27^{hi}CD62L^{+}$ (22, 36), $HLA-DR^{+}$ (37), and $CD45RA^{+}FoxP3^{+}$ (38, 39).

On adoptive transfer, allospecific T_{regs} were found to persist in vivo and could be detected up to 4 weeks after transfer. Because higher numbers of allospecific T_{regs} were found to be localized to donor-allografted tissue after 4 weeks, compared to polyclonal T_{regs} , it could be postulated that either antigen-driven expansion or preferential survival of allospecific T_{regs} occurred in vivo, contributing toward the protective advantage conferred by their use. These findings also reflect previous studies of T_{reg} -mediated transplantation tolerance induction in a mouse model (28). In addition, the accumulation of T_{regs} in graft draining lymph nodes (DLNs) or sites of alloantigen sequestration supports the notion that the superior protection mediated by allospecific T_{regs} observed in vivo may also be associated with their concomitant expression of $CD62L^{+}$ (40).

Development of the humanized mouse has permitted preclinical experimental models to bridge a long-standing gap in the translation of basic research observations to clinical use (18), although much work remains to study the stability, kinetics, and protective mechanisms of human T_{reg} use in vivo. One limitation of this study is that the in vivo model used has focused on direct pathway CD4-mediated damage of allografted skin, whereas other studies have demonstrated a clear effector role for both $CD4^{+}$ and $CD8^{+}$ T cell-mediated human allograft rejection (26, 27). Because a recent study has shown that protection of human skin allografts using polyclonal T_{regs} (19) is associated with a modest decrease in graft infiltrating $CD8^{+}$ T cells, it is possible that the mechanistic basis and protective potential of T_{reg} therapy may differ depending on the immune component targeted. In this respect, the challenge of generating more clinically comparable models of transplantation in the humanized mouse needs to be addressed, allowing us to assess the ability of T_{regs} to inhibit, for example, alloantibody-mediated immunity or regulation of the indirect pathway during graft rejection.

The data we present here have provided in vivo evidence of the relative functional efficacy of human alloantigen and polyclonal T_{regs} in ameliorating immune-mediated allograft pathology. The findings hold important implications for the methods of T_{reg} generation and selection for in vivo clinical use and suggest that efforts should be refocused on the goal of generating allospecific T_{regs} for the induction of antigen-specific immune regulation.

MATERIALS AND METHODS

Cell culture

All cell cultures and assays were performed in complete media [RPMI 1640 supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin, all from Invitrogen],

containing 10% human AB serum (BioSera), and performed in humidified incubators at 37°C and 5% CO₂. Isolated cells were HLA-typed with standard techniques (41).

Purification of human T_{regs}

Human T_{regs} were enriched from peripheral blood TRIMA cones from healthy volunteers, obtained with full informed consent and ethical approval (National UK Blood Service). PBMCs were separated by Lymphoprep density gradient centrifugation (PAA), and erythrocytes were lysed with ACK buffer (Sigma). Total CD4⁺ T cells were isolated by staining PBMCs with a cocktail of mouse anti-human monoclonal antibodies against CD8 (clone FK-18), CD14 (B-A8), CD16 (B-E16), CD56 (B-A19), CD19 (B-C3), TCR (B1), and CD33 (4D3), followed by negative bead selection with pan-mouse immunoglobulin G Dynabeads (from Invitrogen and Telpel). CD4⁺CD25⁺ T_{regs} were then selected by positive isolation with Dynal CD25-conjugated beads (Invitrogen) or cell-sorted based on expression of CD4⁺CD25^{hi}CD127^{lo} (FACSARIAII Cell Sorter, Becton Dickinson). T_{regs} were rested overnight in complete media supplemented with IL-2 (10 ng/ml) (Proleukin, Novartis) before further use. Autologous PBMCs depleted of CD25⁺ cells or CD4⁺CD25⁺ T_{effs} were cryopreserved in human serum containing 10% dimethyl sulfoxide for later use.

Purification of blood- and skin-derived myeloid DCs

Human myeloid CD1c⁺ (BDCA-1) DCs were enriched from PBMCs with a microbead positive selection kit according to the manufacturer's instructions (Miltenyi Biotec). DCs were cultured overnight in complete media with lipopolysaccharide (LPS) (100 ng/ml) (Serotype 055:B5, Sigma-Aldrich) to induce maturation. Cells were then washed thoroughly and then cryopreserved for later use, or irradiated [3000 gray (Gy)] and used directly as allogeneic stimulators. CD1c⁺ dermal DCs were extracted from human skin explants (described below) with a previously described protocol (25). In brief, skin was cut into 1- to 2-mm slices and immersed in DispaseII solution (5 mg/ml) (Roche) for 5 hours at 37°C. After removing the epithelium, the remaining dermis was cultured in six-well plates in complete media for 5 days, allowing dermal DCs to migrate out into the well. Dermal CD1c⁺ and CD14⁺ DCs were further selected by cell sorting. Because skin-derived DCs showed a mature phenotype, they were not further matured by LPS but were irradiated before use.

Flow cytometry and intracellular cytokine staining of human T_{regs}

T_{regs} were screened for expression of markers of T cell activation after polyclonal or allogeneic stimulation for 1 to 5 days by flow cytometry. Activation markers analyzed included CD24 (clone ML5), CD27 (O323), CD38 (HIT2), CD39 (ebioA1), CD62L (DREG56), CD69 (FN50), CD71 (OKT-9), CD73 (AD2), HLA-DR (T36), and TGF-β1 (FAB2463A) (from eBioscience, BD Biosciences, and R&D Systems). For intra-cellular cytokine staining of IFN-γ (B.27) and IL-17 (ebio64), T_{regs} were stimulated with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (1 μg/ml) (both Sigma) for 5 hours with 1× Brefeldin A (eBioscience). CTLA-4 (BN13) and FoxP3 (PCH101) staining was performed with eBioscience Fix/Perm kit under the manufacturer's directions. Dead cells were excluded from analysis by Live/Dead counterstaining (Invitrogen). Flow cytometric data were acquired with an LSRII flow cytometer (Becton Dickinson), analyzed with FlowJo 7.5 software (TreeStar Inc.), and included doublet discrimination.

In vitro MLRs

T_{regs} were polyclonally stimulated with CD3CD28-coated T cell expander beads (25 μml/10⁶ cells) (Invitrogen). Allogeneic MLRs were set up with irradiated peripheral blood- or skin-derived myeloid DCs and T_{regs} at a 1:3 ratio in complete media containing IL-2 (10 ng/

ml) for 1 to 5 days. Markers where cellular expression was found to be modulated compared to unstimulated cells were then further validated in MLR cultures in the presence of an anti-HLA-DR-blocking antibody (clone L243, 10 µg/ml, eBioscience).

Enrichment of antigen-specific T_{reg} s and antigen-specific suppression assays

After polyclonal or allogeneic stimulation, T_{reg} cultures were stained and gated on CD3 (OKT3, eBioscience) and subjected to doublet discrimination to exclude any residual DC stimulator. CD3CD28 beads were removed from polyclonally activated cells before cell sorting. Cells were then sorted into two populations, CD69⁺CD71⁺ and CD69⁻CD71⁻, and either expanded in vitro in the presence of IL-2 (250 U/ml) or rested (48 hours) in complete media with IL-2 (10 U/ml) before performing suppression assays. Antigen specificity of T_{reg} s was tested by in vitro cell proliferation assays. T_{reg} s were labeled with 2.5 µM eFluor670 (eBioscience) and added at ratios of 1:1 to 1:500 relative to autologous T_{eff} s, which were labeled with 5 µM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen). Allogeneic and polyclonal stimulators were used as described above. Assays were performed over 5 days, after which T cell proliferation was analyzed for CFSE expression by flow cytometry. Percentage suppression was calculated using the percentage of divided cells on stimulation, with and without T_{reg} s. Where sufficient allostimulators were not available to perform sequential MLRs to generate alloantigen-specific T_{reg} s and test their suppressive function in vitro, as with some skin-derived DCs, known single HLA-DR-expressing Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines were used as surrogate allostimulators (10th International Histocompatibility Workshop, WS3, WS45, WS29, WS36) selected to match the donor HLA type or specific mismatch, along with an HLA-disparate 3rd Party stimulator.

Animals

NOD/scid/*IL-2R*^{-/-} (NOD.cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) (NSG; The Jackson Laboratory) mice and BALB/c *Rag2*^{-/-} *c*^{-/-} mice (from A. Hayday, King's College London) were used between 4 and 14 weeks at the time of the first experimental procedure. Animals were bred and maintained in the Biological Services Unit of King's College London. All mice were kept under pathogen-specific sterile conditions, and procedures were conducted in accordance with institutional guidelines and the Home Office Animals Scientific Procedures Act (1986).

Humanized mouse model of direct pathway alloimmune-mediated skin injury

Human skin was obtained from routine abdominoplasty and reduction mammoplasty surgery, with informed consent and ethical approval (Guy's & St Thomas' NHS Foundation Trust and King's College London). In brief, split-thickness (500 to 700 µm) skin explants were harvested with a dermatome (Zimmer) and 1-cm² pieces were transplanted onto NSG and *Rag2*^{-/-} *c*^{-/-} mice, as previously described (24). Human skin grafts were fixed epidermal side upward onto similarly sized defects on recipient backs with an absorbable tissue seal (Vet-Bond, Braun Medical). Grafts were covered with Fucidin microbicide (Leo Laboratories Ltd.) and secured with Tegaderm film dressing (3M) for 7 days. Skin was allowed to engraft for 4 to 6 weeks before each experiment was initiated. Graft integrity and healing was assessed by histological analysis 4 weeks after transplantation to ensure that epidermal thickness was comparable to the pre-graft specimen (preservation of rete peg pattern and minimal evidence of inflammation). Allogeneic (one to two HLA-DR 1 mismatches to skin) PBMCs depleted of CD25⁺ cells or enriched CD4⁺CD25⁻ cells (5×10^6) were then adoptively transferred by intravenous injection to induce allograft damage, with or without in vitro-expanded T_{reg} s (1×10^6). Higher doses of PBMC inoculates (10×10^6 to 20×10^6 cells per mouse) routinely resulted in engraftment of both CD4 and CD8 T cells, whereas lower doses resulted in predominant engraftment of CD4 T cells, with no

detectable APC compartments (table S1). No animals developed xenogeneicGVHD symptoms, confirmed by maintenance of stable body weight. Weekly peripheral tail bleeds were performed to monitor human CD45⁺ cell engraftment. Animals were considered as successfully engrafted when human CD45⁺ cells constituted >0.5% of total splenic lymphocytes. Only these animals were selected for further analysis of allografted skin. Skin grafts were subjected to visual and tactile inspection two times weekly for evidence of graft injury, and experiments ended 4 to 6 weeks after T_{eff} transfer, depending on human CD45⁺ engraftment detected in peripheral blood. Splenocytes and DLNs were harvested and analyzed for cellular composition.

Immunohistopathological analysis of skin allografts

Frozen sections (6 to 8 µm) of human skin allografts were prepared after snap freezing in optimum cutting temperature reagent over precooled isopentane. Tissue sections were air-dried and fixed in acetone (immunohistochemistry) or histological fixative saline (immunofluorescence). Nonspecific Fc receptor binding was measured with isotype nonbinding control antibodies. Immunohistochemical staining was performed for human CD45 (clone HI30; eBioscience), human involucrin (SY5; Sigma), and human Ki67 (MM1; Leica) with avidin-biotinylated antibody pairs and developed with the ABC Vectastain Elite kit (Vector Laboratories). Double immunofluorescence stains were performed in the following combinations: CD45, Ki67; FoxP3 (259D/C7), CD3; involucrin (SY5), CD31 (ab28364). Sections were incubated with fluorescence-conjugated secondary antibodies and mounted with Prolong Gold AntiFade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (all from Invitrogen). Samples were analyzed by fluorescence microscopy, and quantitative analysis was performed by counting a minimum of three nonoverlapping visual fields per animal and blinded to treatment protocols. Quantification of CD31⁺ vessels was determined by counting only perfused intact vessels and not single CD31⁺ cells. Apoptotic cells were detected by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) staining according to the manufacturer's directions (Roche). Sections were counterstained with DAPI, and a minimum of six visual fields was quantified for double-positive nuclei.

Statistical analysis

Data shown are means ± SD where indicated. Statistical comparisons of T_{reg} suppression assays were made with two-tailed paired Student's *t* tests. Statistical comparisons of immunohistological parameters CD31, TUNEL, and Ki67 quantitative analysis between treatment groups were made with nonparametric Kruskal-Wallis tests (significance level, 99.9%). Statistical comparisons of FoxP3/CD3 ratio between treatment groups were made with one-way analysis of variance (ANOVA) (significance level, 99.9%), and all significance is denoted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

1. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *Am. J. Transplant.* 2004; 4:378–383. [PubMed: 14961990]
2. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: A critical reappraisal. *Am. J. Transplant.* 2011; 11:450–462. [PubMed: 20973913]
3. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J. Exp. Med.* 2006; 203:1701–1711. [PubMed: 16818678]
4. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat. Rev. Immunol.* 2008; 8:523–532. [PubMed: 18566595]
5. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3⁺ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 2010; 10:490–500. [PubMed: 20559327]
6. Safinia N, Sagoo P, Lechler R, Lombardi G. Adoptive regulatory T cell therapy: Challenges in clinical transplantation. *Curr. Opin. Organ Transplant.* 2010; 15:427–434. [PubMed: 20616725]
7. Trzonkowski P, Bieniaszewska M, Ju ci ska J, Dobyszek A, Krzystyniak A, Marek N, My liwska J, Hellmann A. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4⁺CD25⁺CD127[−] T regulatory cells. *Clin. Immunol.* 2009; 133:22–26. [PubMed: 19559653]
8. Sagoo P, Lombardi G, Lechler RI. Regulatory T cells as therapeutic cells. *Curr. Opin. Organ Transplant.* 2008; 13:645–653. [PubMed: 19060557]
9. Tsang JY, Tanriver Y, Jiang S, Xue SA, Ratnasothy K, Chen D, Stauss HJ, Bucy RP, Lombardi G, Lechler R. Conferring indirect allospecificity on CD4⁺CD25⁺ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J. Clin. Invest.* 2008; 118:3619–3628. [PubMed: 18846251]
10. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, van Meerwijk JP. Prevention of acute and chronic allograft rejection with CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes. *Nat. Med.* 2008; 14:88–92. [PubMed: 18066074]
11. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4⁺CD25⁺ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 2002; 196:389–399. [PubMed: 12163567]
12. Nafady-Hego H, Li Y, Ohe H, Zhao X, Satoda N, Sakaguchi S, Wood K, Uemoto S, Koshiba T. The generation of donor-specific CD4⁺CD25⁺⁺CD45RA⁺ naive regulatory T cells in operationally tolerant patients after pediatric living-donor liver transplantation. *Transplantation.* 2010; 90:1547–1555. [PubMed: 21085066]
13. Davies JK, Nadler LM, Guinan EC. Expansion of allospecific regulatory T cells after anergized, mismatched bone marrow transplantation. *Sci. Transl. Med.* 2009; 1:1ra3.
14. Tang Q, Adams JY, Tooley AJ, Bi M, Fife BT, Serra P, Santamaria P, Locksley RM, Krummel MF, Bluestone JA. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat. Immunol.* 2006; 7:83–92. [PubMed: 16311599]
15. Dijke IE, Weimar W, Baan CC. Regulatory T cells after organ transplantation: Where does their action take place? *Hum. Immunol.* 2008; 69:389–398. [PubMed: 18638654]
16. Peters JH, Hilbrands LB, Koenen HJ, Joosten I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4^{pos}CD25^{high} T cells for immunotherapy. *PLoS One.* 2008; 3:e2233. [PubMed: 18493605]

17. Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, Schiopu A, Taggart DP, Wood KJ. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat. Med.* 2010; 16:809–813. [PubMed: 20473306]
18. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat. Rev. Immunol.* 2007; 7:118–130. [PubMed: 17259968]
19. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation.* 2010; 90:1321–1327. [PubMed: 21048528]
20. Fletcher JM, Loneragan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, Tubridy N, Mills KH. CD39⁺Foxp3⁺ regulatory T cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J. Immunol.* 2009; 183:7602–7610. [PubMed: 19917691]
21. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Höpner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Röttschke O, Falk K. Expression of ectonucleotidase CD39 by Foxp3⁺ Treg cells: Hydrolysis of extracellular ATP and immune suppression. *Blood.* 2007; 110:1225–1232. [PubMed: 17449799]
22. Koenen HJ, Fasse E, Joosten I. CD27/CFSE-based ex vivo selection of highly suppressive alloantigen-specific human regulatory T cells. *J. Immunol.* 2005; 174:7573–7583. [PubMed: 15944257]
23. Hartwig UF, Nonn M, Khan S, Meyer RG, Huber C, Herr W. Depletion of alloreactive T cells via CD69: Implications on antiviral, antileukemic and immunoregulatory T lymphocytes. *Bone Marrow Transplant.* 2006; 37:297–305. [PubMed: 16327814]
24. Conrad C, Boyman O, Tonel G, Tun-Kyi A, Laggner U, de Fougères A, Kotliński V, Gardner H, Nestle FO. Integrin is crucial for accumulation of epidermal T cells and the development of psoriasis. *Nat. Med.* 2007; 13:836–842. [PubMed: 17603494]
25. Nestle FO, Zheng XG, Thompson CB, Turka LA, Nickoloff BJ. Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J. Immunol.* 1993; 151:6535–6545. [PubMed: 7504023]
26. Racki WJ, Covassin L, Brehm M, Pino S, Ignatz R, Dunn R, Laning J, Graves SK, Rossini AA, Shultz LD, Greiner DL. NOD-scid IL2r^{null} mouse model of human skin transplantation and allograft rejection. *Transplantation.* 2010; 89:527–536. [PubMed: 20134397]
27. Turgeon NA, Banuelos SJ, Shultz LD, Lyons BL, Iwakoshi N, Greiner DL, Mordes JP, Rossini AA, Appel MC. Alloimmune injury and rejection of human skin grafts on human peripheral blood lymphocyte-reconstituted non-obese diabetic severe combined immunodeficient β_2 -microglobulin-null mice. *Exp. Biol. Med.* 2003; 228:1096–1104. [PubMed: 14530522]
28. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro-expanded donor alloantigen-specific CD4⁺CD25⁺ regulatory T cells promote experimental transplantation tolerance. *Blood.* 2007; 109:827–835. [PubMed: 17003369]
29. Tsang JY, Tanriver Y, Jiang S, Leung E, Ratnasothy K, Lombardi G, Lechler R. Indefinite mouse heart allograft survival in recipient treated with CD4⁺CD25⁺ regulatory T cells with indirect allospecificity and short term immunosuppression. *Transpl. Immunol.* 2009; 21:203–209. [PubMed: 19446634]
30. Fehse B, Frerk O, Goldmann M, Bulduk M, Zander AR. Efficient depletion of alloreactive donor T lymphocytes based on expression of two activation-induced antigens (CD25 and CD69). *Br. J. Haematol.* 2000; 109:644–651. [PubMed: 10886218]
31. Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. *Stem Cells.* 1994; 12:456–465. [PubMed: 7804122]
32. Porporatto C, Canali MM, Bianco ID, Correa SG. Ability of the polysaccharide chitosan to inhibit proliferation of CD4⁺ lymphocytes from mucosal inductive sites, in vitro and in vivo. *Cell Prolif.* 2009; 42:780–787. [PubMed: 19689471]
33. Stelmazczyk-Emmel A, Jackowska T, Rutkowska-Sak L, Marusak-Banacka M, Wsik M. Identification, frequency, activation and function of CD4⁺CD25^{high}FoxP3⁺ regulatory T cells in children with juvenile idiopathic arthritis. *Rheumatol. Int.* 2011 10.1007/s00296-010-1728-3.
34. Samarasinghe S, Mancao C, Pule M, Nawroly N, Karlsson H, Brewin J, Openshaw P, Gaspar HB, Veys P, Amrolia PJ. Functional characterization of alloreactive T cells identifies CD25 and CD71

- as optimal targets for a clinically applicable allodepletion strategy. *Blood*. 2010; 115:396–407. [PubMed: 19890093]
35. Chen LC, Delgado JC, Jensen PE, Chen X. Direct expansion of human allospecific FoxP3⁺CD4⁺ regulatory T cells with allogeneic B cells for therapeutic application. *J. Immunol*. 2009; 183:4094–4102. [PubMed: 19684083]
 36. Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, Schiopu A, Taggart DP, Wood KJ. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat. Med*. 2010; 16:809–813. [PubMed: 20473306]
 37. Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. *J. Immunol*. 2006; 176:4622–4631. [PubMed: 16585553]
 38. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochoy G, Sakaguchi S. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009; 30:899–911. [PubMed: 19464196]
 39. Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, Atkinson MA, Bluestone JA. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes*. 2009; 58:652–662. [PubMed: 19074986]
 40. Carvalho-Gaspar M, Jones ND, Luo S, Martin L, Brook MO, Wood KJ. Location and time-dependent control of rejection by regulatory T cells culminates in a failure to generate memory T cells. *J. Immunol*. 2008; 180:6640–6648. [PubMed: 18453583]
 41. Bunce M, O'Neill CM, Barnardo MC, Krausa P, Browning MJ, Morris PJ, Welsh KI. Phototyping: Comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens*. 1995; 46:355–367. [PubMed: 8838344]

Fig. 1. Screening of human T_{reg} activation markers by flow cytometry. **(A)** Human T_{reg}s were isolated from whole CD4⁺ T cells from peripheral blood by magnetic bead selection (CD4⁺CD25⁺) or cell sorting (CD4⁺CD25⁺CD127^{lo/-}) and enriched to purities of ~90% and 98% by each method, respectively. Data are representative of nine independent experiments. **(B)** Phenotype of freshly isolated T_{reg}s and autologous T_{eff}s (CD4⁺CD25⁻) was studied by flow cytometry, analyzing typical T_{reg} markers and basal expression of activation markers under investigation. **(C)** Data are summarized, plotting means $\pm$ SD ($n = 6$). **(D)** Expression

of T cell activation markers by purified T_{regs} and autologous T_{effs} after 1 to 5 days of polyclonal stimulation with CD3CD28 beads. Data are representative of six experiments.

Fig. 2.

Analysis of T_{reg} activation marker modulation in response to direct pathway allogeneic stimulation. (A) T_{regs} and autologous T_{effs} were cultured with mature allogeneic CD1c⁺ DCs for 1 to 5 days. Expression of activation markers was analyzed by flow cytometry and is expressed as the change (Δ) in percentage of activation marker–positive cells after in vitro stimulation, relative to unstimulated cells (Fig. 1B). Data plotted show means $\pm$ SD ($n = 7$). (B) Polyclonal and allogeneic stimulation results in coexpression of CD69 and CD71 by T_{regs}. Data are representative of 12 independent experiments. (C) CD69 and CD71 up-regulation induced by allogeneic stimulation was inhibited by addition of α HLA-DR 1–blocking antibody to cultures but did not affect expression in polyclonally stimulated cultures. Data are representative of four experiments. (D) Detailed analysis of CD69⁺CD71⁺ T_{reg} phenotype after allogeneic stimulation (4 days). Inset images show that the activated T_{reg} phenotype is reflected in cell cultures by activated cell clusters ($n = 4$).

Fig. 3.

CD69⁺CD71⁺ enrichment of potent alloantigen-specific T_{regs}. **(A)** Suppressive function of alloantigen (donor) or polyclonal (CD3CD28) activated (CD69⁺CD71⁺) T_{regs} was tested by in vitro CFSE dilution assays after 5 days (upper panels). Flow cytometry data are summarized in line plots (lower panels) as percentage suppression of proliferation mediated by T_{regs} on autologous CD4⁺CD25⁺ T_{eff}s stimulated with Donor or HLA-disparate 3rdParty DCs. **(B)** Summary of five independent experiments showing suppressive capacity of enriched alloantigen-activated CD69⁺CD71⁺ T_{regs} and nonactivated CD69⁻CD71⁻ T_{regs} against the primary allostimulator or 3rdParty allostimulator. **(C)** Number of activated T_{regs} isolated by cell sorting and final cell number expanded in vitro after 4 to 6 weeks for several T_{reg} lines. **(D)** Maintenance of T_{reg} marker expression by two different antigen-specific T_{reg} lines (t1 and t2) after in vitro expansion, and expression of IFN- γ and IL-17 upon stimulation (phorbol 12-myristate 13-acetate/ionomycin) of T_{regs}. High FoxP3 expression is maintained by expanded T_{regs}, compared to polyclonally activated T_{eff}s, which express intermediate FoxP3. **(E)** Alloantigen-specific T_{regs} enriched with CD69⁺CD71⁺ maintained antigen-specific suppressive function after in vitro expansion for 6 weeks. Percentage suppression of autologous T_{eff} proliferation by donor alloantigen-specific T_{regs} (AgS-T_{regs}), in response to primary allostimulator or 3rdParty stimulation, is highlighted within histograms. Data are representative of three independent assays. Statistical significance was calculated with paired Student's *t* tests. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. 4. Assessment of enriched donor alloantigen-specific and polyclonal T_{reg} function with a humanized mouse model of skin allograft injury. **(A)** Strategy for generation of human skin donor alloantigen-specific (AgS-T_{regs}) and polyclonal (Pc-T_{regs}) T_{regs} and in vivo assessment of their protective function with a humanized mouse model of allogeneic CD4⁺ T effector-mediated damage of allografted tissue in NOD/scid/IL-2R^{-/-} (NSG) and BALB/*cRag2*^{-/-} *c*^{-/-} strains. **(B)** Histological assessment of donor-allografted human skin 4 weeks after animals received either PBS (Nil) or 5×10^6 allogeneic human T effectors (T_{effs}). Human CD45 staining shows an intense T_{eff} infiltrate, with focal localization at dermo-epidermal junctions (arrows). Cells across epidermal basal lamina are positive for Ki67, indicating keratinocyte hyperproliferation. Loss of involucrin expression shows epidermal barrier dysfunction in T_{eff}-treated animals.

Fig. 5. More potent protection of skin allografts from alloimmune injury by antigen-specific T_{regs} than polyclonal T_{regs}. (A) Human skin grafts from mice receiving PBS only (Nil), 5×10^6 T_{eff}s alone (T_{eff}s), or in combination with 1×10^6 donor-specific (AgS) or polyclonal (Pc) T_{regs} were analyzed after 4 weeks by staining skin allografts for human CD45, Ki67, and involucrin. (B to E) More detailed characterization of skin allografts, examining (B) epidermal keratinocyte expression of Ki67, (C) TUNEL⁺ nuclei in dermal infiltrates, (D) human CD31 dermal microvasculature, and (E) detection of CD3⁺FoxP3⁺ T_{regs} in perivascular dermal infiltrates was performed. Representative immunofluorescent images from each treatment group are shown (inset images at higher magnification). (F to I)

Quantitative histological analysis of three to five independent visual fields per allograft, where plotted lines represent the mean ($n = 6$ animals per treatment group, pooled data from two independent experiments). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Kruskal-Wallis test. Quantification of CD3⁺FoxP3⁺ cells is expressed as a ratio of total CD3⁺ cells quantified per visual field and was statistically compared by one-way ANOVA.

Table 1

Percentage of direct pathway alloantigen responder T_{regs} . T_{reg} were stimulated with allogeneic myeloid $CD1c^{+}$ DCs for 4 days, after which the percentage of alloantigen responder T_{regs} was calculated by deducting the percentage of $CD69^{+}CD71^{+}$ cells detected in media alone from the percentage detected in MLRs ($n = 8$).

Responder T_{reg} HLA-DR 1	Allostimulator HLA-DR 1	No. of HLA mismatches	% $CD69^{+}CD71^{+}$ responder T_{reg}
01, 04	07, 13	2	1
04, 11	07, 15	2	1.8
01, 07	01, 15	1	2.82
03, 07	07, 17	1	3.82
07, 12	03, 07	1	2
03, 07	03, 13	1	3.6
04, 15	01, 03	2	4.2
03, 13	03, 07	1	1.5
Mean % alloresponder cells			2.59